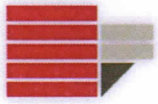
UNIVERSITÀ DELLA CALABRIA



Dipartimento di Farmacia e Scienze della Salute e della Nutrizione

Dottorato di Ricerca in

Biochimica cellulare ed attività dei farmaci in oncologia

Ciclo XXVIII

Con il contributo di "Ministero dell'Istruzione, dell'Università e della Ricerca"

Study of new ligands of steroid receptors: the effect of G-1 (a new ligand of GPER) and development of a novel cell-based androgen screening model

Settore Scientifico Disciplinare BIO13

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RATIONALE

During the first part of the PhD program, it was investigated the role of G-1, a new ligand of the novel Estrogen receptor coupled to G-Protein (GPER) in the progression of adrenocortical carcinoma (ACC). The second part of the program was performed in the University of Michigan (Ann Arbor,USA) where it was completed the first project using Microarray tachnology and was performed a second line aimed to develop a novel cell-based androgen screening model.

GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth in vitro and in vivo

Adrenocortical tumors (ACTs) are common, and most are benign adrenocortical adenomas (ACAs). Malignant adrenocortical carcinoma (ACC) is a rare tumor type and is observed at the rate of one or two cases per million annually. ACTs are classified as either ACAs or ACCs by histopathologic methods that are based on nine Weiss scoring criteria, including the nuclear grade, mitotic rate, presence of necrosis, and others (Erickson et al., 2014). The molecular genetics of adrenocortical tumors remain poorly understood. For decades, molecular studies relied on a small number of samples and were directed to candidate genes. This approach, based on the elucidation of the genetics of rare genetic syndromes in which adrenocortical tumors are a manifestation, has led to the discovery of major dysfunctional molecular pathways in adrenocortical tumors, such as the IGF pathway , the Wnt pathway and TP53(Fassnacht et al., 2013). Interestingly, allelic losses (LOH) at the TP53 locus (17p13) are very frequent and observed in more than 85% of ACC (Bertherat and Bertagna, 2009). Transcriptome analysis suggests also that the Wnt/beta-catenin signalling pathway is activated in ACT. About a third of ACC harbours somatic activating mutations of the betacatenin gene (Bertherat and Bertagna, 2009; Logie et al., 1999). The most consistent and dominant genetic changes in ACC is the perturbation of the insulin-like growth factor II (IGF-II) locus (11p15) that is imprinted. IGF-II is over-expressed in 90% of ACCs determining an autocrine mitogenic effect (Sampaoli et al., 2012). The direct involvement of IGF-II/IGF-IR

system in adrenocortical tumor cell proliferation has been also shown in vitro using adrenal cancer cell line NCI H295R (Logie et al., 1999). Moreover, increased levels of the IGF-IR have been found in advanced human ACC, suggesting an important role for the IGF system in adrenocortical carcinogenesis. For this reason inhibitors for IGF-IR are currently in preclinical trials. However, ACC is a disease extremely heterogeneous and this new pharmacological approach could not be enough for the therapy of all forms of ACC, since several molecular mechanisms trigger ACC development. Thus, progress in the understanding of the pathophysiology of ACC is important to improve diagnosis, prognostic evaluation and treatment of different types of ACC. Usually, ACC are more frequent in women than in men, especially in those exposed to estro-progestin (Barzon et al., 2003; Hsing et al., 1996).

Professor Pezzi's group have demonstrated that ACC are characterized by ERα up-regulation and aromatase (the enzyme involved in the production of estrogens using androgens as substrate) over-expression (Barzon et al., 2008) and that estradiol enhances proliferation of the human adrenocortical carcinoma cell line H295R, whereas antiestrogens upregulate ERβ and inhibit ACC cell growth (Montanaro et al., 2005a).

It is well known that tamoxifen and its active metabolite 4-hydroxytamoxifen (OHT), not only exert antiestrogenic activity [9], but also act as full agonist on the G protein-coupled estrogen receptor GPER (from the GPER gene) (Lappano et al., 2013; Vivacqua et al., 2006a). GPER can mediate rapid E2-induced non-genomic signaling events, including stimulation of adenylyl cyclase, mobilization of intracellular calcium (Ca2+) stores and activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (Ariazi et al., 2010; Prossnitz and Barton, 2009). GPER exhibits prognostic utility in endometrial (Smith et al., 2007), ovarian (Smith et al., 2009), and breast cancer (Filardo et al., 2006) and can modulate growth of hormonally responsive cancer cells (Vivacqua et al., 2006b). Expression of GPER has been characterized in the outer zona glomerulosa (ZG) and in the medulla of the human adrenal (Baquedano et al., 2007), however its expression status in ACC is not known.

A non-steroidal, high-affinity GPER agonist G-1 (1-[4-(6-bromobenzo [1, 3]dioxol-5yl)-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta-[c]quinolin-8-yl]-ethanone) has been developed to dissect GPER-mediated estrogen responses from those mediated by classic estrogen

receptors (Bologa et al., 2006). The biological effects triggered by G-1 appear cell type specific and dependent on the ERs expression pattern (Chimento et al., 2013a; Chimento et al., 2013b; Chimento et al., 2012; Chimento et al., 2010; Chimento et al., 2011). Starting from these observation, by using G-1, we investigated the effects of GPER activation on ACC growth.

Development of a novel cell based androgen screening model

Androgens are hormones that play an essential role in the differentiation and maintenance of primary and secondary male sexual characteristics (Gao et al., 2005). The two main human androgens are testosterone (T), which is involved in the initial virilization phases of the human male embryo, and 5α-dihydrotestosterone (DHT), which is the active hormone in most androgen target tissues (Wiener et al., 1997). T is mainly synthesized by the testicular Leydig cells, in peripheral tissues, as well as to a lesser degree in ovaries and adrenals. T is converted to DHT by 5α-reductases and also can be converted to estradiol by aromatase. DHT is the most active physiologic androgen, inducing ten-fold higher androgen receptor (AR, *NR3C4*) bioactivity than T (Paris et al., 2002; Raivio et al., 2002). In addition, other endogenously produced steroids exhibit various degrees of androgenic activity (Mitchell, 2012; Rege et al., 2013). Several synthetic androgen-related compounds (AR agonists and antagonists) have also been developed to modulate androgen signaling in therapeutic settings (Fang et al., 2003; Larsson et al., 2011).

Androgens mediate their effects through binding and activation of the AR. AR is a member of the steroid nuclear receptor superfamily (Kato and Fujiki, 2008) and acts as a ligand-dependent transcription factor (Lubahn et al., 1988). Among this family, five steroid receptors are known: estrogen (ESR, *NR3A1*), progesterone (PR, *NR2C3*), androgen, mineralocorticoid (MR, *NR3C2*) and glucocorticoid (GR, *NR3C1*) receptors. AR activates a wide range of target genes that encode proteins and noncoding RNAs, including regulatory microRNA species (Narayanan et al., 2010).

Similar to the other steroid receptors, unbound AR is located in the cytoplasm. Upon ligand binding, AR goes through a series of conformational changes, dimerization and translocation to the nucleus, which is mediated by a nuclear localization signal.

Translocated AR binds to androgen response elements (ARE). These ARE are characterized by a consensus (or near consensus) sequence 5'-TGTTCT-3', which is located in the promoter or enhancer regions of AR gene targets. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for GR, MR and PR. The similarity of the response element for AR and the other steroid receptors, and particularly the wide-spread expression of the GR, has been problematic in the development of selective receptor screening assays.

The determination of androgen levels or the discoveries of new androgenic compounds are key elements for the diagnosis of a number of diseases in children and adults. Assays that detect bioactive serum androgens in a sensitive and selective manner benefit the diagnosis and treatment of several pediatric endocrine disorders, such as precocious puberty and ambiguous genitalia. In addition, androgen bioassays provide a screening tool for androgen abuse and endocrine disruptors (Bagchi Bhattacharjee and Paul Khurana, 2014). Over the past 10 years, several bioassays were developed using different methods (Campana et al., 2015). One of the first assays developed relied on a chloramphenicol acetyltransferase (CAT) reporter model (Xu et al., 2008b). This system was limited by experimental variation due to the transient nature of transgene expression. A luciferase reporter bioassay, using MDA-MB453 cells, was developed by Wilson et al (Wilson et al., 2002b). The major caveat of this assay was that it responds to AR as well as to GR agonists. Other androgen-reporter cell lines were developed but most of them were transiently transfected (Kim et al., 2006a; Sun et al., 2007; Vinggaard et al., 1999). Transient transfection assays (He et al., 2000) can provide similar information with stable assays but may not reflect endogenous levels of receptor. A stable expression of AR in the cells can eliminate the need for repetitious transient transfections, reduce the variability associated with these transient assays and moreover be utilized for high-throughput studies. Until now, a selective androgen-responsive transcriptional activation assay has not been widely available.

The aim of this study was to develop a stable cell-based *in vitro* bioassay that expresses the human AR (hAR) gene with sensitive and selective reporter readout. For this purpose, a stable cell line was made with CV1 cells stably transduced with hAR and an MMTV promoter-driven *luciferase* reporter gene. The resulting model is selective for

androgens and does not exhibit reporter activation by other steroid receptors. In addition the model appears useful to determine circulating androgenic bioactivity in human serum samples.

Background

1. Human adrenal gland

1.1 The adrenal gland: general structure

In mammals, the adrenal glands (also known as suprarenal glands) are endocrine glands that sit at the top of the kidneys (Figure 1.1).

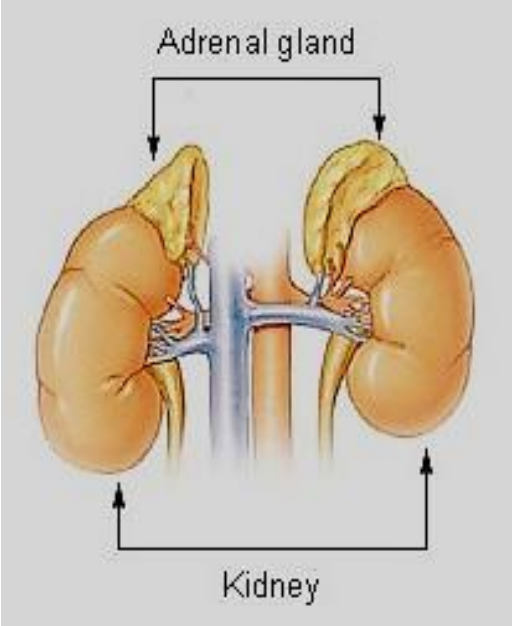


Figure 1.1 Human adrenal gland

The adrenal glands are located bilaterally in the retroperitoneum superior and slightly medial to the kidneys. In humans, the right adrenal gland is triangular in shape, whereas the left adrenal gland is semilunar in shape; in non-humans, they are quadrilateral in shape. The combined weight of the adrenal glands in an adult human ranges from 7 to 10 grams. They are surrounded by anadipose capsule and renal fascia.

It is now known that the adrenal gland consists of two ontogenetically, structurally and functionally distinct endocrine tissues, the cortex and the medulla. The cortex is mesodermal in origin and derived from proliferation of the coelomic epithelium. It produces various steroids with specific functions as will be described later. The medulla, on the other hand, is ectodermal in origin and neural crest-derived. It secretes catecholamines, *i.e.*, adrenaline and noradrenaline, that facilitate the acute mammalian stress or "fight-or-flight" response.

The adrenal glands affect kidney function through the secretion of aldosterone, and recent data suggest that adrenocortical cells under pathological as well as under physiological conditions show neuroendocrine properties; within normal adrenal glands, this neuroendocrine differentiation seems to be restricted to cells of the zona glomerulosa and might be important for an autocrine regulation of adrenocortical function.

Adrenocortical cells, on the other hand, are of mesodermal origin and synthesise steroid hormones that regulate body homeostasis and mediate chronic stress responses, as part of the endocrine hypothalamic-pituitary-adrenal (HPA) axis and renin-angiotensin system.

1.2 Embriology and development

The adrenal gland is two distinct endocrine organs that have separate embryological origins and physiologic functions ; the mesoderm-derived cortex secretes steroid hormones while the neural crest-derived medulla secretes catecholamines (Else and Hammer, 2005). Formation of the adrenal gland occurs in several distinct developmental events (Else and Hammer, 2005; Kim and Hammer, 2007) (Figure 1.2). During the 4th week of gestation in humans (E9.0 in mice), proliferation of mesoderm-derived cells of the coelomic epithelia and underlying mesonephros results in coalescence of the adrenogonadal primordium (AGP), defined by expression of the nuclear receptor NR5a1 (Steroidogenic factor 1, Sf1) (Hatano et al., 1996; Luo et al., 1994). At the 8th week of gestation in humans (E10.5 in mice), the bipotential AGP separates into discrete adrenal primordia (fetal adrenal zone) and gonadal primordial (Hatano et al., 1996; Kim and Hammer, 2007). The segregation of a discrete adrenal primordia from the AGP involves aWilm's tumor 1 (Wt1) and Cited2-mediated upregulation of Sf1 expression (Val et al., 2007). Once separated from the AGP, the adrenal primordial activates Sf1 expression through an entirely different mechanism – the recruitment of the homeobox protein PKNOX1 (Prep1), homeobox gene 9b (Hox) and pre B-cell leukemia transcription factor 1 (Pbx1) to a fetal adrenal-specific Sf1 enhancer (FAdE) (Zubair et al., 2008). Sf1 itself maintains FAdE-dependent expression of Sf1 in the adrenal primordia over time through autoregulation of Sf1 expression. Proliferation of fetal adrenocortical cells is

believed to be under control of fetal pituitary-derived adrenocorticotropic homormone (ACTH) (Mesiano et al., 1997). However, insulin like growth factor 2 (IGF2) is expressed throughout the fetal adrenal cortex and several studies have suggested ACTH mediates some of its effects on proliferation through IGF2 action (Coulter, 2005; Ilvesmaki et al., 1993; Stratta et al., 2003). Concurrent with activation of FAdE-driven Sf1 expression at embryonic day E11.5-12.5 in mice (equivalent to 8–9th week of gestation in humans), neural-crest-derived chromaffin progenitor cells migrate into the central fetal gland. These cells form the adrenal medulla followed by the coalescence of the mesenchymal capsule around the fetal adrenal gland (Else and Hammer, 2005). Before encapsulation is complete, the development of the definitive cortex (definitive zone or adult cortex) is initiated between the capsule and the fetal zone. While the fetal cortex ultimately regresses in all species, the timing of regression is species-specific; in humans the fetal zone regression occurs at birth while in mice the zone persists until puberty in males or the first pregnancy in females (Kim et al., 2009). In humans, functional zonation of the adult cortex into unique concentric steroidogenic regions initiates at birth concurrent with the coalescence of the adrenal medulla (Beuschlein et al., 2002).

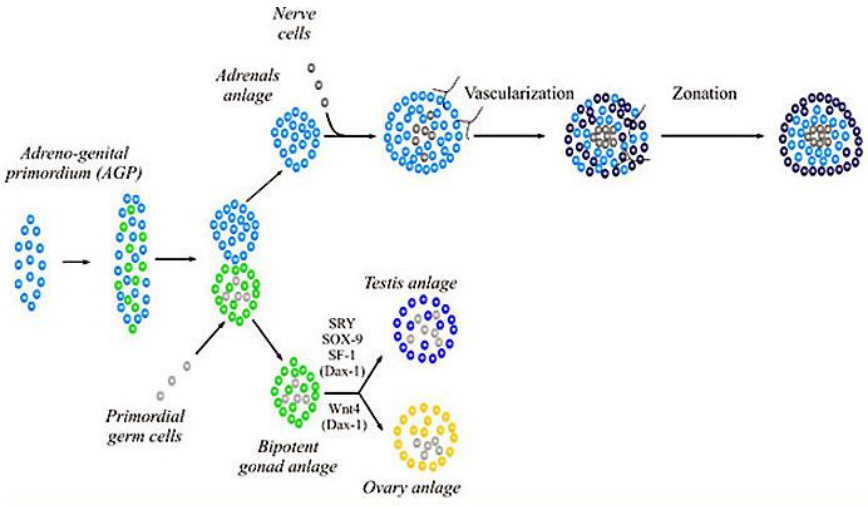


Fig. 1.2 Adrenal and ganad early development

1.3 Histology

The adrenal cortex is composed of three functionally distinct regions, the *zona glomerulosa* (ZG) lying immediately below the capsule and corresponding to approximately 15% of cortical volume characterized by cells organised in rounded clusters around capillary coils or glomeruli, *zona fasciculata* (ZF) corresponding to up to 75% of cortical volume, characterized by cells arranged in radial rows separated by trabeculae and by blood vessels and *zona reticularis* (ZR) that lies next to the medulla, in which cells are located within a uniform reticular net of connective tissue and blood vessels (Miller WL, 2008). The ZG synthesizes mineralocorticoids; the ZF produces cortisol and the ZR secretes the so called adrenal androgens, DHEA and DHEA-sulfate. Each zone is preferentially regulated by different circulating factors that include angiotensin II (Ang II) and potassium (K+) for the ZG, adrenocorticotropic hormone (ACTH) for the ZF, and ACTH plus other yet to be determined factors for the ZR (Wang and Rainey, 2012) (Figure 1.3). It has been established that the reason each zone secretes a unique set of steroids is related to the selective expression of steroid-metabolizing enzymes within each zone (Nguyen and Conley, 2008; Rainey, 1999; Rainey et al., 2002). However, the molecular mechanisms that cause zone-specific expression patterns of enzymes are yet to be resolved. Adrenal steroid production remains an area of active research, which supports the need to develop appropriate cell models that can mimic adrenal physiology or pathology. Primary cultures of adrenocortical cells have proven to be useful for examining the mechanisms controlling many aspects of adrenal physiology (Cardoso et al., 2009; Chen and Hornsby, 2006; Kuulasmaa et al., 2008; Xing et al., 2011; Xing et al., 2010). However, several issues have limited the use of primary adrenal cells as in vitro models. The most common limitations are the constant requirement for fresh tissue and the difficulties associated with the isolation of adequate cortical cells. In addition, cells from different human donors are subject to considerable variability; whereas cells from rodents do not produce cortisol or adrenal androgens due to the lack of steroid 17ahydroxylase (CYP17) expression. To overcome the problems with tissue accessibility and quality, many groups have attempted to establish cell lines from adrenocortical carcinomas. This approach has been somewhat successful leading to adrenal cell lines from several

species and we have previously reviewed the overall development of these models (Mountjoy et al., 1994; Rainey et al., 2004).

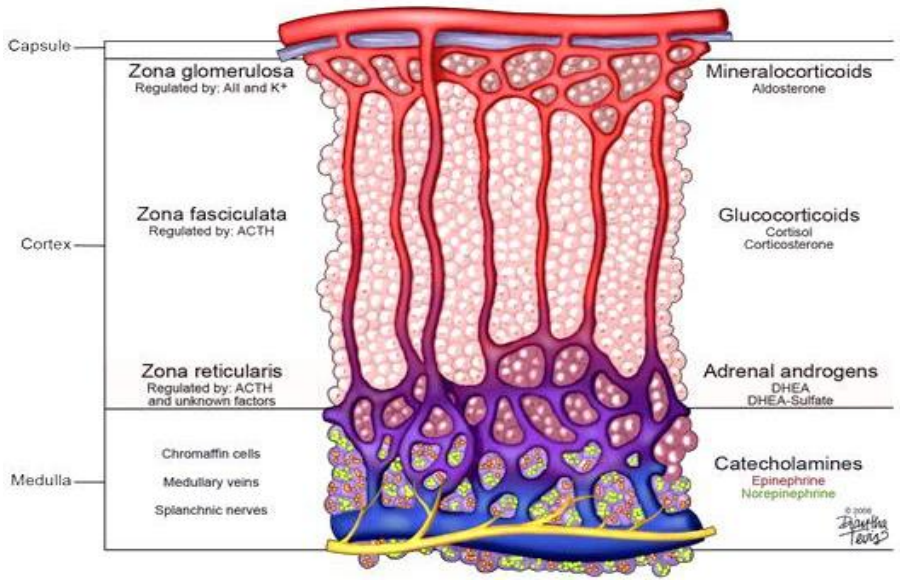


Fig. 1.3 Adrenal cortex regions

1.4 Adrenalcortical steroidogenesis

Steroid hormones regulate a wide variety of developmental and physiological processes from fetal life to adulthood. Steroid hormones are all synthesized from cholesterol and hence have closely related structures based on the classic cyclopentanophenanthrene 4-ring structure. The human adrenal can synthesize cholesterol *de novo* from acetate (Mason and Rainey, 1987), but most of its supply of cholesterol comes from plasma low-density lipoproteins (LDLs) derived from dietary cholesterol (Gwynne and Strauss, 1982). By contrast, rodent adrenals derive most of their cholesterol from high-density lipoproteins via a receptor termed scavenger receptor B1, but this pathway appears to play a minor role in human steroidogenesis. The intracellular cholesterol economy is largely regulated by the sterol response element binding protein (SREBPs), a group of transcription factors that regulate genes involved in the biosynthesis of cholesterol and fatty acids (Horton et al., 2002). Adequate concentrations of LDL will suppress 3-hydroxy-3-methylglutaryl co-enzyme A reductase, the rate-limiting enzyme in cholesterol synthesis. ACTH also stimulates the activity of 3-hydroxy-3-methylglutaryl

co-enzyme A reductase, LDL receptors, and uptake of LDL cholesterol. LDL cholesterol esters are taken up by receptor-mediated endocytosis, and are then stored directly or converted to free cholesterol and used for steroid hormone synthesis (Brown et al., 1979).

The first step in steroidogenesis takes place within mitochondria. The mechanisms by which cholesterol is transported to and loaded into the outer mitochondrial membrane (OMM) remain an active area of research (Chang et al., 2006; Miller, 2007); the principal action of StAR is to facilitate the movement of cholesterol from the OMM to the inner mitochondrial membrane (IMM). Some cholesterol may be incorporated into vesicular membranes that then fuse with other membranes, thus delivering cholesterol from one intracellular compartment to another, but this appears to be a minor pathway (Soccio and Breslow, 2004). Instead, cholesterol is solubilized by binding to proteins.

A steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands (Claahsen-van der Grinten et al., 2011; White and Bachega, 2012). Impaired cortisol and aldosterone production increases adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism.

Most enzymes involved in steroid biosynthesis are either cytochrome P450s (CYPs) or HSDs. These steroidogenic enzymes are functionally, if not absolutely, unidirectional, so the accumulation of products does not drive flux back to the precursor. All P450-mediated hydroxylations and carbon-carbon bond cleavage reactions are mechanistically and physiologically irreversible (Hall, 1986) (Figure 1.4).

Cytochrome P450 is a generic term for a group of oxidative enzymes, all of which have about 500 amino acids and contain a single heme group (Gonzalez, 1988). The human genome includes genes for 57 cytochrome P450 enzymes (Lander et al., 2001; Venter et al., 2001). The genes are now formally termed *CYP* genes. Seven human cytochrome P450 enzymes are targeted to the mitochondria and are termed “type 1”; the other 50

human P450 enzymes are targeted to the endoplasmic reticulum and are termed "type 2." All P450 enzymes activate molecular oxygen using their heme center and add electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). Type 1 enzymes receive electrons from NADPH via a flavoprotein termed ferredoxin reductase and a small iron-sulfur protein termed ferredoxin, whereas type 2 P450 enzymes receive electrons from NADPH via a single 2-flavin protein termed P450 oxidoreductase (POR) (Miller, 2005). Six P450 enzymes are involved in steroidogenesis

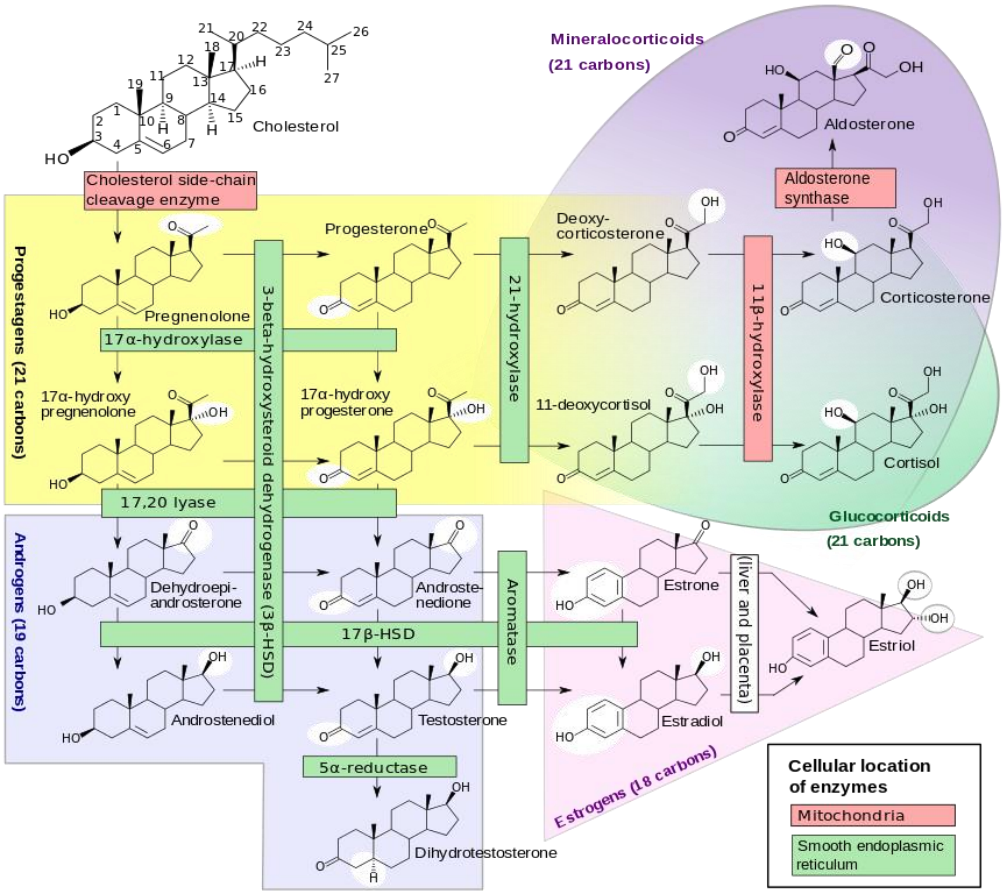


Fig. 1.4 Major human steroidogenic pathways

Mitochondrial P450scc is the cholesterol side-chain cleavage enzyme catalyzing the series of reactions formerly termed "20,22 desmolase." The two isozymes of mitochondrial P450c11, P450c11β (11β-hydroxylase) and P450c11AS (aldosterone synthase), catalyze 11β-hydroxylase, 18-hydroxylase, and 18-methyl oxidase activities. In the endoplasmic reticulum, P450c17 catalyzes both 17α-hydroxylase and 17,20-lyase

activities, P450c21 catalyzes 21-hydroxylation in the synthesis of both glucocorticoids and mineralocorticoids, and P450arom catalyzes aromatization of androgens to estrogens.

The HSDs have molecular masses of about 35 to 45 kDa, do not have heme groups, and require nicotinamide adenine dinucleotide (phosphates) ($NADH/NAD^+$ or $NADPH/NADP^+$) as cofactors; Based on their activities, it is physiologically more useful to classify the HSDs as dehydrogenases or reductases. The dehydrogenases use NAD^+ as their cofactor to oxidize hydroxysteroids to ketosteroids, and the reductases mainly use NADPH to reduce ketosteroids to hydroxysteroids (Agarwal and Auchus, 2005; Sherbet et al., 2007)

1.5 The steroidogenic regulatory protein

Unlike cells that produce polypeptide hormones, which store large amounts of hormone in secretory vesicles ready for rapid release, steroidogenic cells store very little steroid. Thus, a rapid steroidogenic response (*e.g*., adrenal secretion of aldosterone and cortisol in response to stress or the "pulsing" of sex steroids in response to an LH surge) requires rapid synthesis of new steroid. ACTH promotes adrenal steroidogenic cell growth. This growth occurs primarily by ACTH stimulating the production of cAMP, which in turn promotes the synthesis of IGF-II (Mesiano et al., 1993; Voutilainen and Miller, 1987), basic fibroblast growth factor (Mesiano et al., 1991), and epidermal growth factor (Coulter et al., 1996). Together, these growth factors stimulate adrenal cellular hypertrophy and hyperplasia, determining the amount of steroidogenic tissue. Second, acting over days, ACTH acts through cAMP, and angiotensin II acts through the calcium/calmodulin pathway to promote the transcription of genes encoding various steroidogenic enzymes and electron-donating cofactor proteins, thus determining the amount of steroidogenic machinery in the cell. Third, ACTH rapidly stimulates StAR gene transcription (Stocco et al., 2005) and phosphorylation of Ser195 in extant StAR (Arakane et al., 1997) to increase the flow of cholesterol from the OMM to the IMM, where it becomes substrate for the first and rate-limiting enzyme, P450scc. This acute response occurs within minutes and is inhibited by inhibitors of protein synthesis (*e.g*., puromycin or cycloheximide), indicating that a short-lived protein species mediates this

process. All microsomal (type 2) cytochrome P450 enzymes, including steroidogenic P450c17, P450c21, and P450aro, receive electrons from POR, a membrane-bound flavoprotein that is a different protein from the mitochondrial flavoprotein, ferredoxin reductase (Miller, 2005). Nuclear magnetic resonance and x-ray scattering data have recently confirmed this view that POR undergoes these dramatic conformational changes while receiving and then transferring electrons (Ellis et al., 2009)

1.5.1 StAR structure and mechanism of action

The short half-life of the 37-kDa cytoplasmic precursor and the longer half-life of the "mature" 30-kDa intramitochondrial form of StAR initially suggested that the 30-kDa form was the biologically active moiety. When expressed in cytoplasm or added to mitochondria *in vitro*, both the 37- and 30-kDa forms of StAR are equally active (Arakane et al., 1996). When StAR is immobilized on the OMM, it is constitutively active, but StAR is inactive when localized to the mitochondrial intramembranous space or to the matrix (Bose et al., 2002). These data demonstrate that StAR acts exclusively on the OMM (Arakane et al., 1996; Bose et al., 2002), and its activity in promoting steroidogenesis is proportional to its residency time on the OMM (Bose et al., 2002). The interaction of StAR with the OMM involves conformational changes (Baker et al., 2005; Bose et al., 1999) that are necessary for StAR to accept and discharge cholesterol molecules. Although StAR can transfer cholesterol between synthetic membranes *in vitro(Tuckey et al., 2008)*, suggesting that other protein molecules are not needed for its action, this activity can also be seen with the inactive mutant R182L, which is biologically inactive and causes lipoid CAH (Baker et al., 2007). Thus StAR's action to promote steroidogenesis is distinct from its cholesterol-transfer activity.

1.5.2 P450scc

A cell is said to be steroidogenic if it expresses the cholesterol side-chain cleavage enzyme, P450scc, which catalyzes the first step in steroidogenesis. Conversion of cholesterol to pregnenolone in mitochondria is the first, rate-limiting, and hormonally regulated step in the synthesis of all steroid hormones (Koritz and Kumar, 1970; Macchi and Hechter, 1954). This process involves three distinct chemical reactions, the 22-

hydroxylation of cholesterol, 20-hydroxylation of 22(R)-hydroxycholesterol, and oxidative scission of the C20–22 bond of 20(R),22(R)-dihydroxycholesterol (the side-chain cleavage event), yielding pregnenolone and isocaproaldehyde. P450scc can use the hydroxysterol intermediates directly as substrate, providing a useful experimental tool because these hydroxysterols are somewhat water-soluble and do not require StAR for access to P450scc. The reactions catalyzed by P450scc are slow, with a net turnover number of about six (Kuwada et al., 1991) to 20 (Tuckey and Cameron, 1993) molecules of cholesterol per molecule of P450scc per second. In human adrenal gene transcription is regulated by ACTH, by gonadotropins in testis and ovary and by unknown factcors in placenta all activated through cAMP as intracellular second messenger (Kimura and Suzuki, 1967). Each catalytic cycle requires a molecule of NADPH and one molecule of oxygen (Figure 1.5).

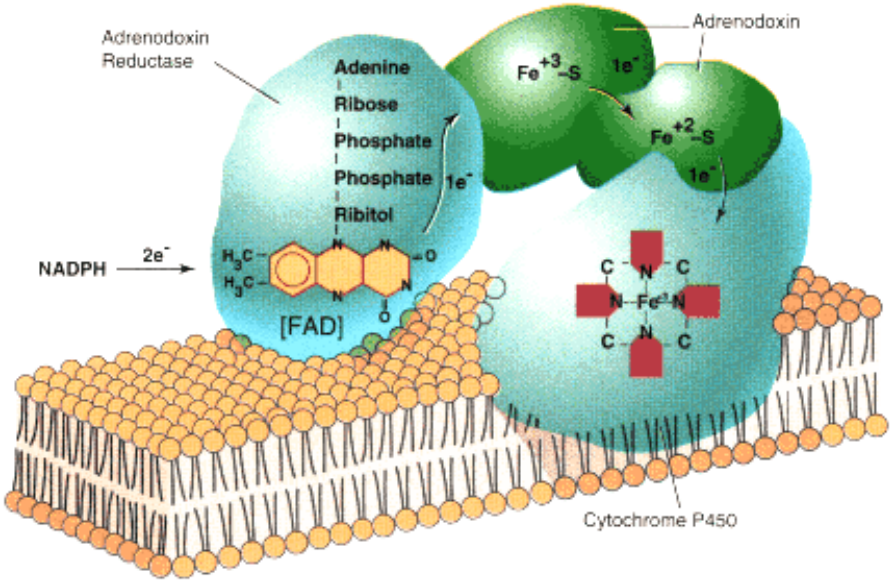


Fig 1.5 Electron transport to mitochondrial forms of cytochrome P450

1.5.3 P450c17: 17α-Hydroxylase/17,20-Lyase

P450c17 is the microsomal P450 enzyme that catalyzes both 17α-hydroxylase and 17,20-lyase activities, principally in the adrenal and gonads. These two activities were once thought to be catalyzed by separate enzymes that differed in the adrenals and gonads. Clinical observations showed that adrenal 17α-hydroxylase activity (reflected by serum cortisol concentrations) was fairly constant throughout life, This dissociation

between adrenal secretion of 17α-hydroxylase products (cortisol) and 17,20-lyase products (DHEA) suggested that distinct enzymes performed the two transformations. the distinction between 17α-hydroxylase and 17,20-lyase is functional and not genetic or structural. P450c17 is encoded by a single gene on chromosome 10q24.3 (Fan et al., 1992; Matteson et al., 1986), which is expressed in the adrenals and gonads (Chung et al., 1987), and not two tissue-specific isozymes as had been thought. This gene, formally called *CYP17A1*, is structurally related to the gene for P450c21 (21-hydroxylase) (Picado-Leonard and Miller, 1987). These 17-hydroxylated steroids then can be cleaved to give C17/20 DHEA and androstenedione, respectively. When the $P450_{C17}$ is absent, as in the zona glomerulosa, the products are C-21 17-deoxy steroids such as aldosterone. When the activity of 17-α-hydroxylase is present products are C-21 17-hydroxysteroids such as cortisol. Instead, when there are 17-α-hydroxylase and 17, 20 $P450_{C17}$ liasica activities the products are C-19 precursors of sex steroid hormones.

1.5.4 Cytochrome b_5 and P450c21 (Steroid 21-Hydroxylase)

Cytochrome b_5 is a small (12–17 kDa) hemoprotein found as a membrane-bound protein in liver and as a soluble protein lacking the C-terminal membrane anchor in erythrocytes. Cytochrome b_5 is expressed in both the adrenals and gonads, where it can interact with P450c17; the adrenal expression is specific to the zona reticularis and may contribute to the genesis of adrenarche (Endoh et al., 1996; Nguyen et al., 2008; Suzuki et al., 2000); Although cytochrome b_5 can receive electrons from POR, the redox potentials of cytochrome b_5 and one electron-reduced P450 are unfavorable for cytochrome b_5-to-P450 electron transfer.

The locus containing the *CYP21* genes is among the most complex in the human genome and explains why 21-hydroxylase deficiency is one of the most common autosomal-recessive diseases. The human P450c21 protein, found only in the adrenals, is a microsomal P450 that employs the same POR used by P450c17 to transport electrons from NADPH. Much less is known about the enzymology of P450c21 than of P450c17, but the available evidence suggests that, unlike P450c17, P450c21 is not very sensitive to the abundance of POR or cytochrome b_5.

1.5.5 Isozymes of P450c11 and Isozymes of 17β-Hydroxysteroid Dehydrogenase

The final steps in the synthesis of glucocorticoids and mineralocorticoids are catalyzed by two closely related mitochondrial enzymes, P450c11β and P450c11AS (Fardella and Miller, 1996; White and Pascoe, 1992). P450C11 is located in the inner mitochondrial membrane. The human genome has two P450 genes located on chromosome 8 between bands q13 and q22 (Kawainoto et al., 1990). These two human isozymes are encoded by tandemly duplicated genes on chromosome 8q21–22 that have 93% amino acid sequence identity (Mornet et al., 1989). P450C11 is encoded by the gene CYP11B1; it is significantly expressed in the fasciculata zone and is the only with 11-β-hydroxylase activity. Disorders of P450c11AS cause aldosterone synthase deficiency, formerly termed corticosterone methyl oxidase (CMO) deficiencies, in which aldosterone biosynthesis is impaired whereas the zona fasciculata and reticularis continue to produce corticosterone and DOC.

Multiple reactions are catalyzed by a group of enzymes collectively known as the 17β-hydroxysteroid dehydrogenases (17βHSDs), sometimes also termed 17-oxidoreductases or 17-ketosteroid reductases (Labrie et al., 1997; Moghrabi and Andersson, 1998). These reactions included the interconversions of androstenedione and testosterone, DHEA and androsta-5-ene-3β,17β-diol, estrone and estradiol, androsterone and 5α-androstane-3α,17β-diol, 5α-androstanedione and 5α-DHT, and others.

1.5.6 P450arom: Aromatase

Estrogens are produced by the aromatization of androgens by a complex series of reactions catalyzed by a single microsomal aromatase, P450aro (Grumbach and Auchus, 1999; Simpson et al., 2002; Simpson et al., 1994). This typical cytochrome P450 is encoded by a single gene on chromosome 15q21.1. This gene uses several different promoter sequences, transcriptional start sites, and alternatively chosen first exons to encode aromatase mRNA in different tissues under different hormonal regulation. The *CYP19A1*gene for P450aro spans over 75 kb (Mahendroo et al., 1991) and contains five different transcriptional start sites (Mahendroo et al., 1993) with individual promoters that permit the tissue-specific regulation of its expression in diverse tissues. P450aro is a glycoprotein, but glycosylation *per se* does not appear to affect activity

(Shimozawa et al., 1993). The p450aro oxidative demethylation action of C_{19} steroids, mainly androstenedione and testosterone, consumes three equivalents of molecular oxygen and NADPH, yielding formic acid and C_{18}-steroids with an aromatic A-ring (Simpson et al., 1994).

1.5.7 Isozymes of 5α-Reductase

The 5α-reductases are important beyond the context of male genital differentiation and androgen action because both isozymes reduce a variety of steroids in degradative pathways. Progesterone, 17OHP, and related C_{21}steroids are excellent substrates for both 5α-reductases, particularly the type 1; cortisol, cortisone, corticosterone, and related compounds are also good substrates (Frederiksen and Wilson, 1971). Such 5α- (and 5β-) reduced steroids may be metabolized further and conjugated for excretion in the urine. Inhibitors of the type 2 enzyme have been developed for the treatment of prostatic hyperplasia and the prevention of its recurrence after surgery(McConnell et al., 1998): finasteride selectively inhibits human 5α-reductase type 2, whereas dutasteride inhibits both isoenzymes. These drugs are approved for treatment of prostatic hyperplasia in the United States.

1.5.8 3βHSD

Once formed, pregnenolone can be converted into 17- idrossipregnenolone by P450C17 or in progesterone by 3-β-hydroxysteroid dehydrogenase Δ4-5Δ isomerase, encoded by the HSD3B gene.

This enzyme presents two activities: 3-β-hydroxysteroid dehydrogenase and isomerase activities.

In humans there are at least two forms of HSD3B, encoded by different genes:
- the gene for HSD3B type I (HSD3B1) is expressed in placenta, skin, mammary gland;
- the gene for HSD3B type II (HSD3B2) is expressed in adrenal glands and gonads.

Both genes are on band p13 of chromosome 1 (Berube et al., 1989).

2. Adrenocortical cancers

2.1 Introduction

Primary carcinoma of the adrenal cortex (Adrenocortical Carcinoma, ACC) is a rare and highly aggressive cancer with a frequently dismal prognosis. It affects worldwide approximately 1–2 new patients per million people a year (Brennan, 1987; Copeland, 1984; Dackiw et al., 2001; Hutter and Kayhoe, 1966; Kebebew et al., 2006; Koschker et al., 2006; Lipsett et al., 1963; Lubitz et al., 1973; Reibetanz et al., 2012), accounting for 0.2% of cancer-related deaths in the United States (Hutter and Kayhoe, 1966; Lipsett et al., 1963). Due to the high and increasing incidence of benign adrenal lesions and incidentalomas, differential diagnosis becomes essential but it's not ever clear preoperatively (Hutter and Kayhoe, 1966) and (Schulick and Brennan, 1999a). With respect to the ability of hormone production, ACCs can be functioning or non-functioning tumors (Bertagna et al., 2008; Schulick and Brennan, 1999b). The rarity of the disease and its dismal prognosis require a multidisciplinary approach to improve results (Bertagna et al., 2008). Indeed diagnosis is often delayed, many patients present at advanced stages and the tumor is quite unresponsive to chemotherapy (Cohn et al., 1986; Crucitti et al., 1996; Wajchenberg et al., 2000). Recurrences, both local and metastatic, are reported in up to 85% of patients after resection (Pommier and Brennan, 1992; Stojadinovic et al., 2003), and overall the prognosis remains poor, with a 5-year survival rate of 16%–47% (Allolio et al., 1989; Dackiw et al., 2001; Fassnacht et al., 2009; Wajchenberg et al., 2000). Radical surgical resection, avoiding tumor rupture, remains the mainstay of therapy and the most important prognostic factor (Kim et al., 2009; Lombardi et al., 2012; Matteson et al., 1986). The very low incidence of the disease has precluded several statistically significant studies that would be needed to improve the management of patients with ACCs. In fact most of recommendations are derived from retrospective series or expert opinions, whereas only few of them are based on prospective clinical trials.

Tumors that originate from the adrenal cortex can be divided into benign adenomas and malignant adenocarcinomas. They differ from other cancers because the cancer may be associated to an endocrine component (Allolio and Fassnacht, 2006).

Secreting forms are responsible for the onset of endocrine syndromes which vary depending on the type of hormone produced in excess:

• Cushing's syndrome, caused by hypersecretion of cortisol;

• Conn's syndrome, caused by aldosterone hypersecretion;

• hirsutism and virilization, caused by hypersecretion of androgens.

ACC can be asymptomatic or can present with symptoms of hormone excess or complaints referable to the mass (Brennan, 1987; Schulick and Brennan, 1999a). Generally ACC present an immature steroidogenesis and almost all of these tumors exhibit hormonal precursor excess but, approximately, 60% of all ACC patients will present with hormone-related signs and symptoms (so-called "functional tumors")(Schulick and Brennan, 1999a; Schulick and Brennan, 1999b).

Differential diagnosis between ACA and ACC is of pivotal clinical relevance, as the prognosis and clinical management of benign and malignant ACTs is entirely different. Imaging techniques including computed tomography, magnetic resonance imaging and positron emission tomography with ^{18}F-2-fluoro-2-deoxy-D-glucose (FDG-PET) can be used for assessing malignancy, but none of these techniques are absolutely reliable (Morelli et al., 2013; Terzolo et al., 2011). It is very difficult to establish malignancy in small adrenal tumors and to exclude it in large tumors with the available imaging techniques. Currently used guidelines propose to remove adrenal tumors with a diameter of >6 cm, as they are associated with a risk of malignancy >25% (Aron et al., 2012). Some hormonal features (eg, androgen secretion characteristic for malignant tumors) can also be exploited in diagnosis. Most recent data using urinary steroid hormone metabolomics showed characteristic patterns of steroid secretion and metabolism in ACC samples (Arlt et al., 2011). The histological diagnosis of malignancy is also often difficult (Patalano et al., 2009) and novel markers of malignancy are intensively searched for using bioinformatics approaches to establish an early and specific differential diagnosis between ACC and ACA.

2.2 Epidemiology

ACC is a rare solid tumor (Kebebew et al., 2006; Wajchenberg et al., 2000). The exact incidence is difficult to determine and most authors estimate an incidence of 1–2 per million population (Allolio et al., 1989; Barzon et al., 2003; Dackiw et al., 2001; Wajchenberg et al., 2000). In contrast, adrenal incidentalomas have a prevalence of at least 3% in a population >50 yr of age (ACC constitute <5% of all adrenal incidentalomas) (Barzon et al., 2003; Bovio et al., 2006; Grumbach et al., 2003; Mansmann et al., 2004; Song et al., 2007). However, ACC prevalence depends on the

size of the tumor, accounting for 2% of lesions <4 cm, 6% of lesions 4–6 cm, and 25% of lesions >6 cm. ACC affects women more commonly than men with a ratio of 1.5:1 (Bilimoria et al., 2008; Dy, 2013; Koschker et al., 2006; Linnard-Palmer, 2012; Roman, 2006; Wooten and King, 1993). Females with ACC are more likely to have functional tumors. Men with ACC tend to have functional tumors before the age of 20 years and non-functional tumors after the age of 40 years (Brennan, 1987; Cohn et al., 1986; Schulick and Brennan, 1999a). Some reports indicate a bimodal age distribution, with a first peak in childhood (<5 years) and a second higher peak in the fourth and fifth decades (Koschker et al., 2006; Schulick and Brennan, 1999a; Wajchenberg et al., 2000). In adults, the mean age of diagnosis is 45 years. The incidence of ACC is 10–15 times higher in children in southern Brazil, which is related to an inherited germline p53 mutation (Michalkiewicz et al., 2004; Ribeiro et al., 1998). Indeed, while ACC most frequently arises sporadically and without known pathogenesis, it has been also associated with a number of familial tumor syndromes, including multiple endocrine neoplasia type 1 or MEN-1 (mutation of the MEN1 tumor suppressor at 11q13), Li-Fraumeni syndrome (p53 mutation on 17p13), Beckwith–Wiedemann syndrome (alterations of gene clusters on 11p15.5 and 15q11–13), and Carney complex (mutation of PRKAR1A gene at 17q23–24 or mutations at 2p16) (Kjellman et al., 1999b; Libe et al., 2007).

2.3 Pathogenesis

The molecular mechanisms involved in adrenocortical tumorogenesis are still poorly understood. Recent study are focused on alterations of the insuline-like growth factor (IGF) system associated with thase tumors. For instance, there are abnormalities ate the 11p15 region, where the IGF-II gene maps, in more than 90% of malignant adrenocortical tumors (Figure 2.1) (Gicquel et al., 2001).

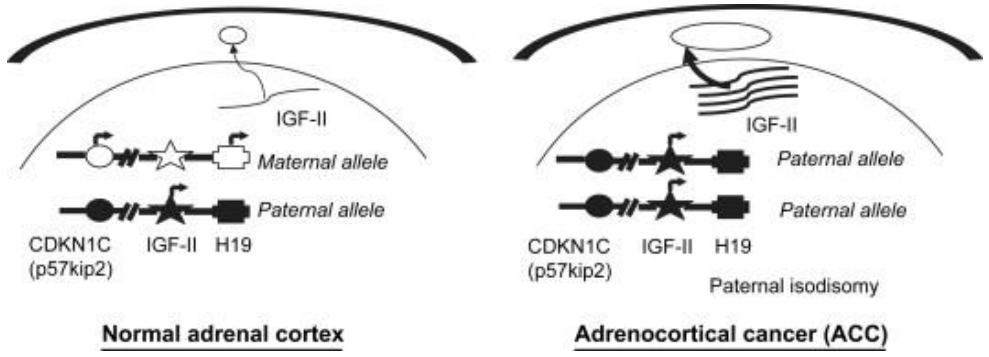


Fig. 2.1 Alterations of 11p15 locus and insulin-like growth factor II (IGF-II) over-expression in adrenocortical cancer (ACC)

The tumors with these abnormalities exhibit strong overexpression of IGF-II gene (Csernus et al., 1999)and large amounts of IGF-II protein (Gicquel et al., 2001; Listrat et al., 1999). The type 1 IGF receptor (Janssen et al., 1997; Wolf et al., 1997) and the IGF-binding protein-2 (IGFB-2) (Boulle et al., 1998) are also specifically overproduced in malignant adrenocortical tumors. These findings strongly implicate the IGF system in adrenocortical tumor progression. The IGF system comprises several elements. The IGFs, IGF-I and IGF-II, are small polypeptides produced in various tissues and cell cultures. They have endocrine and auto/paracrine modes of action (Gockerman et al., 1995). Two structurally different IGF receptors have been described, the type 1 IGF receptor mediating most effects of the IGFs and the IGF-II/mannose-6-phosphate (IGF-II M6P) receptor the function of which should be the internalization and subsequent degradation of IGF-II (Clemmons et al., 1995; Gockerman et al., 1995). IGF-I and IGF-II can also bind with high affinity to IGFBPs. Six high-affinity IGFBPs have been described to date (Clemmons et al., 1995). These IGFBPs modulate the effects of IGFs either positively or negatively depending on their abundance, their affinity for the growth factors and their cellular localization. IGF-II has been implicates in the growth of various tumors including Wilms' tumors, hepatomas, colon carcinomas and pheochromocytomas, suggesting the IGF-II plays a central role in tumorogenesis (Karnieli et al., 1996). Similarly, IGF-II may also be involved in adrenocortical tumors. However, direct proof for this role is lacking.

In adrenocortical carcinoma p53 expression is higher than in adenoma (McNicol et al., 1997) and mutations in the p53 gene were found more frequently in malignant tumors (Reinke V, 1997). Additionally, germ line mutations of p53 predispse to childhood adrenocortical cancer (Sameshima et al., 1992) (Wagner et al., 1994).

Wnt-signaling has recently been identified as a regulator of a number of endocrine functions in health and disease in addition to its original attribution to developmental biology. Wnts are extracellular ligands on frizzled receptors and on lipoprotein receptor-related protein co-receptors. Ligand binding leads eventually to the activation of intracellular signaling cascades; based on the involvement of the transcriptional co-activator beta-catenin it can be distinguished between canonical (i.e. beta-catenin) and non-canonical Wnt-signaling. Recent studies revealed that canonical Wnt-signaling regulates the function of endocrine organs and contributes to a number of endocrine disorders. Wnt signaling molecules can bind to cell-surface receptors called frizzled and to lipoprotein receptor-related protein (LRP) co-receptors (low density LRP). Frizzled receptors are G-protein-coupled seven-transmembrane receptors. Binding to both receptors activates the canonical Wnt-signaling pathway. By targeting a complex containing adenomatous polyposis coli (APC) and axin the activation of this pathway leads to an inhibition of glycogen synthase kinase-3 (GSK3B). This results eventually in the stabilization of β-catenin. Subsequently, β-catenin, a transcriptional co-activator, translocates to the nucleus to activate T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors on canonical Wnt target-genes. Originally, the Wnt signaling pathway has been identified as a regulator of embryogenesis and has thereafter been associated with tumor development (Logan and Nusse, 2004). In addition, the role of Wnt-signaling agonists and antagonists in adipocyte differentiation has been subjected to a number of studies since its original description in 2000 (Bennett et al., 2002; Christodoulides et al., 2009; Longo et al., 2002; You et al., 2002). This has been the evidence linking Wnt-signaling with metabolic regulation.

A mutation that leads to inactivation of the MEN1 germline is found in approximately 90% of families with multiple endocrine neoplasia type 1 (MEN1). Adrenocortical tumors and / or hyperplasia are observed in 25-40% of patients with MEN1 (Kjellman et al., 1999a; Yano et al., 1989). In most cases these are non-functional adrenocortical adenomas. Hyperplasia was found in a typical way in patients with MEN1 who have hypersecretion of ACTH (Cushing's syndrome), while the ACC has been reported rarely in patients with MEN1. The mutation of the MEN1 gene in somatic cells is very rare in adrenocortical tumors (Wales MM, 1995; Yano et al., 1989).

2.4 Adrenocortical adenoma

The adrenocortical adenoma is a benign neoplasm arising from adrenal cortical cells. Dimensions are variable depending on the hormone produced:

- adenoma with hyperaldosteronism is usually unilateral and of yellowish color, around 1.5 cm of size and non-enveloped;
- adenoma with hypercortisolism is unilateral, has dimensions of about 4 cm, is yellow-brown and is encapsulated;
- adenoma with virilization is unilateral, has dimensions of about 5 cm, is red-brown and is encapsulated.

It can present with Cushing's syndrome (it describes the signs and symptoms associated with prolonged exposure to inappropriately high levels of the hormone cortisol) or primary aldosteronism (it is characterized by the overproduction of the mineralcorticoid hormone aldosterone by the adrenal gland).

Is a well circumscribed, yellow tumour in the adrenal cortex, which is usually 2-5 cm in diameter. The color of the tumour, as with adrenal cortex as a whole, is due to the stored lipid (mainly cholesterol), from which the cortical hormones are synthesized. These tumors are frequent incidental findings at post mortem examination, and appear to have produced no significant metabolic disorder; only a very small percentage lead to Cushing's syndrome. Nevertheless, these apparently non-functioning adenomas are most often encountered in elder obese people. There is some debate that they may really represent nodules in diffuse nodular cortical hyperplasia. Very occasionally, a true adrenal cortical adenoma is associated with the clinical manifestations of Conn's syndrome, and can be shown to be excreting mineralocorticoids.

More frequent with advancing age, adrenocortical adenomas have a peak between 50 and 70 years and the most affected are women (58%) and the right side.

2.5 Adrenocortical carcinoma

ACC is a rare and highly aggressive malignancy with an annual incidence of 0.7–2.0 cases per million population (Kebebew et al., 2006; Kerkhofs et al., 2013). ACC can occur at any age, with a peak incidence between 40 and 50 years, and women are more often affected (55–60%). The incidence in children is particularly high in southern Brazil due to the high prevalence (0.27%) of a specific TP53 germline mutation (R337H) (Custodio et al., 2012). Histologically standpoint are detectable: solid or

trabecular areas with fibrous bands interposed between the tumor nodules, necrosis, the presence of large cells with vacuolated cytoplasm, nucleus atypical and hyperchromatic, prominent nucleoli, frequent mitosis, evidence of vascular and capsular invasion.

It is highly aggressive: about 60% of patients have metastases at diagnosis, with a 5-year survival rate of 8% for recurrent and inoperable disease.

Cushing's syndrome is most frequently associated with endocrine cancer. The therapeutic approach of choice for adrenocortical carcinoma is surgery. Surgery should be conducted only after appropriate preoperative diagnostic tests, including biochemical evaluation and imaging. In the setting of adrenal imaging characteristics not clearly excluding malignancy, surgeons are obligated to approach the resection as a cancer operation.

In patients with localized adrenal tumor, suspicious for ACC, surgical resection should be considered. Suspicious features to consider are: tumors size >4 cm, functional tumor, radiologic suspicious characteristics. For tumors invading surrounding tissue or organs, concomitant resection of kidney, liver, spleen, pancreas, stomach, colon and wall of the vena cava should be considered (Schteingart et al., 2005) even if, in primary ACCs, it is quite infrequent that the tumor invades the liver or adjacent kidney. Obviously this is not always predictable during surgery, as in our case in which the tumor was strongly adherent to the kidney, such as to look like a single mass, although histological examination has then denied the spread to the renal parenchyma. The evidence that patients with ACC remain at high risk for tumor recurrence despite complete surgical tumor excision has fueled the search for adjuvant therapies. Even with ostensibly complete resections, rates of local recurrence have typically ranged from at least 19% to 34% in those patients with no residual disease after surgery (Bellantone et al., 1997; Gonzalez et al., 2007). The role of cytotoxic chemotherapy is continuously under investigation. The recommended first-line cytotoxic treatment regimens are etoposide, doxorubicin, cisplatin plus mitotane (Berruti et al., 2005), or streptozotocin plus mitotane (Khan et al., 2000). Mitotane remains the only drug approved by the U.S. Food and Drug Administration and European Medicine Executive Agency for treatment of ACC (Schteingart et al., 2005). The pharmacological mechanism by which mitotane exerts its adrenolytic effect is still not completely understood. Very recently it has been proposed by Sbiera et al, a mechanism where the mitotane causes endoplasmic reticulum stress and profound alteration of lipid-related genes; it was demonstrated that mitotane down-regulates steroidogenesis by inhibition of sterol-O-acyl-transferase 1

(SOAT1) and confers adrenal-specific cytotoxicity leading to lipid-induced ER-stress (Sbiera et al., 2015). Mitotane leads with relative specificity to a destruction of the inner zones of the adrenal cortex, the zona fasciculata, and zona reticularis. Several studies have evaluated the efficacy of mitotane as an adjuvant therapy or for advanced ACC as a single treatment or in combination with chemotherapy. Adjuvant treatment is routinely started within 3 months after surgery.

In most patients mitotane abolish steroid secretion but, since uncontrolled hormone secretion might worsen significantly quality of life and may even be life threatening, sometimes additional measures are required to control endocrine symptoms, such as adrenostatic drugs (metyrapone, etomidate). About follow-up, it's repeated every 3 months for the first two years, including abdominal CT or MRI and hormonal markers, and kept on for at least 10 yr. However, the results of the treatment of advanced forms of carcinoma with mitotane are conflicting: some reports attest durable and complete remissions, while others attribute to mitotane a modest antineoplastic activity. Treatments with cisplatin and etoposide in combination with mitotane are placed among the most active for in advanced cancer. Other cytotoxic agents were used in the treatment of this disease such as vincristine, 5-fluorouracil and streptozotocin giving variable results (Berruti A, 1998; Bonacci R, 1998).

A radionucleotide-based approach to therapy of ACC is the use of [131I]IMTO. [123I]IMTO single-photon emission CT imaging showed high tracer uptake in tissue of adrenocortical origin (Hahner et al., 2008), suggesting that [131I]IMTO represents a suitable compound for targeted radionuclide therapy. The clinical utility of this technology, however, needs further evaluation with prospective clinical trials.

Control of the deleterious effects of elevated hormone levels in ACC patients is important. In general, several inhibitors of steroidogenesis as well as direct hormone receptor antagonists can be used to achieve this goal. Several inhibitors of steroidogenesis are in use. During treatment with any of the steroidogenesis inhibitors, patients need to be regularly evaluated for adrenal insufficiency and should be regarded as adrenal-insufficient in times of physical stress (febrile illness or significant injury/surgery). Mitotane inhibits CYP11A1 and CYP11B1 and together with its adrenolytic effects may lead to some control of hormonelevels. Ketoconazole and metyrapone are commonly used to control glucocorticoid excess. Ketoconazole inhibits CYP17A1, CYP11A1, and to some extent CYP11B1 . The usual starting dose is 200 mg twice daily and can be increased to 1200 mg/d. During treatment with ketoconazole,

liver enzymes need to be carefully watched. Because it is an inhibitor of several hepatic drugmetabolizing enzymes (eg, CYP3A4, CYP2C9, and CYP1A2), drug interactions need to be carefully reviewed. Another powerful inhibitor of steroidogenesis at the level of CYP11B1 is metyrapone (Hartzband et al., 1988), and 250 mg twice daily is the usual starting dose and can be increased to 2 to 3 g/d in 250-mg intervals. Due to the inhibition of CYP11B1, a relative increase in adrenal androgens may occur, possibly worsening symptoms related to hyperandrogenemia. Other steroidogenesis inhibitors such as aminoglutethimide or etomidate are not in widespread use.

Etomidate is an anesthetic compound often used for rapid induction for intubation or short-term procedures. Even at doses much lower than those used for anesthesia, etomidate is a powerful inhibitor of CYP11B1 and CYP11B2 (Drake et al., 1998). For this effect, it can be used in the inpatient setting. Some centers have experience with a steady low-dose perfusor, which is a last-resort option. Steady infusion can be safe because doses used are only 1/10 of the anesthetic dose (2–3 vs 20–30 mg/h). A direct antagonist used for glucocorticoid excess is mifepristone. However, neither ACTH nor glucocorticoid levels can be used to guide therapy.

Spironolactone can also be used to control androgen effects in women with androgen-secreting tumors and mineralocorticoid effects in patients with mineralocorticoid-secreting tumors. As with other malignancies, local control of ACC is important both for effecting the possibility of a disease cure and for improving symptomatic outcomes. Although traditionally considered ineffective for ACC, radiotherapy has been shown in several recent series to offer a significant improvement in disease control in both the adjuvant and palliative settings (Fassnacht et al., 2006; Hermsen et al., 2010), although such an improvement has not been universally demonstrated (Habra et al., 2013).

In recent years, considerable advances toward understanding the pathogenesis of ACT have been made.

Different strategies have enabled these achievements:

1. Identification of genetic alterations in rare familial syndromes and evaluation of whether the same defects are present in sporadic tumors.
2. Investigation of signaling pathways that were proved important in other tumors types.
3. Employment of high-throughput techniques such as genome wide expression profiling, methylation profiling and microRNA profiling to interrogate novel signaling pathways.

4. Studies with animal models with one or more genetic defects in known signaling pathways.

2.6 Role of ERα and ERβ activation on ACC development

A possible involvement of estrogen in tumor development was been suggested by epidemiological evidence and experimental studies: adrenal tumors, especially those secreting, are more frequent in women and the use of estrogen-progestin is a risk factor for tumor development.

Recently, in several tumoral cells the presence of a cross-talk has been reported between the IGF system and estrogens, which is able to activate the same pathway through the action of estrogen receptors (Hamelers and Steenbergh, 2003).

It has been largely demonstrated that the effects of estrogens are mediated by the ERα and ERβ, which act as transcription factors (Nilsson et al., 2001). In the human fetal adrenal gland the mRNA of ERβ was much more expressed than that of ERα and the ERβ protein was detected in the definitive zone of the adrenal cortex (Takeyama et al., 2001). The highly estrogenic environment during pregnancy has been reported to influence steroidogenesis of the primate fetal gland (Albrecht et al., 1999; Hirst et al., 1992) and it has been suggested that the effects of estrogens via ERβ may play an important role in modulating the development of both human and primate fetal adrenal glands (Albrecht et al., 1999; Takeyama et al., 2001).

H295R proliferation seems to be supported by the presence of an autocrine mechanism mediated by E2 through its receptors (Sirianni et al., 2012).

The assessment of response to estrogen receptor antagonists (such as ICI 182 780 and OHT (4-OH tamoxifen)) confirms the E2 involvement in H295R cell proliferation (Montanaro et al., 2005b).

This showed a dose-dependent inhibition of basal and E2-dependent cell proliferation. In particular, OHT induced morphological changes characteristic of apoptosis up-regulating the expression of FasL and inducing autocrine activation of caspases while ICI caused a cytostatic effect that could be explained by the inhibitory effects exerted by ICI on IGF signaling pathway, which is strongly activated in H295R by autocrine IGF-II action through the IGFIR (Sirianni et al., 2012).

ICI mediated inhibition of cell growth is therefore not solely attributable to competition between estrogen and ICI for the estrogen receptor but also to the interruption of the IGF signaling pathway (Montanaro et al., 2005a).

ERα and ERβ belong to the steroid/thyroid hormone superfamily of nuclear receptors, members of which share a common structural architecture (Evans, 1988; Katzenellenbogen and Katzenellenbogen, 1996; Tsai and O'Malley, 1994). They are composed of three independent but interacting functional domains: the NH_2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain. Binding of a ligand to ER triggers conformational changes in the receptor and this leads, via a number of events, to changes in the rate of transcription of estrogen-regulated genes. These events, and the order in which they occur in the overall process, are not completely understood, but they include receptor dimerization, receptor-DNA interaction, recruitment of and interaction with coactivators and other transcription factors, and formation of a preinitiation complex (Katzenellenbogen and Katzenellenbogen, 1996; McKenna et al., 1999). Another striking difference between the two receptors is their distinctive responses to the synthetic antiestrogens tamoxifen, raloxifene, and ICI-164,384. On an ERE-based reporter gene, these ligands are partial E2 agonists with ERα but are pure E2 antagonists with ERβ (Batistuzzo de Medeiros et al., 1997; McDonnell et al., 1995; McInerney et al., 1998).

The COOH-terminal, E/F-, or ligand-binding domain (LBD) mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression (Eudy et al., 1998; Giguere et al., 1988; Tsai and O'Malley, 1994).

Crystallographic studies with the LBDs of ERα and ERβ revealed that the AF2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and that the position of helix 12 is altered by binding of ligands. When the ERα LBD is complexed with the agonists, E2 or diethylstilbestrol (DES), helix 12 is positioned over the ligand-binding pocket and forms the surface for recruitment and interaction of coactivators (Shiau et al., 1998; Wurtz et al., 1996). In contrast, in the ERα- and ERβ-LBD complexes with raloxifene (Pike et al., 1999) or the ERα-LBD 4-OH-tamoxifen complex (Shiau et al., 1998), helix 12 is displaced from its agonist position over the ligand-binding cavity and instead occupies the hydrophobic groove formed by helix 3, 4, and 5. It is evident that different ligands induce different receptor conformations (McDonnell et al., 1995; Paech et al., 1997) and that the positioning of helix 12 is the key event that permits discrimination between estrogen agonists (E2 and DES) and antagonists (raloxifene and 4-OH-tamoxifen).

Levels of ERβ significantly lower, ERα up-regulation and aromatase over-expression are characteristic of the tumoral condition. In addition, the expression of ER was

correlated with the expression of nuclear hormone receptors, suggesting that they may be involved in the modulation of ER. Results of this study suggest that estrogen produced locally by aromatase can induce the proliferation of adrenocortical cells through autocrine and paracrine mechanisms and open new perspectives on the potential use of anti-estrogens and aromatase inhibitors as therapeutic agents against adrenocortical carcinoma.

3. G Protein Coupled Estrogen Receptor: GPER and its ligand

3.1 Introduction

Estrogen promotes different biochemical actions that involve different kinetics. Categorically, these signaling events are described as rapid or "pregenomic" events (second messenger and protein/lipid kinase activation) that occur within minutes of estrogen exposure and delayed or genomic transcriptional responses that are most conveniently measured using an hourly metric. Estrogen receptors (ER), ERα and ERβ, which belong to the nuclear steroid hormone receptor superfamily, function as hormone inducible transcription factors and induce estrogen dependent gene transactivation. Selective estrogen receptors modulators such as tamoxifen (TAM), designed to block estrogen-ER binding, are widely and effectively used clinically in the treatment of breast cancer. The hydroxytamoxifen, the active metabolite of TAM, is also capable to activate a seven-transmembrane G-protein-coupled receptor (GPCR), named GPER, that has been identified as a transmembrane estrogen receptor able to mediate rapid estrogen signaling in different cell types (Prossnitz and Maggiolini, 2009).

The signaling mechanisms employed by GPER that allow for stimulation of adenylyl cyclase and release of membrane tethered epidermal growth factor (EGF) like polypeptides are not particular to GPER and are familiar to many other GPCR (1, 2). Alternative models of estrogen action have been suggested and involve intact ER protein, or derivatives of, as well as the possibility that ER and GPER may act coordinately for this purpose (3). In the past, numerous studies, have demonstrated estrogen signaling in GPER positive, ER negative cells indicate that GPER can act as a "stand alone" receptor. In addition to the fact that ER and GPER are linked to different signaling mechanisms in reproductive cancers, their actions are independent by several measures, including the facts that in breast tumors and in cultured breast cancer cells lines (4, 5) ER and GPER show independent expression, different binding affinities for various estrogens and are differentially activated by them (5–7) and some of GPER agonists serve as ER antagonists (2).

3.2 Characterization of GPER-dependent cellular functions

GPER was first discovered as an orphan member of the 7-transmembrane receptor family by multiple studies (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). Based on the amino acid sequence homology, GPER is similar to the chemokine subfamily of GPCRs. Comparing expression of GPER ina number of ER-positive MCF7 and ER-negative MDA-MB-231 cell lines and tissue revealed a strong positive correlation between ER and GPER expression, suggesting a positive link to physiologic responses in estrogen-responsive tissues and cancers (Carmeci et al., 1997).

Filardo et al (Filardo et al., 2000) investigated the role of GPER in the rapid activation of MAPKs by estrogen in breast cancer cells. Estrogen-mediated activation of Erk 1/2 in ER-negative SKBr3 cells as well as in MDA-MB-231 (GPER-transfected) demonstrated that expression of GPER correlated with the functional response to estrogen. In addition to estrogen, the ER antagonist, ICI 182, 780, also stimulated MAPK activation via GPER. Overall, the results suggested that ER-negative cells could maintain responsiveness to estrogen through the expression of GPER (Filardo, 2002). Over the past years, a small number of papers related to GPER reported that progestin upregulates GPER expression in MCF-7 cells (Ahola et al., 2002) and this expression is essential for progestin-mediated growth inhibition which is involved in part as a result of Erk inactivation (Ahola et al., 2002). A second phase of GPER-dependent signaling suggested that GPER promotes estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting Bcl-2 expression (Kanda and Watanabe, 2003b), promotes cell growth by stimulating of cyclin D expression (Kanda and Watanabe, 2004) and upregulates nerve growth factor production in macrophages through *c-fos* induction (Kanda and Watanabe, 2003a). The upregulation of *c-fos* by estrogen and phytoestrogens was also shown in brest cancer cells (Maggiolini 2004); however, there was no demonstration that GPER directly initiated the observed effects but is correlated with responsiveness to estrogen.

Two reports provided evidence that GPER binds estrogen. The binding of tritiated estrogen to membrane of SKBr3 and GPER-transfected HEK cells was described by Thomas et al (Thomas et al., 2005). Estrogen treatment of GPER-transfected cell membrane also activates GTP-binding proteins and the production of cAMP. Revankar et al described the binding and colocalization of a fluorescent estrogen to GPER in both GPER-transfected cells as well as endogenously expression cells (Revankar et al.,

2005). The binding affinity of GPER represented 10 fold higher vlue than that determined for ERα. Interestingly, expression of a GFP-tagged GPER as well as antibody staining of endogenously expressed GPER revealed that the vast majority of GPER was localized to intracellular membranes, predominantly the endoplasmic reticulum, suggesting a novel site of action for GPER function. The ability of GPER to bind estrogen was confirmed by staining with the fluorescent estrogen, suggesting that this pool might be functionally active. Finally, although estrogen-mediated activation of PI3K could also be mediated by ERα, this mechanism did not involve EGFR transactivation, which was required for GPER. Thus, although both ER and GPER are both capable of activating PI3K in response to estrogen treatment, the two receptors utilize distinct signaling pathways and respond differentially to tamoxifen (Revankar et al., 2005).

3.3 Identification and characterization of GPER-selective ligands

Several pharmacological studies have a common problem to find GPER-selective ligands because the lack of specificity of estrogen that, it is capable to bind and to activate classical estrogen receptors and GPER. On the other hand, the ER antagonist/SERMs tamoxifen and ICI182/870 have been shown to act a GPER agonists (Filardo et al., 2000; Revankar et al., 2005) for an in depth review of GPER ligand binding properties. Bologa et al (Bologa et al., 2006) screened a library of approximately 10,000 compounds for chemical similarity to estrogen and the top 100 compound were tested for GPER activity. One of these displayed activity against GPER, serving as an agonist of the receptor. In addition, this compound, termed G-1, was inactive against classical estrogen receptors and thus represented the first selective GPER ligand. Recently studies revealed that G-1 was capable of eliciting calcium mobilization as well as PI3K activation cells expressing GPER but not in cells expressing either ERα and ERβ (Bologa et al., 2006). Furthermore, G-1, like estrogen, mediated an inhibition of chemotaxis towards EGF/serum in both MCF-7, which express classical ERs and GPER, and SKBr3 cells, which express only GPER. Other groups, subsequentially, have utilized G-1 to examine the role of GPER in multiple systems because in different cell contexts, the pathways utilized by estrogen may vary depending on the complement of receptors expressed (Sathya et al., 2015; Yan et al., 2015).

Pang et al, confirmed a role of GPER in the control of meiotic arrest. They cloned GPER from the Atlantic croaker (Pang et al., 2008). Treatment of croaker and zebrafish *in vitro* with estrogens and G-1, reduced progestin-induced oocyte maturation. Furthemore, injection of GPER antisense oligonucleotides in zebrafish oocyte blocked the inhibitory effects of estrogen on oocyte maturation, confirming a role for GPER in the control of meiotic arrest. A role for GPER has also been documented in the strogen-mediated stimulation of primordial follicle formation in the hamster ovary (Wang et al., 2008), where GPER is expresses in both granulose and theca cells and its expression is regulated by gonadotropins.

The role of GPER was examinated by Teng et al in urothelial cell proliferation, where estrogen is known to stimulate cell proliferation through the classical estrogen receptors and the response is reduced at high estrogen concentrations (Teng et al., 2008). Urothelial cells were also shown to express high levels of GPER, raising the question as to the specific roles of individual estrogen receptors in these cells. In these cells, G-1 stimulation inhibited cell proliferation, in contrast to the effects of estrogen. Interesting, overexpression of GPER inhibited, estrogen-induced cell proliferation suggesting that the inhibitory effects of estrogen on cell proliferation correlate with GPER expression. Furthemore, G-1 failed to induce *c-fos*, *c-jun* or cyclin D1 expression and GPER overexpression abolished estrogen-induced *c-fos, c-jun* or cyclin D1 expression whereas GPER downregulation enhanced expression of the same genes. These results suggested that with the classical estrogen receptor stimulating proliferation and GPER serving to inhibit proliferation via downregulation of the AP-1 components *c-fos* and *c-jun* with decreases in cyclin D1 expression.

Kuhn et al examined the role of GPER in the mechanical hyperalgesia through PKC activation (Kuhn et al., 2008). They demonstrated that G-1 and not classical ER agonists activated PKC in neurons of dissociated dorsal root ganglia. ICI182, ER antagonist and GPER agonist, was able to mimic the mechanical hyperalgesia of G-1 when injected into the paws of adult rats, further substantiating a role of GPER in this pathway. In another study, Alyea et al, have investigated the contribution of membrane estrogen receptors in the estrogen-mediated modulation of dopamine transporters in nerve growth factor differentiated PC12 pheochromocytoma cells (Alyea et al., 2008). Knockdown studies using siRNA revealed the ERα depletion blocked estrogen-mediated efflux, whereas GPER depletion increase efflux (with ERβ depletion having no effects. Treatment G-1 alone had no effect on efflux but, co-administration of

estrogen and G-1 resulted in substantial inhibition of the estrogen response, suggesting that in this system GPER serves to antagonize the stimulatory effect of ERα.

One of the major feedback targets for estrogen in the brain is the gonadotropin-releasing hormone (GnRH) neurons, which regulate gonadal function and fertility in mammals. Romano et al, determined that G-1 does not show any effect on the calcium dynamics of GnRH neurons, where estrogen and ERα selective agonists displayed activity (Romano et al., 2008). Otto et al (Otto et al., 2009) performed *in vivo* experiments with G-1 to examine the effect on mammary and uterine tissue. In their study, estrogen, but not G-1, regulated expression of Wnt-4, Frizzled-2, IGF-1 or cycline E1. G-1 failed t induce ductal growth and endbud formation in the mammary gland. It was unclear from such study whether the appropriate conditions were employed as GPER may exhibit altered kinetics or responses from those primarily evoked by classical estrogen receptors. Two more studies characterized GPER knockout mice. In one case revealed no obvious defects in reproductive organs (Otto et al., 2009) but in the other revealed alterations in glucose tolerance, bone growth, blood pressure and serum insulin-like growth factor-I levels (Martensson et al., 2009), In the latter study, aged female GPER knockout mice were hyperglycemic with impaired glucose tolerance, associated with decreased insulin expression and release, both *in vivo* and in isolated pancreatic islets. Other more recent publications descried the ability of G-1 to induce vasorelaxation with resulting decreases in blood pressure (Haas et al., 2009) as well as a role of G-1 in ameliorating the effects of multiple sclerosis in an animal model of autoimmune encephalomyelitis (Wang et al., 2009). In both studies, G-1 actvity was absent in GPER knockout mice, confirming the physiological activity of G-1 through GPER.

It also possible that for some of estrogen-mediated activity, GPER and ERs work in concert, as in estrogen induced thymic atrophy. Wang et al, using a GPER knockout mice and G-1, reported that GPER contributed, along with ERα, to estrogen-induced thymic atrophy (Wang et al., 2008). In this study, GPER was indispensable for thymocyte apoptosis, preferentially in T cell receptor β chain$^{-/low}$ double-positive thymocytes. Kamanga-Sollo et al in their study concluded that, whereas GPER mediates the estrogen-stimulated increase in IGF-I mRNA, ERα mediates the proliferative effect. They examined the mechanism involved in the estrogen-mediated stimulation of IGF-I mRNA and muscle growth, using bovine muscle satellite cell culture, that although G-1 stimulated the induction of IGF-I mRNA (as ICI 182,780).

With the wealth of synthetic and natural estrogen substances in existence, it is perhaps not surprising that a large number have been shown to interact with GPER. Of the therapeutic anti-estrogens, ICI182,780 (a selective estrogen receptor downregulator, SERD) was first demonstrated to interact with GPER, but surprisingly, as opposed to its antagonistic action towards ERα/β, ICI182,780 acted as an agonist towards GPER (Filardo et al., 2000). Subsequent binding studies revealing an affinity of ~30 nM (Thomas and Dong, 2006). Similarly, 4-hydroxytamoxifen (the active metabolite of tamoxifen, a selective estrogen receptor modulator, SERM) also acts as a GPER agonist (Vivacqua et al., 2006b), and recently raloxifene has also been demonstrated to activate GPER in cells deficient for ERα (Petrie et al., 2013), consistent with the actions of a series of benzothiophene SERMs in neuroprotection (Abdelhamid et al., 2011). Many synthetic compounds from the pesticide and plastics industries known to have estrogenic effects have also been demonstrated to bind and/or activate GPER, including atrazine (Albanito et al., 2008a), bisphenol A (Chevalier et al., 2012; Dong et al., 2011; Sheng et al., 2013), daidzein (Kajta et al., 2013), zearalonone, nonphenol, kepone, p,p'-DDT, o,p'-DDE and 2,2′,5′,-PCB-4-OH (Thomas and Dong, 2006). Finally, a number of phytoestrogens display agonist activity towards GPER, including genistein (Maggiolini et al., 2004; Vivacqua et al., 2006a), quercetin (Maggiolini et al., 2004), equol (Rowlands et al., 2011), resveratrol (Dong et al., 2013), oleuropein, and hydroxytyrosol (Chimento et al., 2013b).

3.4 Transcriptional activations mediated by GPER

It is often the observation that the rapid signaling events mediated by GPER can also lead to the transcriptional response of estrogens with the ligand-dependent genomic model of ER activity. Kanda et Watanabe demonstrated that E2, through GPER, upregulates nerve growth factor inducing by *c-fos* expression via cAMP in macrophages (Kanda and Watanabe, 2003a; Kanda and Watanabe, 2003b). The same authors demonstrated that E2 induces cyclin D2 and Bcl-2 expression via protein kinase A-mediated CREB phosphorylation in Keratinocytes (Kanda and Watanabe, 2004). It was shown that E2 attenuates hepatic injury after trauma-hemorrhage by upregulating Bcl-2 expression through a GPER and PKA-dependent pathway (Hsieh et al., 2007).

C-fos expression, used as an early molecular sensor for estrogen activity, provided further evidence of GPER-dependent transcriptional activation by E2 in ER-positive MCF-1 and ER-negative SKBr3 breast cancer cells (Maggiolini et al., 2004). In this

study they proved that GPER signaling requires EGFR and occurs through rapid ERK 1/2 phosphorylation in triggering the genomic response to estrogen notably in tumor cells devoid of ERs. E2, the phytoestrogen genistein and the 4-hydroxylated metabolite of the SERM tamoxifen (OHT) induced the expression of *c-fos* through the GPER/EGFR/ERK signaling pathway and also induced proliferation of thyroid tumor cells lacking ER (ARO cells) or cells expressing a non-transcriptionally active variant of ERα (FRO and WRO cells) (Vivacqua et al., 2006a). The GPER pathway may represent a new window to examine the classical ER-mediated biological thyroid cell response.

In endometrial cancer cells with WT ERα (Ishikawa) or its splice variant (Hec1A) (Vivacqua et al., 2006b) was shown the agonist activity of E2 and OHT elicited through the GPER/EGFR/ERK signaling pathway. In these cell contexts, OHT shows the antagonist property on ERα activation by E2, mediated induction of c-*fos* and cell proliferation in a GPER-dependent fashion similar to E2. These findings provided further insight into the molecular mechanisms potentially involved in the increased incidence of endometrial cancer in women treated with tamoxifen for breast tumors (van Leeuwen et al., 1994). In patients with endometrial carcinoma, GPER overexpression correlated with EGFR levels, occurred more frequently in high-grade, biologically aggressive histological subtypes and was associated with poorer survival rates (Smith et al., 2007).

The discovery of G-1, GPER-specific agonist, represented a key experiment to study the GPER activity (Bologa et al., 2006). Taking advantage of the lack of any detectable activity of G-1 on the classical ER and using ovarian cancer cells that express both ERα and GPER, Albanito et al observed that G-1, like E2, up-regulated diverse estrogen-responsive genes including c-*fos*, pS2 and cyclins A, D1 and E; however, it failed to increase the ERα-target gene PR, which only responded to E2 treatment (Albanito et al., 2007). These data were checked using ER-negative and GPER-positive SKBr3 cells, where G-1 like E2 stimulated c-*fos* expression, but had no effect on PR expression (Albanito et al., 2007). These results suggested that GPER, possibly together with ERα, mediates the transcriptional activation of the other genes, while estrogen-activated PR expression occurs specifically through ERα. In addition, in ovarian cancer cells, E2 and G-1 used in combination did not show any increase in the transcriptional activation of *c-fos* compared to either compound alone, suggesting that a common pathway mediates the genomic response. Otherwise, knocking down GPER or ERα revealed a cross-talk between these estrogen receptors in the stimulation of c*fos* by G-1 and E2.

In SKBr3 cells, which express GPER and not ER, the knock-down of GPER was sufficient to block the growth stimulation by G-1 and E2. Nevertheless, GPER can be sufficient to signal alone in absence of ER as in SKBr3 breast cancer cells. While, when both receptors are co-expressed, ERα and GPER, the findings indicate that cooperation between them may take place (Sukocheva et al., 2006). In these latter cells, it was shown (Albanito et al., 2008a; Albanito et al., 2008b) that atrazine, the environmental contaminant and endocrine-disruptor, activates GPER dependent signaling, although in ovarian cancer cells, both GPER and ERα were required to induce c-*fos* expression and cell proliferation in line with the results obtained using E2 and G-1. Previous studies have shown that atrazine may exhibit an estrogen-like action increasing aromatase expression and activity without any direct agonism or antagonism of the classical ERs (Fan et al., 2007a; Fan et al., 2007b). Atrazine, in the ovarian cancer cells (Albanito et al., 2008a), acted through both GPER and ERα via the EGFR/MAPK signaling pathway to trigger transcriptional activation and cell proliferation. The authors concluded that a co-work between GPER and ERα contributes to atrazine activity.

From these data it is reasonable to argue that the evaluation of estrogenic activity of phyto- and xenoestrogens should be extended to their potential ability to activate GPER signaling alongside the well-known agonist effects exerted through the classical ER-mediated genomic response. Madak-Erdogan *et al.* evaluated the the action of E2 and estrogen-dendrimer conjugates (EDCs), which are unable to cross the nuclear membrane (Harrington et al., 2006), in a genome-wide cDNA microarray analysis of MCF-7 breast cancer cells (Madak-Erdogan et al., 2008), to examine the effect of extranuclear estrogen-mediated pathways. A physiological role for GPER-mediated transcriptional responses through cross-talk with ERα was founded in mouse spermatogonia GC-1 cells, which served to investigate the estrogen-mediated regulation of testicular function (Sirianni et al., 2008). Sirianni *et al.* investigated the potential involvement of an estrogen-binding receptor, GPER, in estrogen signaling. The authors demonstrated that E2 and G-1 activate the EGFR/ERK pathway causing the stimulation of c-*fos* and cyclin D1 expression as well as GC-1 cell growth. Interestingly, using ICI182,780 or silencing GPER exression, the proliferative effects induced by E2 and G-1 were abrogated. The results obtained are consistent with Bouskine *et al.* They demonstrated that E2, through the activity of a Gi protein, could induce rapid activation of ERK1/2 and PKA signaling pathways, which are involved in the proliferation of human germ cell tumors (Bouskine et al., 2008).

Interestingly, the cell membrane-associated form of ER has been reported to couple with and activate different G proteins, thereby triggering biological responses via EGFR transactivation (Razandi et al., 2003). Moreover, E2 activation of GPER activates the EGFR signaling cascade (Filardo et al., 2000) similar to other GPCR ligands (Thomas et al., 2006). Albanito et al. provided a loop between EGFR and GPER in ER-negative breast cancer cells, where, EGF, by up-regulating GPER expression, engages E2 to potentiate the biological response to EGFR signaling (Albanito et al., 2008b).

The clinical observation that GPER overexpression is associated with lower survival rates in endometrial cancer patients (Smith et al., 2007) and higher risk of developing metastatic disease in patients with breast tumor (Filardo et al., 2006) suggests an important involvement in carcinogenesis. Therefore, the expression levels of GPER, which are regulated by EGF/EGFR signaling may characterize the estrogen sensitivity of these tumors in addition to predisposing tumors to an altered responsiveness to endocrine therapy. Recently, a set of genes has been identified that may contribute to the proliferative activities of GPER (Pandey et al., 2009). Of particular interest was the connective tissue growth factor (CTGF).

It is well known that tamoxifen can act as full agonist of GPER (Lappano et al., 2013; Vivacqua et al., 2006a). GPER can mediate rapid E2-induced non-genomic signaling events, including stimulation of adenylyl cyclase, mobilization of intracellular calcium (Ca2+) stores and activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (Ariazi et al., 2010; Prossnitz and Barton, 2009). However, GPER exhibits prognostic utility in different cell lines while its expression status in ACC has not been investigated.

4. Cell-based assays for screening androgen receptor ligands

4.1 Introduction

Androgens represent a broad group of steroid hormones that mediate their effects through binding and activation of the androgen receptor (AR, *NR3C4*). The AR is expressed in a variety of tissues, including the heart, pituitary, skeletal muscle, uterus, and thyroid, with the highest expression level observed in the prostate, adipocyte and liver (Sar et al., 1990). The AR can be activated by several physiologic ligands (mainly testosterone and dihydrotestosterone, DHT) that bind the AR with different affinities and bioactivity (Keller et al., 1996; Wilson et al., 2002a). DHT is the most active physiologic androgen with a tenfold higher androgen receptor bioactivity than testosterone (Paris et al., 2002; Raivio et al., 2002).

Binding of androgen to the cytosolic AR (Marcelli et al., 1991) results in a conformational change in the receptor that causes dissociation of heat shock proteins, transport from the cytosol into the cell nucleus and dimerization of the androgen-AR complexes (Nemoto et al., 1994; Wong et al., 1993). The AR dimer then binds to a specific sequence of DNA known as the androgen response element (ARE) that enhances transcription of AR-responsive genes (Chamberlain et al., 1994; de Gooyer et al., 2003; De Vos et al., 1994; Mowszowicz et al., 1993; Quigley et al., 1992; Simental et al., 1991; Tomura et al., 2001; Tyagi et al., 2000; van Steensel et al., 1995; Zoppi et al., 1992). AREs are identified by the presence of six-nucleotide half-site consensus sequences spaced by three random nucleotides in the promoter region of target genes: 5'-TGTTCT-3 (Claessens et al., 2008; Gao et al., 2005). Conversely, anti-androgens like casodex or hydroxyflutamide, bind to the AR and cause nuclear translocation but no transcriptional activation (Tomura et al., 2001; Tyagi et al., 2000). AR has a characteristic structure: two activation functions (AF1 and AF5) in the N-terminal domain (NTD), a DNA-binding domain (DBD) which contains the dimerization domain, a nuclear localization signal (NLS), a hinge region, and a carboxy-terminal ligand-binding domain (LBD) which contains a third activation function domain (AF2). All the AR regions are highly conserved except the NTD which is important in transcriptional regulation.

AR cell based models have been applied to a variety of discovery-based projects. Many focus on defining novel androgens that might play a role in human diseases of androgen excess (premature adrenarche or polycystic ovary syndrome) (Moran et al., 2015;

Utriainen et al., 2015). In addition to endogenous steroid hormones, AR cell based assays have been used to define androgenic activity in legumes, soybeans, yams and industrial chemicals with concerns of their ability to act as endocrine disruptors and/or toxicants (Eertmans et al., 2003). Finally, AR bioassays have become an alternative method for the detection of designer androgens in laboratories testing serum for sports doping (Cooper et al., 2013).

Historically, androgens have been measured as individual steroids using selective immunoassays. While these assays perform relatively well and provide a degree of high throughput, such assays can be flawed by cross-reactivity with steroids of similar structure. In addition, as there are a number of different steroids that can activate the androgen receptor, the immunoassay approach of measuring one steroid at a time does not provide a broad view of the circulating androgen milieu. Over the past ten years there has been an expansion in the use of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for measurement of natural and synthetic androgens. These methods have an improved specificity over most antibody based immunoassays. In addition, these methods allow a broader analysis of multiple steroid hormones and may be important for disease diagnosis. However, these methods are not useful in the identification of unknown synthetic or naturally occurring androgenic steroids or other substances; for these types of studies, investigators have relied on *in vitro* cell-based AR bioassays.

Cell-based steroid receptor reporter assays have become an important resource for compound profiling and drug discovery because of their ability to provide quantitative and functional information within a short time span. The cells used for developing cellular AR assays have two specific requirements: abundant expression of the AR and a reporter system driven by an ARE.

The principles of the reporter gene assays are quite simple and rely on AR ligand entry into the cells, binding to the cytoplasmic AR, translocation of the AR complex into the nucleus, binding to the ARE, resulting in an increase in reporter gene expression. Importantly, the activity of a ligand can be elucidated in samples without the need to have any information on chemical structure. A variety of reporter genes have been used for model development, including β-galactosidase, luciferase, lactamase, and green fluorescent protein (GFP). In the next paragraph will be discussed the cell-based AR bioassays currently available for detection of androgenic and anti-androgenic activity (Figure 4.1).

4.2 Yeast-based systems using a β-galactosidase reporter

In yeast cells, steroid bioactivity of substances can be determined without the presence of any other mammalian proteins/pathways influencing the AR activity. These cells have the advantages of easy handling, fast growth, inexpensive media components and robustness towards toxic effects of the tested chemicals or solvents (Roy et al., 2008). These attributes make the yeast AR screen a fast and easy tool. Some disadvantages of yeast assays include laborious pre-assay cell preparation and complex cell lysis steps. Using yeast assays to express mammalian proteins also raise concerns regarding phosphorylation, glycosylation, folding and post translational modifications.

β-galactosidase (β-gal) is encoded in *E. coli* by the *lacZ* gene of the lac operon. The enzyme function in bacteria is to cleave lactose to form glucose and galactose.

Chlorophenol red-β-D-galactopyranoside (CPRG), a chromogenic substrate, described

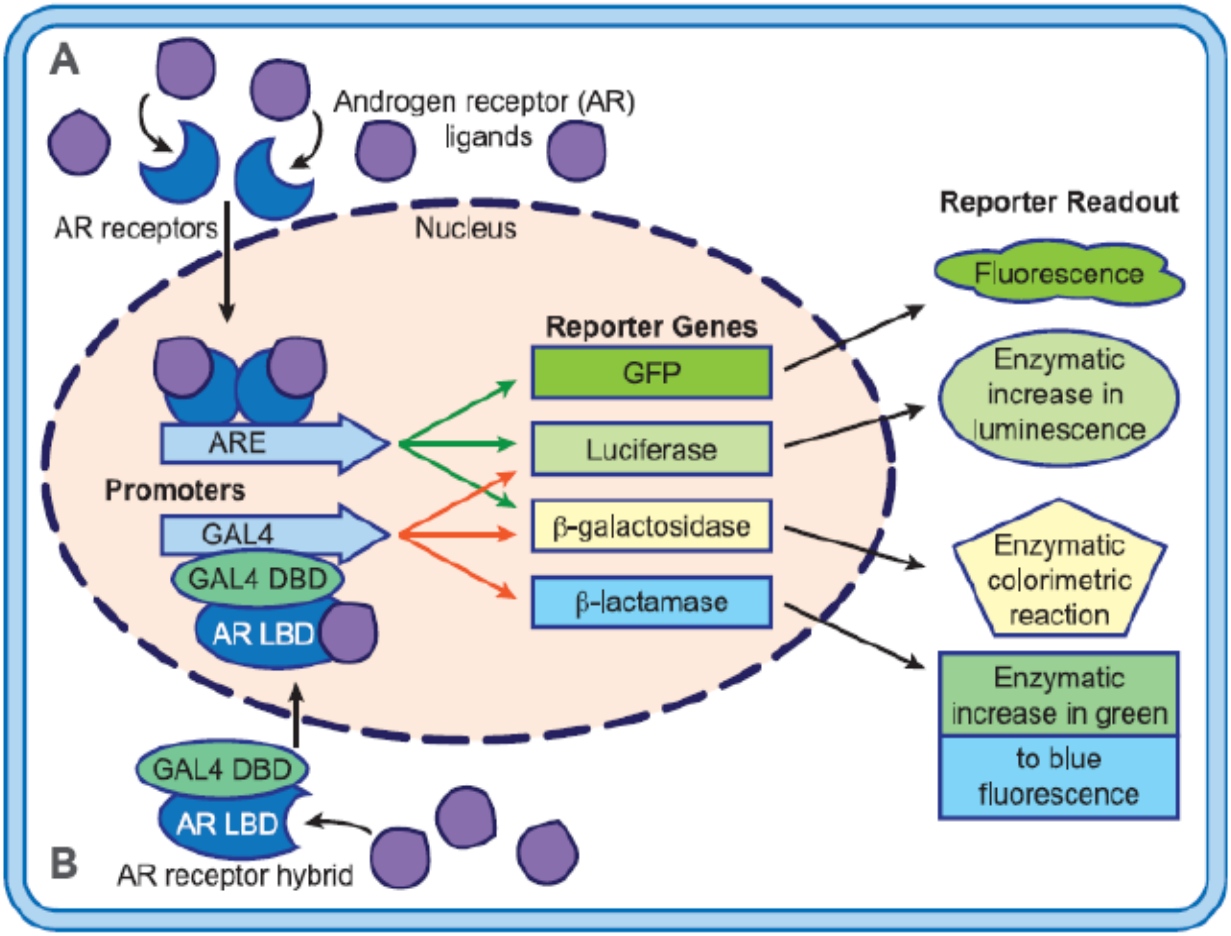


Fig. 4.1 Cell-based bioassays for the study of AR activity. (A) Models using native androgen receptor rely on ligand/antagonist effects on AR regulation of reporter gene transcription. AR binding causes translocation of the cytosolic AR into the nucleus and its binding to androgen responsive elements that drive a variety of reporters. Models are available that use GFP, luciferase or β-galactosidase. **(B)** Models using yeast-two-hybrid system for determining androgen activity and activation of reporter gene transcription. Androgen binding causes the translocation of cytosolic AR receptor hybrid, [GAL4 DNA binding domain (DBD)/AR ligand binding domain (LBD)] into the nucleus and its binding and activation of GAL4 promoter-driven luciferase, β-galactosidase or β-lactamase reporter systems.

by Seeber and Boothroyd, (Seeber and Boothroyd, 1996) and the synthetic compound, o-nitrophenyl-β-D-galactoside (ONPG), described by Li *et al* (Li et al., 2008b) are used for spectrometric detection of β-gal. Both substrates are colorless but became colored once hydrolyzed by β-gal. For the ONPG/ β-gal assay the time required for yeast exposure to the tested compounds is 6 h. The cells are then lysed and an aliquot of the extract is mixed with the β-gal reaction substrate in a buffer containing sodium phosphate and magnesium chloride. The assay ends with the spectrophotometric measurement of the yellow reaction product (o-nitrophenol). The production of o-nitrophenol, per unit time, is proportional to the concentration of β-gal, allowing the intensity of the yellow color produced to determine the enzyme concentration (Li et al., 2008b). The use of XGal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) for β-gal detection requires at least 16 h of yeast exposure to the test compounds (Jiao et al., 2008; Li et al., 2008a). XGal, a chromogenic substrate for β-gal, produces a blue color that can be detected visually over background. Using the XGal substrate, β-gal assays provide a more sensitive reporter for activity, but XGal is not as quantitative as the β-gal assay (Mockli and Auerbach, 2004).

As shown in Table 1, for most of the β-gal assays examined, as exposure times increase, EC_{50} values decrease. In 1991, Purvis *et al. (Purvis et al., 1991)* developed an androgen-inducible expression system for *Saccharomyces cerevisiae.* The PGKare-lacZ (PGK promoter followed by ARE and lacZ sequence) was integrated into the *S. cerevisiae* genome at the ura3-52 locus. The resulting strain was then stably transfected with human AR (hAR) expression plasmids. The transfected cells were incubated in the presence of different concentrations of DHT and assayed for β-gal activity. EC_{50} was 1nM for DHT treatment with a steroid exposure time of 40 h. A similar AR assay with comparable steroid exposure time and EC_{50} was developed by Sohoni P. and Supter PJ (Sohoni and Sumpter, 1998). Based on the hypothesis that one chemical may activate multiple steroid receptors, they used two recombinant yeast strains: one containing a gene for the human estrogen receptor (also containing a plasmid carrying an estrogen responsive element regulated lacZ reporter) and the other yeast strain expressing the human AR (also containing an ARE regulated lacZ reporter). When an active ligand bound to either receptor, lacZ was transcribed/translated and then secreted into the medium. The medium could then be used for the chromogenic substrate CPRG. They confirmed previously reported anti-androgenic and estrogenic activity of vinclozolin and p,p'-1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) (Bitman et al., 1968;

Kelce et al., 1995) and they found estrogenic activity in several reported anti-androgenic compounds namely o,p'- 1,1,1,-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), bisphenol A and butyl benzyl phthalate.

Chatterjee S. *et al.* (Chatterjee et al., 2007) constructed a yeast-based AR bioassay to evaluate the androgenic activity of endocrine disruptors from pulp and paper mill effluents. The system consisted of hAR and ARE -driven *lacZ* transformed in *S. cerevisiae*. Production of *lacZ* was shown to be driven by the CYC1 yeast promoter and β-gal activity was detected using XGal. The assay detection required at least 16 h of exposure to the tested chemicals; EC_{50} was 16 nM for testosterone and 4 nM for DHT, which was consistent with the performance of other, previously constructed, assays (Leskinen et al., 2005; Michelini et al., 2005).

Table 1. Androgen Receptor Bioassays using the β-Galactosidase Reporter

AR type	Promoter	Exposure time	EC_{50} value	Assay cells	Reference
hAR[2a]	ARE	40 hours	1 nM DHT	*S. Cerevisiae*	Purvis et al. 1990
hAR[2a]	ARE	40 hours	1 nM DHT	*S. Cerevisiae*	Sohoni and Supter 1998
hAR[2a]	ARE	Overnight	3.5 nM DHT	*S. Cerevisiae*	Gaido et al. 1996
hAR[2a]	ARE	16 hours	4 nM DHT	*S. Cerevisiae*	Chatterjee et al. 2007
AR Yeast two-hybrid protein based models					
hAR-LBD	GAL4	16 hours	4.8 nM DHT	EGY48	Lee et al. 2003
GAL4DBD-ARLBD	GAL4	4 hours	10 nM DHT	Y190	Nishikawa et al. 1998
GAL4DBD-ARLBD	GAL4	2 hours	13 nM DHT	Y187	Li et al. 2008

[1]endogenous, [2]stable, [3]transient

[a]clonal, [b]mixed population

A recently reported AR cell bioassay, more selective then those previously described, was developed by Lee JH *et al*. (Lee et al., 2003) The group developed a detection system for androgenic and anti-androgenic compounds, which was based on yeast two-hybrid protein interactions. A yeast strain, ARhLBD-ASC1, was established by co-transformation of yeast cells harboring lacZ reporter plasmid. ARhLBD-ASC1 is a dual vector expressing system containing the LexA fused hinge–ligand binding domain (hLBD) of the human AR, and B42 fused to ASC-1 that interacts with the AR-hLBD in an androgen-dependent manner. In this yeast strain, androgens, but not other hormones,

stimulated β-gal activity. β-gal activity was measured as a colorimetric reaction following 16 h of incubation. This system allowed relatively high throughput and could be done in 96 well dishes.

In order to study the effect of endocrine disruptors on AR, Nishikawa J *et al.* (Nishikawa et al., 1999) developed a yeast model system with short exposure time (4 h) but relatively low sensitivity ($EC_{50\ around}$ 10 nM DHT). The major goal of the study was to develop a novel screening method to examine chemical effects on several steroid receptors. Y190 yeast cells were transformed with the pGBT9–LBD of the estrogen and androgen receptors, GAL4-receptor DBD and GAL4AD–coactivator fusion proteins. The major goal of the study was to develop a novel screening method to examine chemical effects on several steroid receptors. Because the yeast strain Y190 harbors a GAL4 binding site upstream of a lacZ reporter gene, GAL4DBD-ER binds to the regulatory region of the lacZ gene. If GAL4DBD-ER interacts with GAL4AD-coactivator, GAL4AD recruits the basal transcriptional machinery to the promoter region of the lacZ gene resulting in β-gal production. The system was adapted for other receptors by exchanging the ER portion of GAL4DBD fusion with other receptors. In addition, the models were improved by including mammalian nuclear receptor co-factors. For the development of the AR bioassay, the ER-LBD was changed to AR-LBD and the β-gal reporter responses were enhanced by adding a vector containing mammalian nuclear receptor coactivators. Based on these studies, the steroid receptor models were most effective using the following co-factors: ER–TIF2, AR–SRC1, PR–TIF2, GR–SRC1 and MR–SRC1.

Different combinations of plasmids in the yeast Y187 were used by Li *et al.* (Li et al., 2008a). Plasmids used were pGBT9, the AR-LBD and pGAD424 GRIP1/FL (described by Doesburg *et al.* (Doesburg et al., 1997)) or pGBT9 ERRγ and pGAD424 GRIP1/FL. This model has a low compound exposure time (2 h) but limited sensitivity (EC_{50} around 13 nM DHT). These investigators developed the models to study endocrine disruptors in pesticides which were suspected of modulating the endocrine systems in humans. The endocrine disruptors examined for their ability to interact with the ER, AR, PR or ERRγ included p, p'- dichlorodiphenylethane (p, p'-DDE), p, p'-dichlorodiphenyltrichloroethane (p,p'-DDT), hexachlorobenzene (HCB) and r-hexachlorocyclohexane (r-HCH). The results showed that p,p'-DDE was an ER agonist and an AR and PR antagonist (PR > AR), while p,p'-DDT was an ER agonist and AR antagonist. HCB and r-HCH were antagonists for AR and ERR, while r-HCH was a PR

antagonist and a weak antagonist of ERR; the endocrine disruptor, r-HCH was able to reverse the ERR inhibition induced by 4-hydroxytamoxifen.

Yeast-based assays assessing chemical interactions with the estrogen, androgen, and progesterone receptor were developed by Gaido *et al*. (Gaido et al., 1997). For the AR bioassay, the EC_{50} was around 3.5 nM DHT with an exposure time of 18 h. The yeast contained two separate plasmids: an expression plasmid containing the CUP1 metallothionein promoter fused to the human nuclear receptor cDNA, and a reporter plasmid carrying two ARE upstream of the structural gene for β-gal; this system overexpressed two proteins (RSP5 or SPT3) in yeast containing either the progesterone or the androgen receptor, respectively.

One of the earliest reporter gene assays, chloramphenicol acetyltransferase (CAT reporter), has also been used for AR bioassay development. The CAT enzyme is normally found in prokaryotes but not eukaryotes. It transfers the acetyl group from the acetyl CoA molecule to chloramphenicol, causing its detoxification. Xu *et al*. used this reporter system to develop a hAR reporter assay using the CV-1 cell line (African Monkey kidney cell line) (Xu et al., 2005). The CV-1 cell line was transiently transfected with an ARE driven reporter gene plasmid (pMMTV-CAT) and a hAR expression plasmid AR/pcDNA3.1. An EC_{50} of 0.39 nM was observed for DHT following an incubation period of 24 h. Using this AR reporter model the group investigated Bisphenol A (BPA), 4-octylphenol.4-nonylphenol and a number of pesticides for agonistic and antagonistic activities. The caveat of this system is its experimental variation due to the transient nature of transgene expression.

4.3 β-Lactamase Reporter Model

The β-lactamase (BLA) reporter system, which can be used for studying gene expression in living cells, uses the bacterial enzyme TEM-1 BLA which lacks the periplasmic secretory signal sequence. BLA is encoded by the ampicillin resistance gene, a 29- kDa enzyme, and is active either as a monomer or when fused N or C terminally to a heterologous protein (Zlokarnik, 2000). It can cleave β-lactam-containing molecules with simple kinetics and high catalytic efficiency. Overexpression of BLA does not show toxicity in eukaryotic cells. CCF2 and CCF4 (coumarin cephalosporin fluorescein), the β-lactamase fluorescent substrates, can be detected by fluorescence resonance energy transfer (FRET). In the intact molecule, excitation of coumarin at 408 nm leads to efficient FRET to the fluorescein derivative and produces

green fluorescence. Cleavage of CCF2/4 by β- lactamase separates the two fluorophores, causing loss of FRET and excitation at 408 nm that result in blue fluorescence detectable at 460 nm. Thus, based on the change in the fluorescence emission signal, live cells expressing BLA can be distinguished by epifluorescence microscopy, fluorescent plate reader, or flow cytometry.

Wilkinson *et al.* (Wilkinson et al., 2008) developed a panel of steroid hormone receptor bioassays by stably engineering expression of Gal4-DBD, with specific nuclear receptor LBD, using the HEK293 cell line with stable insertion of a GAL4 promoter driven β-lactamase reporter. Plated cells were incubated for 16 h with ligands or test compounds. Lactamase substrate was then added and fluorescence signal read using a fluorescent plate reader. After subtracting the average fluorescence intensity from the cell-free controls, the 460 nm/530 nm emission ratio was calculated. The response ratio corresponds to the 460 nm/530 nm emission ratio of the stimulated wells divided by the 460 nm/530 nm emission ratio of the unstimulated wells. The AR lactamase bioassay exhibits high sensitivity to DHT with an EC_{50} of 1 nM. The particular utility of this assay is its potential for high throughput screening and a high degree of selectivity for the AR.

4.4 Luciferase Reporter Model

Firefly luciferase is one of several bioluminescent reporters that have achieved broad use for molecular biology studies. Compared to the tests previously discussed, some of the luciferase AR models have higher sensitivities then those with lactamase, particularly with the mammalian cell models that can detect picomolar levels of DHT. The details, including the sensitivity of these AR-cell based assays, are reviewed in Table 2. The most commonly used luciferase is from the firefly *Photinus pyralis.* This gene encodes a 61 kDa enzyme that oxidizes D-luciferin in the presence of oxygen, ATP and Mg^{2+}; the fluorescent product of the reaction can be quantified by measuring the released light using a luminometer. The assay is rapid, simple, relatively inexpensive, sensitive, and possesses a broad linear range. Cells transfected with a luciferase reporter plasmid are lysed using a detergent-containing buffer. The substrate can be mixed with the lysate; some luminometers directly inject the reagents into the lysate and the fluorescence is read at a defined time after mixing. The luciferase reporter is most often used as a read-out of gene expression to study transcriptional control mechanisms (promoter studies) or to study activity of transcription factors (as is the

case for the AR models). Both yeast and mammalian cell line AR-driven luciferase reporter models have been developed(Lee et al., 2003).

Table 2. Androgen Receptor Bioassays using a Luciferase Reporter

AR type	Promoter	Reporter	Exposure time	EC_{50} value	Assay cells	Reference
Yeast cell -based AR Models						
hAR[2]	ARE	Firefly Luciferase	3 hours	10 nM DHT	*S. Cerevisiae*	Michelini et al. 2004
hAR[2]	ARE	Firefly Luciferase	2.5 hours	5.5 nM DHT	*S. Cerevisiae*	Leskinen et al. 2005
hAR[2]	ARE	Bacterial Luciferase	3-4 hours	9.7 nM DHT	*S. Cerevisiae*	Eldridge et al. 2007
Mammalian cell-based AR Models						
hAR[1]	MMTV	Firefly Luciferase	24 hours	0.063 nM DHT	22Rv1	Kim et al. 2006
hAR[3]	MMTV	Firefly Luciferase	24 hours	0.008 nM DHT	PC3	Kim et al. 2006
hAR[1]	MMTV	Firefly Luciferase	24 hours	0.075 nM DHT	LNCaP	Kim et al. 2006
hAR[1a]	MMTV	Firefly Luciferase	48 hours	~0.2 nM DHT	MDA-MB-453	Hartig et al. 2002
hAR[2a]	MMTV	Firefly Luciferase	48 hours	~0.2 nM DHT	CV-1	Hartig et al. 2002
hAR[3]	MMTV	Firefly Luciferase	24 hours	3.6 nM DHT	CV-1	Sun et al. 2007
hAR[3]	MMTV	Firefly Luciferase	24 hours	~0.5 nM R1881	CHO	Vinggaard et al. 1998
hAR[2a]	MMTV	Firefly Luciferase	24 hours	~0.5 nM R1881	CHO	Roy et al. 2003
hAR[1a]	ARE	Firefly Luciferase	24 hours	115 nM DHT	T47D	Blankvoort et al. 2001
hAR[1a]	MMTV	Firefly Luciferase	Overnight	0.14 nM DHT	MDA-kb2	Wilson et al. 2001
hAR[2a]	ARE	Firefly Luciferase	24 hours	0.13 nM DHT	U2OS	Sonneveld et al. 2004
hAR[2b]	MMTV	Firefly Luciferase	24 hours	0.01 nM DHT	U2OS	Sedlák et al.2011
hAR[2b]	GRE	Firefly Luciferase	24 hours	0.01 nM DHT	U2OS	Sedlák et al.2011
AR yeast two-hybrid protein models						
GAL4D BD-AR LBD	GAL4	Firefly Luciferase	24 hours	0.1 nM DHT	U2OS	Sedlák et al.2011

[1]endogenous, [2]stable, [3]transient

[a]clonal, [b]mixed population

4.4.1 Yeast-AR luciferase models

Yeast systems are inexpensive and do not contain all the mammalian enzymes, activators and coregulators and hence may not support maximal transcriptional activity for all receptors. However, the low costs and quick cell expansion capabilities make it a good choice for experimental goals.

Michelini *et al.* developed a bioluminescent yeast-based bioassay for androgens (Michelini et al., 2005). The bioassay is based on *S. cerevisiae* cells, modified to express hAR, and contain ARE sequences to regulate expression of luciferase. The bioassay responds to testosterone in a concentration-dependent manner from 0.05 to 1000 nM. The EC_{50} of DHT is 10 nM. This assay is also able to respond to progesterone and 17β-estradiol, with an EC_{50} of 20 and 50 nM respectively, apparently via an AR mechanism. An *S. cerevisiae* strain, expressing hAR, estrogen receptor α or estrogen receptor β, with luciferase controlled by the receptors' respective hormone responsive elements, was developed by Leskinen *et al*. (Leskinen et al., 2005). These investigators describe the construction and use of a set of bioluminescent yeast strains for the detection of compounds that regulate androgen or estrogen receptor mediated hormonal signaling. The luciferase coding sequence was inserted into the vector pRS316/GPD-PGK (Sikorski and Hieter, 1989), between the GPD promoter and PGK terminator yielding pRS316luc. Sample analysis can be performed in one day and there is no requirement for cell lyses or centrifugation. Yeast cells were incubated with test compounds or complex samples for 2.5 h, resulting in an EC_{50} value of 5.5 nM DHT.

Another yeast AR bioassay, using a bacterial luciferase reporter, was developed by Eldridge *et al* (Eldridge et al., 2007). An EC_{50} of 9.7 nM was observed for DHT using a S. cerevisiae strain engineered to respond to androgenic chemicals. The strain contained stable expression of the hAR and a reporter controlled by an ARE between two promoters (GPD and ADH1). Co-transformation of this plasmid with a second plasmid (pUTK404), containing the genes required for aldehyde synthesis (luxCDE) and FMN reduction (frp), yielded a bioluminescent reporter system that is responsive to a wide variety of bioactive androgens.

4.4.2 Mammalian cell AR luciferase models

Mammalian cell-based bioassays have been developed in immortalized cell lines which are relatively easy to culture, maintain, and show higher sensitivity than the yeast system. However, a careful characterization of model systems is necessary. It is

imperative that the parent cell line selected for the development of the bioassay does not contain steroid-metabolizing enzymes since that could give inaccurate luciferase response results. Secondly, parent cells containing other steroid receptors could pose a problem since the ARE consensus DNA sequence has almost 80% similarity to cis-regulatory elements of glucocorticoid (GR, *NR3C1*), mineralocorticoid (MR, *NR3C2*) and progesterone receptors (PGR, *NR3C3*) (de Gooyer et al., 2003; Paris et al., 2002). In these models, reporter gene expression can be activated by hormone ligands leading to false positives. Numerous mammalian cell lines, including prostate carcinoma cells (LNCaP, 22Rv1, PC3 and DU-145) (Terouanne et al., 2000) or other cells (HepG2, CV-1, COS-1, COS-7 and CHO) have been engineered to develop androgen reporter assays (Hartig et al., 2002; Stone et al., 1978).

Kim *et al.* used three prostate cancer cell lines (22Rv1, PC3, and LNCaP) to develop AR-regulated reporter gene assays (Kim et al., 2006a). While 22Rv1 and LNCaP cell lines have an endogenous AR, the PC3 cell line, reported to be AR negative (Alimirah et al., 2006), was transiently transfected with a hAR expression vector. Among the three cell lines that were transiently transfected with pMMTV-luc, DHT stimulated proliferation only in LNCaP cells. It is important to note that the endogenously expressed AR in LNCaP cells contains a mutation in the ligand binding domain that alters steroid binding selectivity and can lead to activation by a variety of steroids that normally do not activate AR (Veldscholte et al., 1992). EC_{50} values of DHT for 22Rv1, PC3/AR+, and LNCaP were 0.063 nM, 0.008 nM, and 0.075 nM, respectively. While the sensitivity was good, each line showed endogenous expression of GR and therefore increased luciferase reporter in response to cortisol.

Hartig *et al.* (Hartig et al., 2002) used a human breast carcinoma cell line (MDA-MB-453) and the African green monkey kidney cell line (CV-1). MDA-MB-453 cells were transduced with a luciferase reporter regulated by the MMTV. The MDA-MD-453 cell model expressed endogenous GR and AR. CV-1 cells were transduced as above with MMTV-luc and also a hAR. While the CV-1 exhibited relative selectivity for AR activation, the MDA-MB-451 transduced reporter responded to both glucocorticoids and androgens.

In 2007 Sun *et al.* used CV-1 cells that had been transiently transfected with hAR and MMTV-driven luciferase (Sun et al., 2007). These investigators tested the effects of three common pyrethroids (fenvalerate, cypermethrin, permethrin) and their metabolite 3-phenoxybenzoic acid (3-PBA) for anti-androgenic and androgenic activity (Sun et al.,

2007). The assay displayed appropriate response to known AR agonists (EC_{50} 3.6 nM with DHT) as well as AR antagonists.

A transient AR reporter assay for detection of anti-androgenic chemicals was used by Vinggaard *et al*. (Vinggaard et al., 1999) Chinese Hamster Ovary cells (CHO) were co-transfected with vectors containing hAR and MMTV-luc by non-liposomal transfection. Cells were treated for 24 h with the synthetic androgen receptor agonist, R1881 (10 nM), resulting in a 30- to 60-fold induction of luciferase activity. CHO cells were subsequently used to develop a stable cell line (Roy et al., 2004). For stable line development, CHO cells were co-transfected with plasmids encoding MMTV-luc, neomycin and hAR. After selection with neomycin and cloning, an active, responsive clone was obtained that stably expressed both the hAR and the luciferase reporter. Stimulation of the cells with androgens for 24 h resulted in about a 15-fold stimulation of luciferase activity, with the minimum effective dose of testosterone being 0.1 nM resulting in an EC_{50} around 0.5 nM with R1881. Sixty different chemicals (pesticides or their metabolites, and common industrial chemicals) were screened with the cell line for their ability to activate or inhibit reporter as compared to a positive control. The most potent anti-androgenic compounds identified were bisphenol A, α-hexachlorocyclohexane, vinclozolin and 4,4-DDE.

An androgen reporter system that utilizes an endogenously expressed AR was developed by Blankvoort *et al*. (Blankvoort et al., 2001). The human breast cancer cell line T47D was stably transfected with a luciferase gene under transcriptional control of the PB-ARE-2 promoter. The model system was called AR-LUX (Androgen Receptor-mediated LUciferase eXpression) and was evaluated for its responsiveness to a number of androgens, anti-androgens, non-androgenic steroids, and to compounds modulating the AR itself. Following 24 h of treatment, an EC_{50} value of 115nM was determined for DHT. Luciferase responses were also elicited by high concentrations of the steroids progesterone, 17β-estradiol, aldosterone, and dexamethasone. The ability to selectively examine AR activation was a concern of this model.

The MDA-kb2 cell line, containing an endogenous AR, was developed by Wilson *et al*. (Wilson et al., 2002b). Cells were transformed with an androgen-responsive luciferase reporter plasmid driven by MMTV, selected with geneticin, and cloned. The active clone was chosen and the resulting line termed, MDA-kb2. The MDA-kb2 has been a useful tool for studying the activation of both AR and GR because both receptors are present and both receptors can activate the MMTV promoter. Following 24 h of

treatment, an EC_{50} of 0.14 nM was determined for DHT. This model is relatively easy to use, grows well and is stable but responds to both AR and GR agonists.

The U2OS cell line was used by Sonneveld *et al*. (Sonneveld et al., 2005). These investigators developed the AR CALUX (Chemically Activated LUciferase eXpression) bioassay. It contains the human androgen receptor and a luciferase reporter construct containing three AREs coupled to a TATA promoter. The EC_{50} of DHT was found to be 0.13 nM. The sensitivity of AR CALUX was assessed by measuring the luciferase activity induced by a series of natural steroids (DHT, testosterone and androstenedione).

In 2011 Sedlák *et al.* developed two panels of U2OS-based luciferase reporter cell lines using two different reporter formats (Sedlak et al., 2011). In the first model, the activity of the receptor was monitored by a reporter vector containing synthetic promoter with multimerized ARE or the MMTV upstream of the luciferase gene. The second model relied on the chimeric steroid receptor, where the N-terminal part of the receptor containing AF1 and the DBD was replaced by the DBD from the yeast transcription factor Gal4. This construct was co-transfected with reporter vector containing 9 copies of GAL4 response element (used even in HEK293) (Paguio et al., 2010). The investigators compared the two panels using several ligands and concluded that, in general, both systems generated a similar qualitative response. Both systems (AR/GRE or AR/MMTV) and AR-LBD/9XGal4UAS showed high sensitivity to DHT with an $EC_{50,}$ after 24 h treatment, of 0.01 nM and 0.1 nM, respectively.

4.5 Green Fluorescent Reporter Model

Compared to luciferase assay, fluorescent protein assay offers cheap and faster direct detection using spectrofluorometer or fluorescence microscope. The main advantage of green fluorescent protein (GFP) is that it does not require enzymatic substrates for detection. In addition, the use of different fluorescent proteins enables an investigator to track the expression of two (or more) genes in the same cell (multiplexing). GFP and its genetically enhanced variations are quantitative reporters with high levels of photo-stability and brightness. It is an auto-fluorescent protein initially derived from the Jellyfish *Aequorea Victoria* and can be used for a variety of biotechnological applications (March et al., 2003). Most of the steroid bioassays that make use of GFP reporter were designed for the determination of estrogenic activity using yeast as the cell model (Bovee et al., 2004; Gaido et al., 1997; Lyttle et al., 1992; Xu et al., 2008a). The benefit of using GFP is the ability for direct quantification using either a

fluorescence microscope or a luminometer. The details, including the sensitivity of these AR-cell based assays are reviewed in Table 3.

Table 3. Androgen Receptor Bioassays using Fluorescent Proteins as a Reporter

AR type	Promoter	Reporter	Exposure time	EC_{50} value	Assay cells	Reference
Yeast cell AR bioassay model						
hAR[2a]	ARE	yEGFP	24 hours	50 nM T	*S. Cerevisiae*	Bovee et al. 2008
hAR[2a]	ARE	GFP	24 hours	16 nM T	*S. Cerevisiae*	Beck et al. 2008
Mammalian cell AR bioassay model						
hAR[2b]	MMTV[b]	dsEGFP	24 hours	0.1 nM DHT	PC-3	Dennis et al. 2008
Mammalian cell AR nuclear translocation model						
hAR[1b]	NA	Nuclear AR-GFP	2 hours	0.08 nM R1881*	HeLa	Marcelli et al. 2006
hAR[1a]	NA	Nuclear AR-GFP	2 hours	0.96 nM R1881*	HeLa	Szafran et al. 2008

[1]mutated, [2]WT

[a]stable, [b]transient transfection

*EC_{50} nuclear translocation

NA= not applicable

Bovee *et al.* (Bovee et al., 2008) constructed recombinant yeast that expresses hAR and a yeast enhanced GFP (γEGFP), as a measurable reporter protein, in response to androgens. They stably integrated, in the *S. Cerevisiae* genome, the reporter vector and the receptor expression vector. The γEGFP reporter gene is optimized for yeast expression under control of the CYC1 promoter which contains two ARE sequences. The hAR coding region is constitutively expressed under the control of a GDP promoter. The investigators also demonstrated that *S. Cerevisiae* did not metabolize test compounds, displayed no crosstalk for non-androgen steroids and had a relative androgenic potency. Androgen activity can be quantified directly in a cytofluorimeter using excitation at 485 nm and measuring fluorescent emission at 530 nm. The EC_{50} value for testosterone activation of yEGFP expression was 50 nM following 24 h of incubation.

GFP was introduced as an alternative reporter gene in the androgen assay system developed by Beck *et al*. (Beck et al., 2008). The hAR coding sequence was inserted into expression plasmid YEpBUbi–FLAG1, resulting in the plasmid YEpBUbiFLAG–AR, and the ERE on the reporter vector YRpE2 was substituted with an ARE, resulting

in the plasmid YRpE2–ARE. The vector YRpE2-GFP was used as a backbone to create the reporter plasmid YRpE2-GFP-ARE, using GFP as a reporter gene. For evaluation of the reporter system, β-galactosidase, as a primary reporter gene, was added. Several known AR agonistic compounds (5a-dihydrotestosterone, testosterone, androstenedione, 17a-methyltestosterone, progesterone, epitestosterone, and norgestrel) were tested to evaluate both reporter systems. The model shows an EC_{50} of 16nM with testosterone.

In 2008 Dennis *et al.* developed an assay for the assessment of AR transcriptional activity using destabilized enhanced GFP (dsEGFP) in PC3 cells (Dennis et al., 2008). Confocal images were collected using microscopy and the EGFP quantification was measured by the HyperCyt® high-throughput flow cytometry. PC3 cells were transiently co-transfected with an expression vector for the wild-type hAR (pDsRedhAR) and an MMTV promoter EGFP (pMMTVdsEGFP). Agents with established androgenic and anti-androgenic activity were used for validation of the multifunctional androgen receptor screening assay. HyperCyt analysis requires 24 h treatment with compounds followed by cell centrifugation. A lack of selectivity was demonstrated after treatment with R1881, DHT, E2, progesterone, bicalutamide, nilutamide and androstenedione; all compounds induced significant increases in the percent of cells expressing dsEGFP compared to unstimulated wells. The sensitivity of the assay in response to AR was evaluated on EC_{50} of R1881 (1.34 pM) and DHT (0.1 nM).

4.6 Fluorescent AR translocation bioassay

In recent years several cell-based models have been developed to monitor androgen activity by imaging AR nuclear translocation in response to ligands (Tomura et al., 2001; Tyagi et al., 2000). GFP-tagged AR is the only assay that currently allows detection with fluorescent microscopy and automated image analysis to quantify changes in AR nuclear translocation, intracellular dynamic and solubility in response to compounds and AR mutations. AR transgenes containing GPF or its spectral variants cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), allow tracking of the dynamic events that occur following ligand binding, using real-time microscopy.

To investigate the cellular translocation of GFP-AR after treatment with agonists and antagonists, Marcelli *et al.* (Marcelli et al., 2006) utilized an AR (A619Y) containing a mutation in the DNA binding domain (Nazareth et al., 1999) that inactivates the transcriptional activity of the receptor. A619Y is able to form distinct foci upon

exposure to active compounds. The model relies on transient transfection of HeLa cells with tagged AR. This investigative group has used GFP-AR as well as CFP-AR that allows for dual examination with proteins tagged with YGF. Incubation of cells for 2 h with ligand (R1881, Casodex, Flutamide, and Estradiol) was sufficient to allow the quantification of AR nuclear translocation (EC_{50} 0.08 of R1881). A high throughput microscopy (HTM) system was used to automate fluorescent image acquisition and analysis of AR nuclear translocation and nuclear foci formation, while the CytoShop software was utilized to quantify the translocation. The results demonstrated that agonist addition resulted in a translocation of the receptor from the cytoplasm to the nucleus where it became organized into stable foci. Interestingly, AR antagonist also caused some nuclear translocation but without the resultant focal distribution (also called hyperspeckling). Fluorescence recovery after photobleaching (FRAP) also revealed that agonist-bound GFP-AR exhibited reduced mobility relative to un-liganded or antagonist-bound GFP-AR.

A different high throughput (HT) image-based assay that quantifies AR subcellular and subnuclear distribution and transcriptional reporter gene activity on a cell-by-cell basis was developed by Szafran *et al*. (Szafran et al., 2008). This assay permitted the analysis of cell cycle dependent changes in AR function in unsynchronized cell populations, allowing for the determination of cell cycle position with simultaneous analysis of DNA. HeLa cell lines were generated to stably express wild type (GFP-AR), mutant GFP-ART877A (LNCaP mutation) (Veldscholte et al., 1990) or GFP-ARF764L (AIS mutation)(Marcelli et al., 1994). R1881, mibolerone, and DHT were tested to demonstrate the utility of the AR bioassay. All three compounds induced GFP-AR nuclear translocation in a dose-dependent manner. Using R1881, the calculated EC_{50} concentration for nuclear translocation was 0.96 nM. The AR agonists DHT and mibolerone demonstrated similar effects when compared to R1881. An automated microscope was used to capture the images, and CytoShop and Pipeline Pilot image analysis software was used to quantify.

5. MATERIALS AND METHODS

5.1 GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth in vitro and in vivo

5.1.1 Cell culture and tissues

H295R cells, a cell line established from a human adreno-cortical carcinoma, were obtained from Dr W.E. Rainey (University of Michigan at Ann Arbor, USA) (Rainey et al., 1994) and cultured in Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12; 1:1; Eurobio, Les Ulis, France) supplemented with 1% ITS Liquid Media Supplement (100×; Sigma), 10% calf serum and antibiotics (Eurobio), at 37 °C in an atmosphere of humidified air containing 5% CO_2. Cell monolayers were subcultured onto 100 mm dishes for phosphatase activity and laddering assay (8 x 10^6 cells/plate), 60 mm dishes for protein and RNA extraction (4 x 10^6 cells/plate) and 24 well culture dishes for proliferation experiments (2 x 10^5 cells/well) and grown for 2 days. Prior to experiments, cells were starved overnight in DMEM/F-12 medium without phenol red and containing antibiotics. Cells were treated with (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (G-1) (1 µM) (Tocris Bioscience, Bristol, UK) in DMEM/F-12 containing FBS-DCC 2,5% (fetal bovine serum dextran-coated charcoal-treated). Inhibitors PD98059 (PD) (10 µM) (Calbiochem, Merck KGaA, Darmstadt, Germany) was used 1h prior to G-1. Adrenocortical tumors, removed at surgery, and normal adrenal cortex, macroscopically dissected from adrenal glands of kidney donors, were collected at the hospital-based Divisions of the University of Padua (Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients, in accordance with the Declaration of Helsinki guidelines as revised in 1983. Diagnosis of malignancy was performed according to the histopathologic criteria proposed by Weiss et al. (Weiss et al., 1989) and the modification proposed by Aubert et al. (Aubert et al., 2002). Clinical data of the six ACC patients included in this study are shown in Table 1. Patient C6 terminated mitotane treatment six months after beginning of therapy for severe gastrointestinal side effects. Patients C1 and C2 were treated with chemotherapy EAP protocol (etoposide, doxorubicin, and cisplatin) + mitotane.

Table 1: Clinical data of the 6 ACC patients analyzed in this study

Sample ID	Age(years)	Gender	Stage at surgery	Syndrome	Weiss score	Size (cm)	Outcome
C1	41	M	IV	Cushing	9	16	Died, 1 year
C2	17	F	IV	Cushing	9	14	Died, 18 months
C3	43	F	III	None	4	9	Died, 8 years
C4	46	M	III	None	3	18	Remission, 7 years
C5	47	M	IV	Cushing	9	14	Died, 1 year
C6	57	M	II	SubclinicalCushing	5	14	Remission, 4 years

5.1.2 RNA extraction, reverse transcription and real time PCR

TRizol RNA isolation system (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from H295R, SKBR3 and ACCs. Each RNA sample was treated with DNase I (Invitrogen), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 µl using the ImProm-II Reverse transcription system kit (Promega Italia S.r.l., Milano, Italia); cDNA was diluted 1:2 in nuclease-free water, aliquoted, and stored at -20°C. The nucleotide sequences for GPER amplification were forward, 5'-CGCTCTTCCTGCAGGTCAA-3', and reverse, 5'-ATGTAGCGGTCGAAGCTCATC-3' ; the nucleotide sequences for GAPDH amplification were forward, 5'-CCCACTCCTCCACCTTTGAC-3', and reverse, 5'-TGTTGCTGTAGCCAAATTCGTT-3'. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad Laboratories S.r.l., Milano, Italia) using 0.1 µmol/L of each primer, in a total volume of 30 µl reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad) with the dissociation protocol was used for gene amplification; negative controls contained water instead of first-strand cDNA. Each sample was normalized to its GAPDH content. The relative gene expression levels were normalized to a calibrator (normal tissue for ACC tissues or SKBR3 for H295R cells). Final results were expressed as n-fold differences in gene expression relative to GAPDH and calibrator, calculated using the ΔΔCt method as previously shown (Sirianni et al., 2009) .

5.1.3 Western Blot analysis

Fifty µg of protein was subjected to western blot analysis (Sirianni et al., 2007). Blots were incubated overnight at 4 °C with antibodies against GPER, Cyclin E (CCNE), Cyclin B1 (CCNB1), phospho-Rb, Cytochrome c, Bax, Bcl-2, Parp1, pERK1/2-

ERK2 (all from Santa Cruz Biotechnology, Santa Cruz CA, USA). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and immunoreactive bands were visualized with the ECL western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). To assure equal loading of proteins, membranes were stripped and incubated overnight with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology).

5.1.4 Histopathological and Immunohistochemical analysis

Tumors were fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm (6-7 serial sections for each sample) and stained with hematoxylin and eosin, as suggested by the manufacturer (Bio-Optica, Milan, Italy).

Paraffin-embedded sections, 5 mm thick, were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (seven to eight serial sections). Immunohistochemical experiments were performed using mouse monoclonal Ki-67 primary antibody at 4°C over-night (Dako Italia Spa, Milano, Italy). Then, a biotinylated goat-anti-mouse IgG was applied for 1h at room temperature, to form the avidin biotin-horseradish peroxidase complex (Vector Laboratories, CA, USA). Immunoreactivity was visualized by using the diaminobenzi-dine chromogen (Vector Laboratories). Counterstaining was carried out with hematoxylin (Bio-Optica, Milano, Italy). The primary antibody was replaced by normal rabbit serum in negative control sections.

5.1.5 Cytochrome c detection

Cells were treated for 24 h, fractioned and processed for Cytochrome c detection as previously reported (Chimento et al., 2012). Briefly, cells were harvested by centrifugation at 2500 rpm for 10 min at 4 °C. Pellets were resuspended in 50 μl of sucrose buffer (250 mM sucrose; 10 mM Hepes; 10 mM KCl; 1.5 mM MgCl2; 1 mM EDTA; 1 mM EGTA) (all from Sigma-Aldrich, Milano, Italy) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF and 0.05% digitonine (Sigma-Aldrich). Cells were incubated for 20 min at 4 °C and then centrifuged at 13,000 rpm for 15 min at 4 °C. Supernatants containing cytosolic protein fraction were transferred to new tubes and the resulting mitochondrial pellets were resuspended in 50 μl of lysis buffer (1% Triton X-100; 1 mM EDTA; 1 mM EGTA; 10 mM Tris–HCl, pH 7.4) (all from Sigma-

Aldrich) containing 20 µg/ml aprotinin, 20 µg/ml leupeptin, 1 mM PMSF (Sigma-Aldrich) and then centrifuged at 13,000 rpm for 10 min at 4°C. Equal amounts of proteins were resolved by 11% SDS/polyacrylamide gel as indicated in the Western blot analysis paragraph.

5.1.6 Cell cycle analysis and evaluation of cell death

Subconfluent monolayers growing in 60 mm plates were depleted of serum for 24 h and treated for an additional 24 h with G-1 1 µM. The cells were harvested by trypsinization and resuspended with 0.5 ml of Propidium Iodide solution (PI) (100 µg/ml) (Sigma-Aldrich) after treatment with RNase A (20 µg/ml). The DNA content was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data acquired using CellQuest software. Cell cycle profiles were determined using ModFit LT program. Subconfluent monolayers growing in 60 mm plates were depleted of serum for 24 h and treated for 24 and 48 h with G-1. Trypsinized cells were incubated with Ligation Buffer (10 mM Hepes (pH = 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 1.8 mM CaCl2) containing Annexin-V-FITC (1:5000) (Santa Cruz) and with Propidium Iodide. Twenty minutes post-incubation at room temperature (RT) protected from light, samples were examined in a FACSCalibur cytometer (Becton Dickinson, Milano, Italy). Results were analyzed using CellQuest program.

5.1.7 Caspases 9 and 3/7 Activity Assay

H295R cells after treatments were subjected to caspases 9 and 3/7 activity measurement with Caspase-Glo 9 and 3/7 assay kits (Promega) and modified protocol. Briefly, the proluminescent substrate containing LEHD or DEVD sequences (sequences are in a single-letter amino acid code) are respectively cleaved by Caspases 9 and 3/7. After caspases cleavage, a substrate for luciferase (aminoluciferin) is released resulting in luciferase reaction luminescent signal production. Cells were trypsinized, harvested and then suspended in DMEM-F12 before being incubated with an equal volume of Caspase-Glo reagent (40 µl) at 37°C for 1 h. The luminescence of each sample was measured in a plate-reading luminometer (Gen5 2.01) with Synergy H1 Hybrid Reader.

5.1.8 TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assay

Cells were grown on glass coverslips, treated for 24 h and then washed with PBS and fixed in 4 % formaldehyde for 15 min at room temperature. Fixed cells were washed with PBS and then soaked for 20 min with 0.25% of Triton X-100 in PBS. After two washes in deionized water, they were stained using the Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen) according to the manufacturer's protocol. Co-staining with Hoechst33342 was performed to analyze the nuclear morphology of the cells after the treatment. Cell nuclei were observed and imaged under an inverted fluorescence microscope (200X magnification).

5.1.9 Determination of DNA fragmentation

To determine the occurrence of DNA fragmentation, total DNA was extracted from control and G-1 (1 μM) treated (48h) cells as previously described (Chimento et al., 2012). The attached and detached cells floating in the medium were collected by scraping and centrifuging (1500 rpm for 5 min at 4 _C). Pellets were washed three times with PBS and then resuspended in DNAladdering lysis buffer (10% NP40, 200 mMEDTA, 0.2 MTris–HCl pH 7.5). Lysates were centrifuged at 3000 rpm for 5 min at 4 °C. The recovered DNA was incubated with RNase A (final 5 lg/ll) in 1% SDS for 2 h at 56 °C. After addition of proteinase K (final 2.5 lg/ll) samples were incubated for an additional 3 h at 37 °C. DNA precipitation was performed using ethanol/ammonium acetate precipitation O/N at -80 °C. The following day samples were centrifuged at 12,000 rpmfor 20 min at 4 _C and washed with 80% ice-cold ethanol. DNA pellets were resuspended in nuclease-free water. Equal amounts of DNA were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Sigma–Aldrich).

5.1.10 Assessment of cell proliferation

[^{3}H]Thymidine incorporation assay. H295R cell proliferation after G-1 treatment was directly evaluated as previously described (Sirianni et al., 2010). Cells were cultured in complete medium in 24 well plates (200,000 cells/well) for 24 h, then treated in serum-free medium for 48 h. Control cells were treated with the same amount of vehicle alone (dimethylsulfoxide), which never exceeded the concentration of 0.01% (vol/vol). [3H]thymidine incorporation was evaluated after a 6 h incubation period with 1 lCi [3H]thymidine per well (Perkin–Elmer Life Sciences, Boston, MA, USA). Cells were

washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 1 ml 0.1 N NaOH at 37 °C for 30 min. The total suspension was added to 5 ml optifluor fluid and radioactivity determined in a b-counter. Each experiment was performed in triplicate and results are expressed as percent (%) of basal.

MTT assay. The effect of G-1 on cell viability was measured using 3-[4,5-Dimethylthiaoly]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Sirianni et al., 2012). Briefly, cells were treated for different times as indicated in figure legends. At the end of each time point fresh MTT (Sigma-Aldrich), re-suspended in PBS, was added to each well (final concentration 0.33 mg/ml). After 30 minutes incubation, cells were lysed with 1 ml of DMSO (Sigma-Aldrich). Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer.

5.1.11 Gene silencing experiments

For the gene silencing experiments, H295R cells were plated in 12 well plates ($1x10^5$ cells/well) for proliferation experiments or in 6 well plates (2×10^5 cells/well) for Western blot analysis; cells were transfected with control vector (shRNA) or shGPER in 2,5 % DCC-FBS medium using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendations. First, cells were plated in growth medium, after 48 h were trasfected with 5.5 µg of plasmid in serum-free medium. After 6 hours, 500 µL of growth medium was added at the cells for 48 hours. For proliferation experiments cells were transfected for 24 h and then treated for 48 h before performing MTT assay.

5.1.12 Xenograft model

Four-week-old nu/nu−Forkhead box $N1^{nu}$ female mice were obtained from Charles River Laboratories Italia (Calco, Lecco, Italy). All animals were maintained in groups of five or less and quarantined for two weeks. Mice were kept on a 12 h/12 h light/dark regimen and allowed access to food and water *ad libitum*. H295R cells, 6×10^6, suspended in 100µl PBS (Dulbecco's Phosphate Buffered Saline), were combined with 30µl of Matrigel (4mg/ml) (Becton Dickinson) and injected subcutaneously in the shoulder of each animal. Resulting tumors were measured at regular intervals using a caliper, and tumor volume was calculated as previously described (Seshadri et al., 2007), using the formula: $V = 0.52\ (L \times W^2)$, where L is the longest axis of the tumor

and *W* is perpendicular to the long axis. Mice were treated 21 days after cell injection, when tumors had reached an average volume of about 200 mm^3. Animals were randomly assigned to be treated with vehicle or G-1 (Tocris Bioscience) at a concentration of 2 mg/kg/daily. Drug tolerability was assessed in tumor-bearing mice in terms of: a) lethal toxicity, i.e. any death in treated mice occurring before any death in control mice; b) body weight loss percentage=100−[(body weight on day x/body weight on day 1)×100], where x represents a day during the treatment period (Hollingshead, 2008; Johnson et al., 2001). Animals were sacrificed by cervical dislocation 42 days after cell injection. All animal procedures were approved by Local Ethics Committee for Animal Research.

5.1.13 In vivo magnetic resonance analyses

Mice were anesthetized with 1-2% isofluorane in O2, 1 L/min (Forane, Abbott SpA, Latina, Italia) and underwent MRI/MRS study. MR analyses were performed at 4.7 T on Agilent Technologies system (Palo Alto, CA, USA). T2-weighted MRI was acquired using a spin echo sequence with the following parameters: TR/TE= 3000/70 ms, section thickness of 1.0 mm, number of acquisitions = 4, point resolution of 256 μm.

5.1.14 Scoring system

The immunostained slides of tumor samples were evaluated by light microscopy using the Allred Score (Allred et al., 1998) which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to <1/10; 3 = 1/10 to <1/3; 4 = 1/3 to 2/3; 5 = >2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0 = none; 1 = weak; 2 = moderate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six to seven serial sections were scored in a blinded manner for each sample.

5.1.15 Microarray analysis

H295R cells were plated for 48 h, starved O/N in DMEM-F12 before induction or not with G-1 1 μM and 100 nM in DMEM-F12 with 2.5% choarcol-stripped for 24 h. Total RNA from triplicate samples was extracted with RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and purity

were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The arrays were scanned at high resolution on the iScan system (Illumina). Results were analyzed by GeneSpring GX (version 12.1) software (Silicon Genetics) by customizing to the Illumina single-color analysis. To identify the differences among the control and treatment, a list of apoptotic markers was created by using transcripts satisfying the conditions including fold expression differences and statistical differences between the respective condition. Differences in the GO term were defined based on a list of genes having a 1.5-fold or greater and $P < .05$ difference in control and treatment expression using GeneSpring GX version 12.1 software. For the pathway analysis, a list of transcripts up-regulated in G-1 was created (≥1.25-fold and $P < .05$).

5.1.16 Data analysis and statistical methods

All experiments were performed at least three times. Data were expressed as mean values + standard error (SE), statistical significance between control (basal) and treated samples was analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Control and treated groups were compared using the analysis of variance (ANOVA) with Bonferroni or Dunn's post hoc testing. A comparison of individual treatments was also performed, using Student's t test. Significance was defined as $p < 0.05$.

5.2 Development of a novel cell based androgen screening model

5.2.1 Materials

T, DHT, cortisol (Cort), progesterone (Prog), aldosterone (Aldo), androstenedione (AD4), hydroxyflutamide (OHF) and the 11-keto and 11-hydroxy forms of androstenedione and T were purchased from Sigma (Missouri, USA). Coelenterazine used for the luciferase assay was purchased from Promega (Wisconsin, USA). Penicillin, streptomycin, hygromycin, geneticin (G418) and DMEM/F12 medium were purchased from Life technologies (New York, USA).

5.2.2 Cell line

The CV1 monkey kidney cell line was obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) from GE Healthcare Life Science (Utah, USA) and

antibiotics including 1% penicillin/streptomycin. The cells were incubated under a humid atmosphere of 5% CO_2, at 37°C, and the medium was changed every 3 days. CV1 cells were plated at a density of 20,000 cells/well (48 well dish) in growth medium and grown to 60% confluence after which they were treated for steroids activity.
CV1-ARluc cells were plated in a 48 wells culture plate in 500 µl of growth medium (10% FBS/DMEM-F12, G418 and Hygromycin). The cells were incubated under a humid atmosphere of 5% CO_2, at 37°C. All treatments were performed with charcoal-stripped FBS serum to eliminate contaminating steroids.

5.2.3 Stable Transduction

CV1 cells (20,000 cells/well) were plated about 18 h before transduction in a 48 well-dish. The lentivirus pBM14-MMTV with the *Gaussia Luciferase* gene was diluted 1:10 in DMEM/F12 medium and added to the flask with 8 µg/mL of polybrene. The flask was centrifuged at 1200 rpm for 80 min and after 4 h in a humidified 5% CO_2 incubator the cells were supplemented with 1 ml of DMEM/F12 containing 10% FBS without any antibiotics. After 48 h, the cells were selected in medium containing 1200 µg/ml of G418. The medium was changed three times a week. The obtained cells, named CV1-luc, were transduced with a lentivirus containing the hAR gene and the hygromycin selective gene. The stable transfection was performed as described above, using a multiplicity of infection (MOI) of 10 and 8 µg/mL of polybrene. 50 clones were obtained after 14 days of dual antibiotic (G418 and Hygromycin) selection. The clones were isolated using cloning rings (Sigma, Missouri, USA) and re-seeded and grown in a 48-well dish. After reaching 60% confluence, the cells were treated in DMEM/F12 containing 10% charcoal-stripped FBS and 10 nM of testosterone. After 24 h the treated cells were assayed for luciferase activity using the appropriate luminescence kit (Coelenterazine, Promega). The clone with the largest T induced luciferase activity was named CV1-ARluc and was used for further studies.

5.2.4 Isolation of RNA and qPCR analysis

The cells (25.000 cells/well) were grown for 24 h in 48 well culture. Total RNA was isolated from the cells previously plated using an RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and purity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For cDNA generation, 100 ng of total RNA was reverse transcribed

using the High Capacity Kit (Applied Biosystems, Foster City, CA, USA). For qPCR, 12 ng of prepared cDNA was mixed with Fast Universal PCR Master Mix (Applied Biosystems). AR and peptidylprolyn isomerase A (PPIA) primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). PPIA was used as the housekeeping control gene.

5.2.5 Protein extraction and protein assay

Cells were lysed in 200 µL Mammalian Protein Extraction Reagent (Pierce Chemical Co., Illinois, USA), and the protein content was estimated by the bicinchoninic acid (BCA) protein assay using the BCA protocol (Thermo Scientific, Illinois, USA).

5.2.6 Western analysis

CV1 and CV1-ARluc cell lines were plated at a density of 75,000 cells/well (24 well-dish), in growth medium. Samples were lysed with lysis buffer (2% sodium dodecyl phosphate, 62.5 µM Tris, 0.04% bromophenol blue, 0.5 % dithiothreitol) and heated at 95 °C for 5 min. Proteins were then loaded (20 µg) on 10% Bis-Tris gel and electrophoresed for 1 h before transferring to polyvinylidene difluoride membranes. The membranes were then blocked with 5% BSA for 1 h and incubated with primary antibody (AR, polyclonal rabbit antihuman, 1:1000 BSA, Sigma) and secondary antibody (goat anti-rabbit, 1:5000, Life Technologies).The Pierce ECL Western Blotting Substrate kit (Life technologies) was then used for signal development.

5.2.7 AR translocation study

CV1-ARluc cells were grown on microscope slides from Globe Scientific (previously treated with 50 µg/ml of Poly D-Lysine at room temperature for 1 h) in 100-mm plates for 24 h in growth medium. Cells were treated with DHT for 18 h and subsequently fixed with methanol at -20 °C for 20 minutes and washed three times with PBS. Slides were then incubated overnight with a rabbit anti-human AR antibody (Sigma) and then with a secondary goat anti-rabbit antibody (Sigma) for 1h at room temperature. Prolong Gold mounting medium with DAPI was used to visualize the cell nucleus.

5.2.8 Gaussia Luciferase analysis

CV1 and CV1-ARluc cells were plated at a density of 25,000 cells/well (48 well-dish), in growth medium for 24 h and then treated for indicated time points. The treated

medium was collected and 25 µL was mixed with 50 µL of coelenterazine (previously diluted 1:100 in 50 mM Tris, 150 mM NaCl and water). Luminescence was then measured by FLUOstar OPTIMA microplate reader according to the manufacturer's instructions (Life Technologies).

5.2.9 Sera

Charcoal dextran stripped human serum was obtained by Equitech-Bio. In the present study, we analyzed human serum from 20 healthy adults (10 females and 10 males), age 20-35 years. All samples were collected under protocols approved by the Institutional Review Board (IRB) at the University of Michigan.

5.2.10 Extraction method

The indicated concentrations of steroids were prepared separately in DMEM/F12 medium with10% charcoal-stripped FBS, and stripped human serum using ethanol as a carrier solvent. Several extraction methods were tested and were found to have variable abilities to disrupt androgen regulation of reporter activity (data not shown). The method that exhibited androgenic activity most similar to unextracted medium was solid phase extraction using Sep-Pak Cartridges column from Waters (Chromatography division Millipore Corporation, MA, USA). The columns were activated with 4 ml of methanol and subsequently washed with 4 ml of deionized water. Standards made separately in DMEM/F12 with 10% charcoal stripped FBS and stripped human serum were dispensed at a volume of 600 µl in the columns, followed by another wash and elution with 2 ml of 100% methanol. Samples were evaporated at 37°C using a thin stream of nitrogen gas and the dried extract was then re-suspended in 300 µl of DMEM/F12 medium with 10% charcoal stripped FBS.

6. RESULTS

6.1 GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth in vitro and in vivo

6.1.1 G-1 treatment decreases H295R cell growth in vitro and in vivo

We first examined GPER expression in human ACCs and in H295R cells. By western blot analysis (Fig. 1. A) and real time RT-PCR (Fig. 1. B-C) we demonstrated that GPER is expressed in normal adrenal, in human ACCs and in H295R cells at variable levels. Effects of G-1 on cell viability and proliferation were tested using increasing concentrations (0.01-0.1-1μM) for different times (24-48-72 h) (Fig. 1. D-E). Of the different doses tested only 1μM caused a time-dependent reduction in H295R cell growth. Doses higher than 1μM did not show any more pronounced effect (data not shown). Knocking down of GPER gene expression, using a specific shRNA, (shGPER) was assessed by western blot analysis and revealed a substantial decrease in protein content compared to the control shRNA (insert, Fig. 1. F). However, GPER silencing was able to only partially abrogate the inhibitory effects exerted by G-1 on H295R cell proliferation (Fig. 1. F).

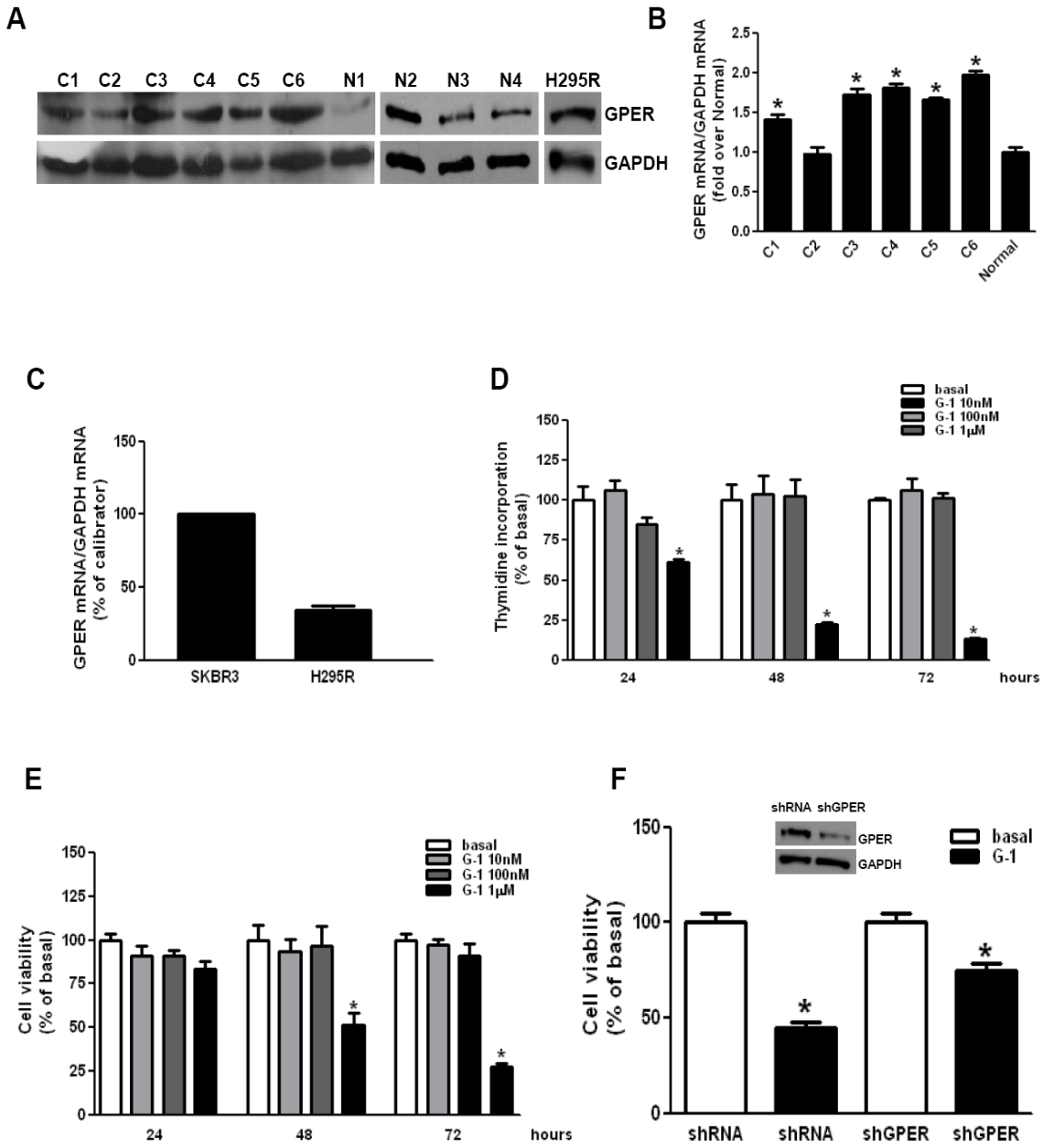


Figure 1: G-1 treatment decreases H295R cell growth *in vitro*. (A), Western blot analysis of GPER was performed on 50 μg of total proteins extracted from normal adrenal, ACCs and H295R cells. GAPDH was used as a loading control. (B-C), GPER mRNA expression in normal adrenal and ACCs (B), H295R and SKBR3 (positive control) cells (C) was analyzed by real time RT-PCR. Each sample was normalized to its GAPDH RNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator. Data represent the mean+SE of values from at least three separate RNA samples; *, P<0.05, versus calibrator). (D-E), H295R cells were treated with G-1 (0.01-1μM) for different times (24, 48 and 72 h). Cell proliferation was evaluated by [^{3}H]Thymidine incorporation (D) and MTT (E) assays. Results were expressed as mean+SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*, P<0.05 versus basal). (F) MTT assay was performed on H295R cells, which were previously transfected for 72 h in the presence of control vector (shRNA) or shGPER. Twenty-four hours after transfection cells were treated in 2.5 % DCC-FBS medium for 48 h with G-1 (1μM). Results were expressed as mean+SE of three independent experiments each performed in triplicate. (*p < 0.05 versus basal). The insert shows a Western blotting assay on H295R protein extracts evaluating the expression of GPER receptor in the presence of shRNA or of shGPER. GAPDH was used as a loading control.

H295R cells were used to generate xenograft tumors in athymic nude mice. Twenty one days after tumor grafting all mice developed a detectable tumor and were randomized to be treated with either vehicle or G-1. G-1 administration produced a statistically significant decrease in tumor volume from day 14 post treatment (Fig. 2. A). A trend of growth inhibition was observed thereafter. The drug was well tolerated without lethal toxicity or body weight loss during treatment (data not shown). Multi-slices T2-W MRI indicated larger tumor volume in vehicle treated animals compared to tumors from G-1 treated mice. Hyperintense large cystic area and haemorrahagic regions, that appear as dark areas in the tumor sections, were present in vehicle treated animals (Fig. 2. B). Grafted tumors harvested after three-week treatment with G-1 showed a significant decrease in tumor weight compared to vehicle treated animals (Fig. 2. C). Hematoxylin and eosin staining of xenograft tumors revealed some picnotic nuclei only in G-1 treated tumors (Fig. 2. D). Ki-67 immunostaimning was significantly lower in G-1-treated tumors compared to control mice (value score control: 6,6±0,89 (SD); value score G-1 treated cells: 3,1±0.55 * (SD) (*$p<0.05$) (Fig. 2. E). Collectively these events support the idea of cells exiting G_1 but remaining stuck in G_2 phase. In agreement with the observation that inappropriate accumulation of B type cyclins is associated with the initiation of apoptotic pathways (Ling et al., 2002), we found that G-1 caused cell death by apoptosis. Cells were treated for 24 or 48h with vehicle or G-1, incubated with an Annexin-V specific antibody and sorted by flow cytometry. As shown in figure 3 D the number of dead cells increased in a time dependent manner reaching about 40% of apoptotic cells 48 h after G-1 treatment (Fig. 3. D).

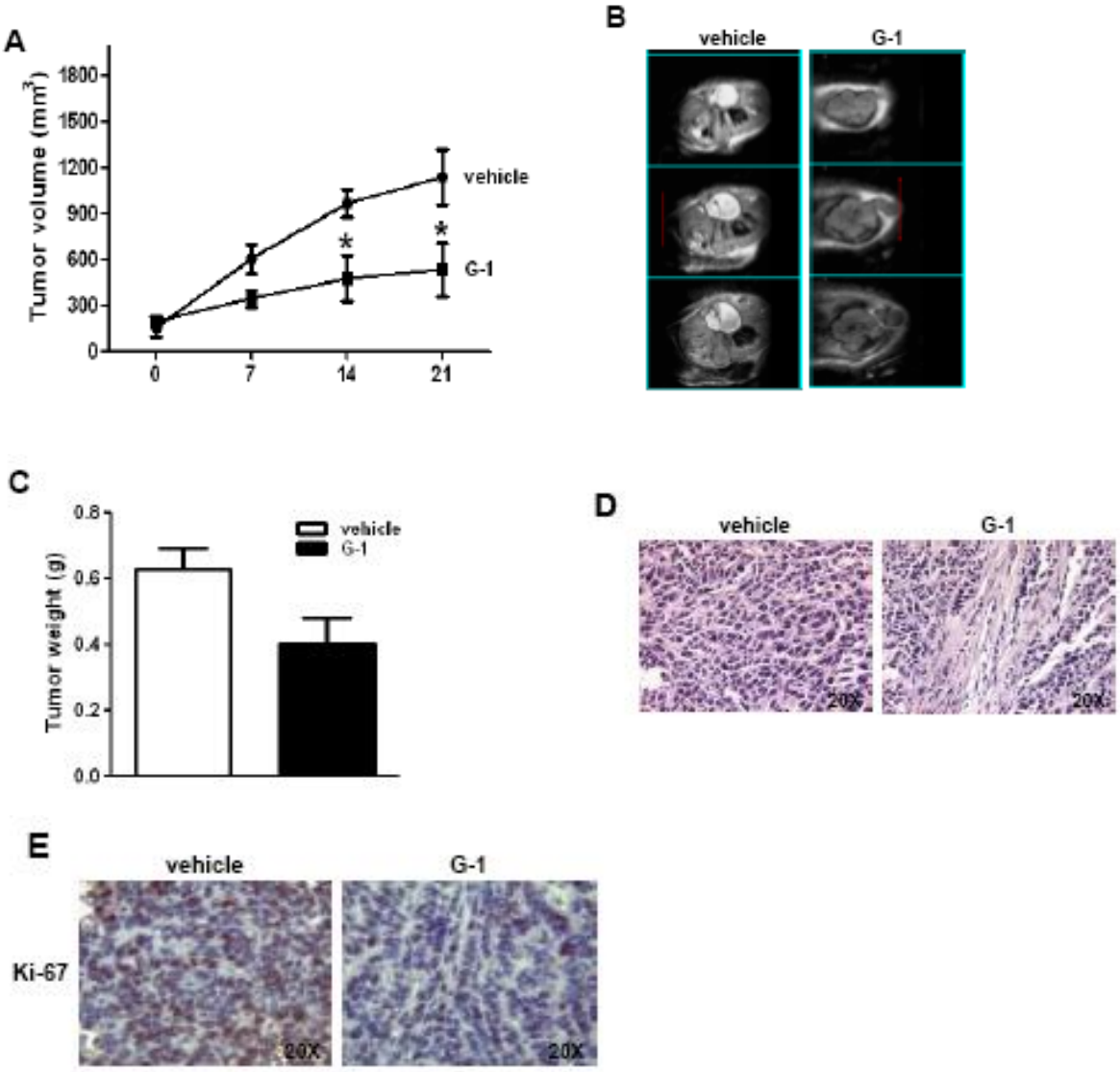
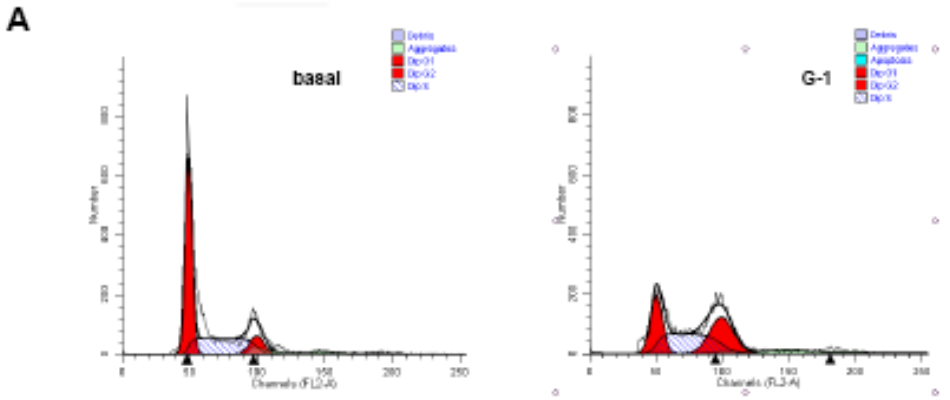


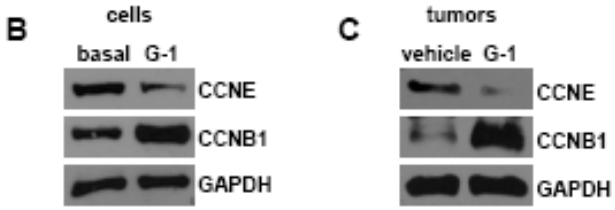
Figure 2: G-1 treatment decreases H295R cell growth *in vivo*. (A), 6 x 10^6 H295R cells were injected subcutaneously in the flank region of immunocompromized mice and the resulting tumors were grown to an average of 200 mm^3 twenty one days after inoculation. Tumor volumes were calculated, as indicated in Materials and Methods. Values represent the mean+SE of measured tumor volume over time in the control group (filled circles, n=10) and in the G-1-treated group (filled triangles, n=10). Data represent pooled values from two independent experiments. (*P<0.05 versus control at the same day of treatment). (B), *In vivo* coronal T2-weighted spin-echo MR image of primary ACCs. Examples of multi-slices T2-W MRI (section thickness of 1 mm) tumors from vehicle treated mice (control tumors) show a larger volume compared to tumors from G-1 treated mice. Hyperintense large cystic area and haemorrhagic regions that appear as dark areas in the tumor sections, are present in the control tumors. (C), After 3-week treatment tumors were harvested and weighed. Values represent the mean+SE of measured tumor weight (n=10) (*, P<0.05 versus vehicle). (D), Hematoxylin and eosin stained histologic images of H295R xenograft tumors. (E), Representative pictures of Ki-67 immunohistochemical staining of H295R xenograft tumors. NC, negative control.

6.1.2 G-1 induces H295R cell cycle arrest and cell death

Cell cycle analysis of H295R cells after 24 h of G-1 treatment demonstrated a cell cycle arrest in the G_2 phase (Fig. 3. A). This effect was further confirmed by a change in the expression of cyclins, after G-1 treatment (Fig. 3. B). Specifically, by western analysis we observed that G-1 treatment caused a decrease in Cyclin E (CCNE), while Cyclin B1 (CCNB1), involved in the regulation of G_2 phase, was increased. CCNE and CCNB1 had similar expression pattern in protein samples extracted from xenografts tumors (Fig. 3. C).



	H295R cell population (%)		
Treatment	G1	S	G2/M
basal (24h)	55,08±5,52	34,38±3,44	10,54±1,05
G-1 (24h)	26,90±1,34	40,07±2,0	33,03±1,65



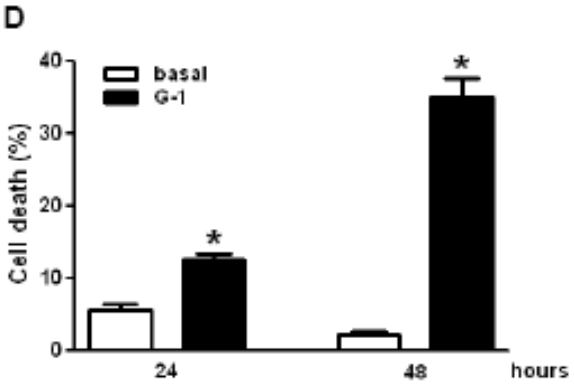


Figure 3: Effects of G-1 treatment on cell cycle distribution and on cell death. (A), H295R cells were synchronized in serum-free media for 24 h and then exposed to vehicle (basal) or G-1 (1 µM) for the indicated times. The distribution of H295R cells in the cycle was determined by Flow Cytometry using Propidium Iodide stained

nuclei. Table shows the distribution of H295R cell population (%) in the various phases of cell cycle. (B-C), Western blot analyses of Cyclin E (CCNE) and Cyclin B1 (CCNB1) were performed equal amounts of total proteins extracted from H295R cells treated with G-1 (1 μM) for 24 h (B) and xenografts tumors (C). Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. (D), Subconfluent H295R monolayers starved for 24h were treated for the indicated times with G-1 (1 μM). Then cells were stained with Annexin V/ FITC plus PI and examined by flow cytometer. Graph represents the percentage of cell death at the different times of treatment. (*, P <0.05 versus basal).

6.1.3 G-1 causes cell nuclei morphological changes, DNA damage and apoptosis

G-1 ability to trigger apoptosis in H295R cells was further confirmed by evaluation of DNA fragmentation. TUNEL staining demonstrated the presence of increased positive cells in H295R cells treated with G-1 (Fig. 4. A). In addition, Hoechst staining evidenced that untreated H295R cells had round nuclei with regular contours; while nuclei from cells treated with G-1 appeared shrunken and irregularly shaped or degraded with condensed DNA. DNA gel electrophoresis extracted from G-1 treated H295R cells revealed a classic laddering pattern of inter-nucleosomal DNA fragmentation that was absent in control cells (Fig. 4. B). This event was associated with an increase in Parp-1 cleavage (Fig. 5. A). The presence of G-1 increased Bax expression while decreased Bcl-2 (Fig. 5. B). Similarly, data obtained from western blot analysis of tumors samples overlap with those obtained in H295R cells (Fig. 5. C). When the intrinsic apoptotic mechanism is triggered, Cytochrome c (Cyt c) is released from the mitochondria into the cytosol (Oberst et al., 2008). Therefore we fractionated G-1 treated H295R cell lysates into cytosolic and mitochondrial fractions and evaluated Cytochrome c release by western blot analysis (Fig. 5. D). Cytochrome c levels increased in the cytosolic fraction of treated samples while decreased in the mitochondrial compartment. Cytochrome c release from mitochondria into the cytosol triggers caspase activation. After G-1 treatment we detected active Caspase 9 (Fig. 5. E) as well as the executioner Caspase 3/7 (Fig. 5. F).

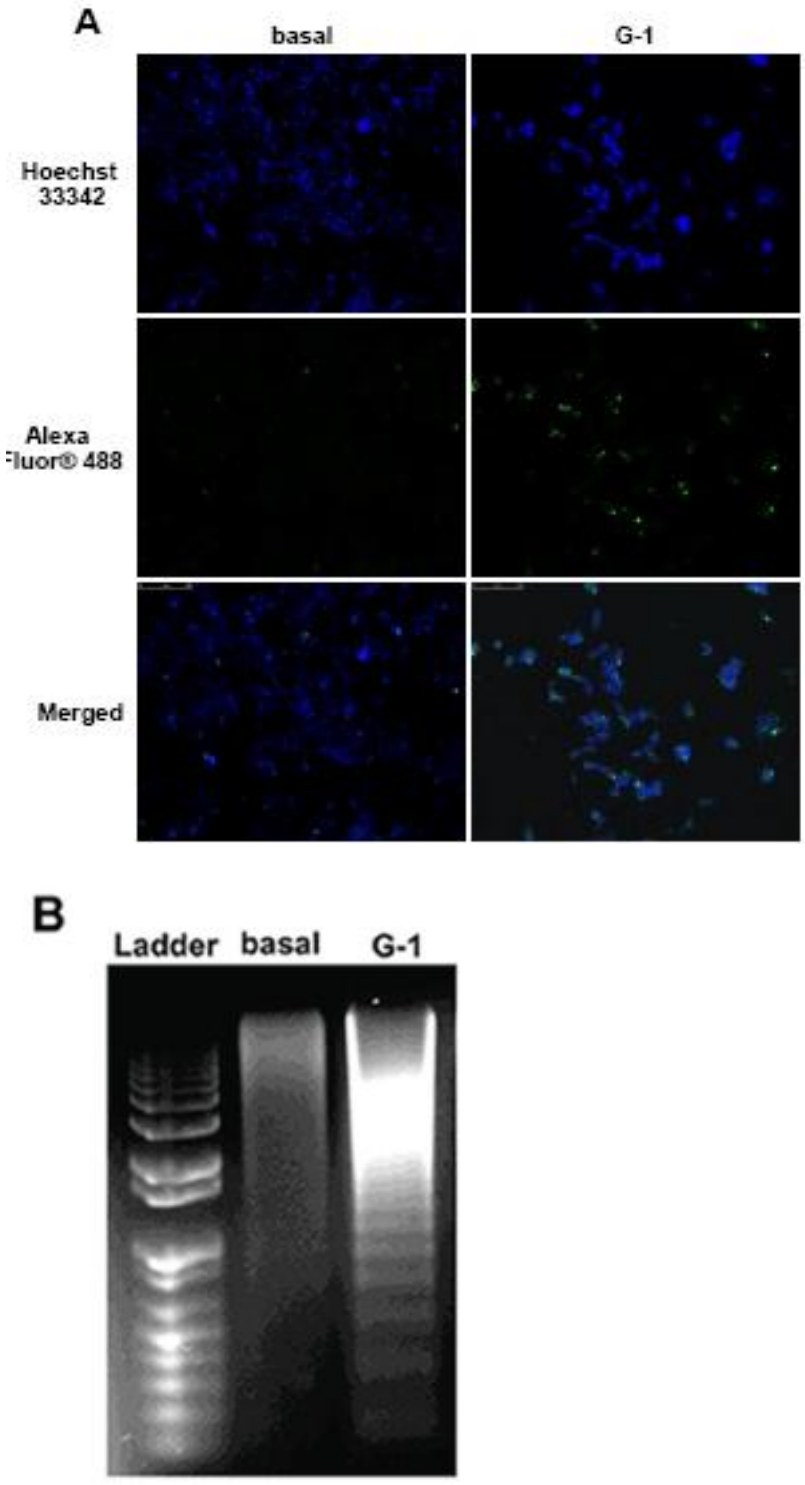


Figure 4: G-1 treatment induces apoptosis in H295R cells. (A), Cells were left untreated (basal) or treated with G-1 (1 µM) for 24 h; after treatment cells were fixed with paraformaldehyde and processed for TUNEL staining. Nuclei counterstaining was performed using Hoechst 33342. Fluorescent signal was observed under a fluorescent microscope (magnification 200X). Images are from a representative experiment. (B), After 48 h treatment DNA was extracted from cells and analyzed on a 1.5% agarose gel. Images are from a representative experiment. (C-E), H295R cells were treated with G-1 (1 µM) for 24 h.

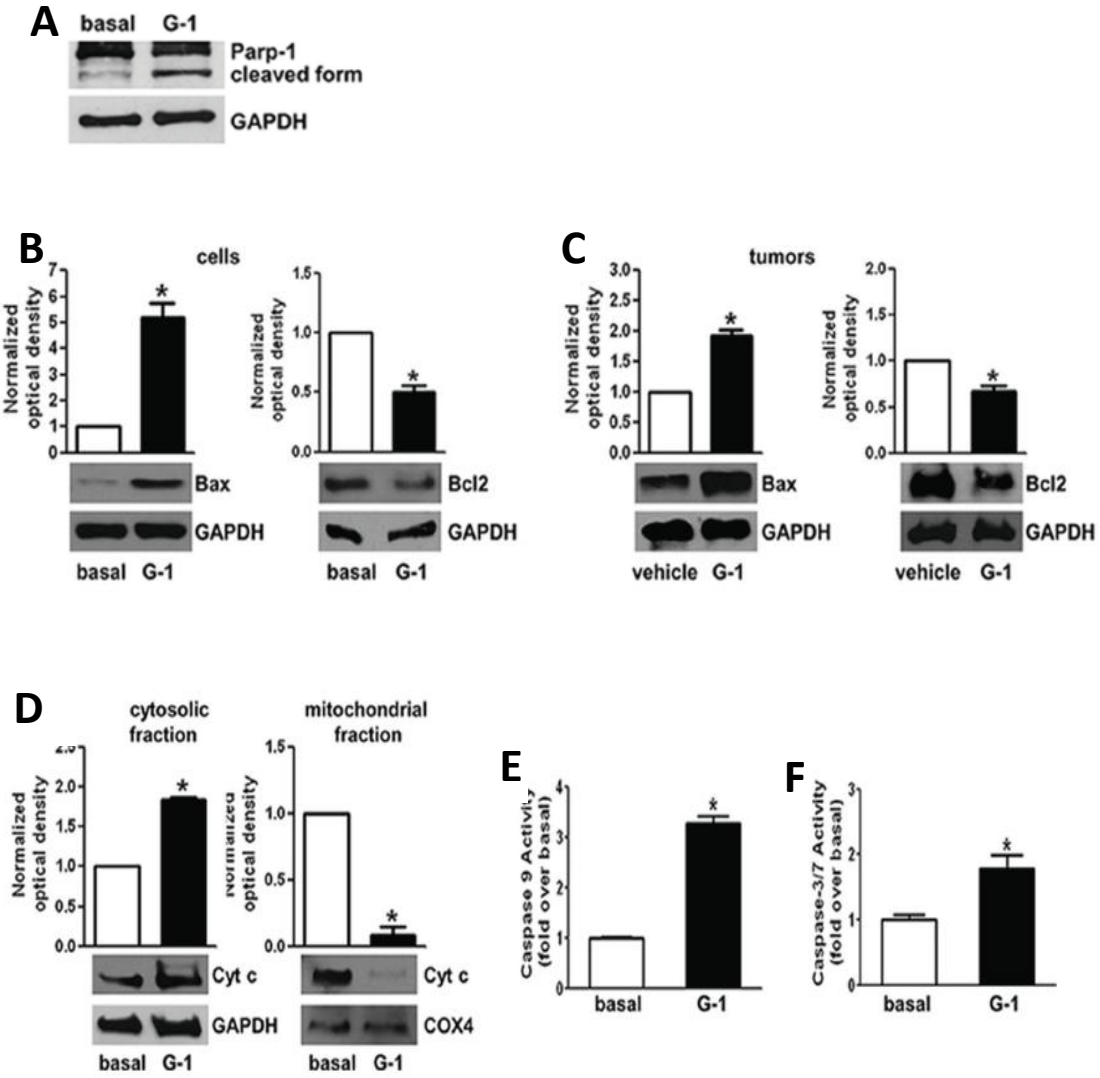


Figure 5: G-1 treatment modulates apoptotic markers in H295R cells. Western blot analyses of Parp-1 (A), Bax and Bcl-2 (B), Cytochrome c (D) were performed on equal amounts of total proteins. Blots are representative of three independent experiments with similar results. (C), Bax and Bcl-2 were analyzed on total proteins extracted from xenograft tumors. GAPDH was used as a loading control. (A-D), H295R cells were treated with G-1 (1 µM) for 24 h. Caspase 9 (E) and caspase 3/7 (F) activity was determined by a luminescent assay. Results were expressed as percentage of enzyme activity. Graphs represent mean+SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*, $P<0.05$ versus basal).

6.1.4 G-1 treatment causes sustained ERK1/2 phosphorylation

In order to define the molecular mechanism associated with G-1-induced apoptosis, we investigated the activation of MAPK family members extracellular signal-regulated kinase 1/2 (ERK1/2), which have been demonstrated to be involved in apoptosis if activated for a prolonged time (Chen et al., 2005). As shown in figure 6 A, G-1 treatment activated ERK1/2 in a time-dependent manner as seen by the increased levels of their phosphorylation status. Activation started after 30-min of G-1 treatment and

persisted for up to 24 h (Fig. 6. A). ShGPER, that partially reversed G-1 effects on cell proliferation (Fig. 6. B) did not affect ERK1/2 activation. Involvement of ERK1/2 in G-1-induced apoptosis of adrenocortical cancer cells was confirmed by the observation that MEK1 inhibitor, PD98059, prevented the up-regulatory effect exerted by G-1 on Bax expression (Fig. 6. C).

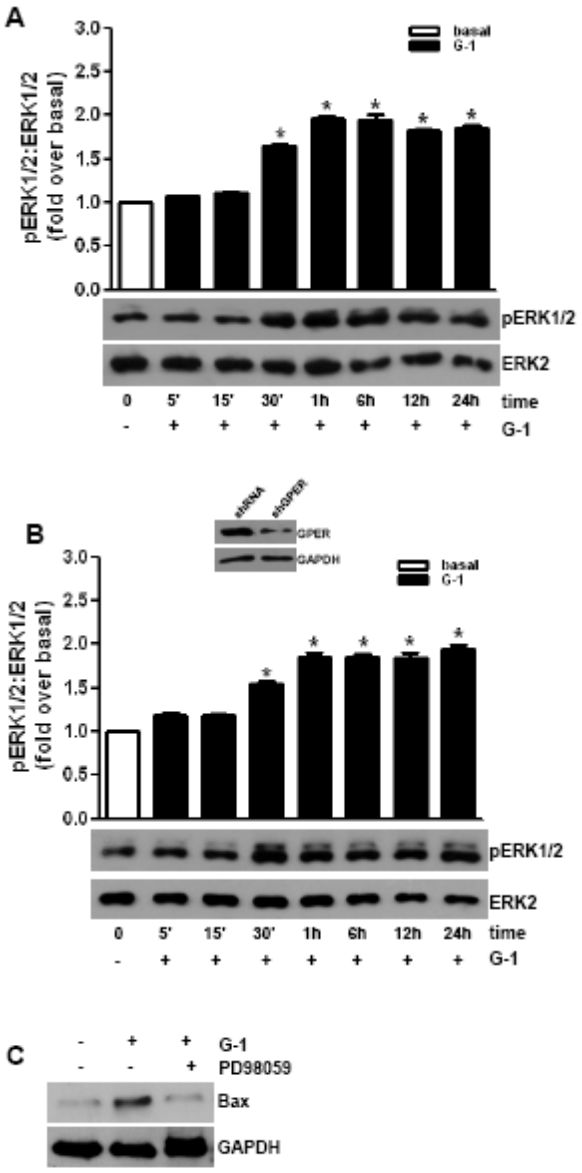


Figure 6: G-1-induced MAPK activation correlates with an increased protein expression of proapoptotic Bax. H295R cells were transfected with shRNA (A) or shGPER (B) for 72 h. Forty-eight hours after transfection cells were untreated (0) or treated for at the indicated time with G-1 (1μM). Western blot analyses of pERK1/2 were performed on 10 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. The insert in Fig. 5. B shows a Western blot on H295R protein extracts evaluating the expression of GPER receptor in the presence of shcontrol or of shGPER. GAPDH was used as a loading control. (A-B up panels) Graphs represent means of normalized optical densities from three experiments, bars represent SE. *$p < 0.05$ versus basal. (C), H295R cells were treated for 24 h with vehicle (-) or G-1 (1 μM) alone or

combined with PD98059 (10 μM). Western blot analysis of Bax was performed on equal amounts of total proteins. GAPDH was used as a loading control. Blots are representative of three independent experiments with similar results.

6.1.5 Microarray data

To further investigate the gene involved in G-1 action, as an alternative GPER-independent pathway, we measured several gene expressions, via microarray analysis. The genes examinated were 75,000. The mRNA levels of a total of 300 genes were induced at least 1.3-fold by G-1 1 μM compared with untreated control cells while, microarray analysis applied on H295R cells treated with 100 nM of G-1 did not showed any substantial changes over control. As a quality control step, the expression values were fit in a principal components analysis (PCA) and then plotted the first two principal components (PCs). PCA can be used to visualize the overall structure of high dimensional data. We expect samples with similar expression profiles to group near each other. As showed in Figure 7, G1 μM is the sample that shows the largest source of variation respect of basal.

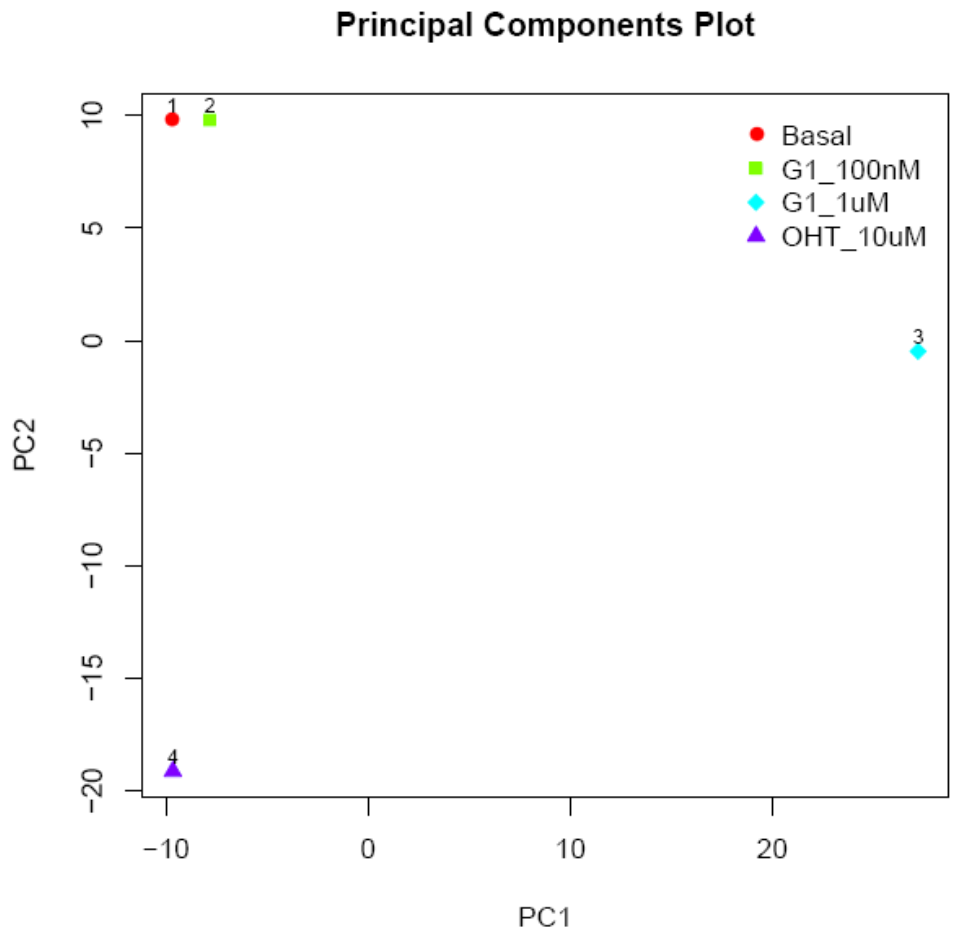


Figure 7: PCA plot. Quality control step of microarray analysis.

As showed in Figure 8, a panel with the major changes in gene expression were reported.

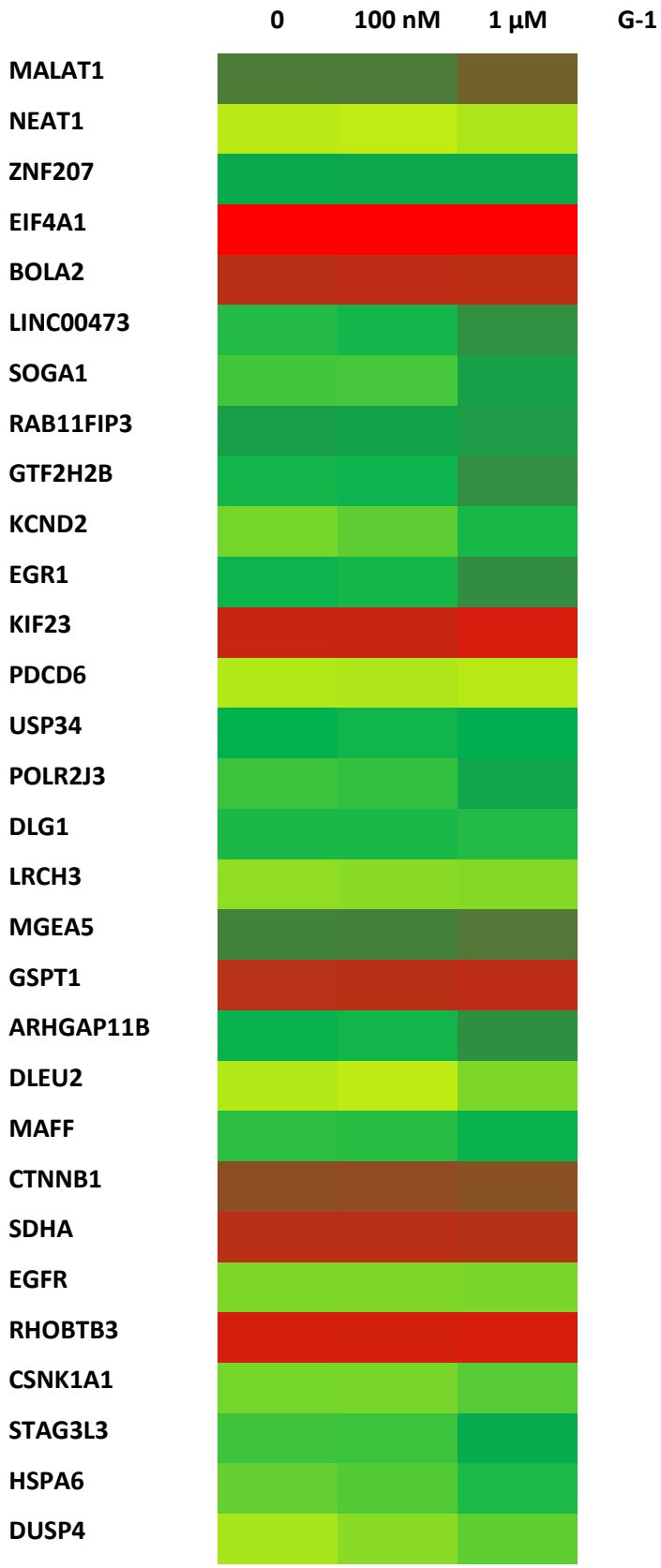


Fig. 8 Microarray data. For each gene and condition, the colour indicates the ratio of the values obtained for the treated and untreated samples

However we revealed that G-1 1 uM is able to modify the expression of 431 genes. Among these, the data analysis revealed that EGR-1 gene was upregulated by 7 fold in G-1 treated sample respect to untreated control cells.

6.2 Development of a novel cell based androgen screening model

6.2.1. AR expression in transduced CV1 cell line

A double transduction with the lentiviral hAR and Gaussia luciferase constructs was used to obtain a CV1 cell line stably expressing the hAR and the androgen responsive gaussia luciferase gene. A total of 48 geneticin and hygromycin resistant clones were obtained. Out of the androgen responsive clones, to the most responsive to testosterone exhibited an 80-fold induction of reporter gene activity. All experiments were conducted using this clone, CV1-ARluc. The untransduced parent CV1 and the transduced CV1-ARluc cell lines were initially tested for human AR expression by qRT-PCR analysis and western blot (Fig.1).

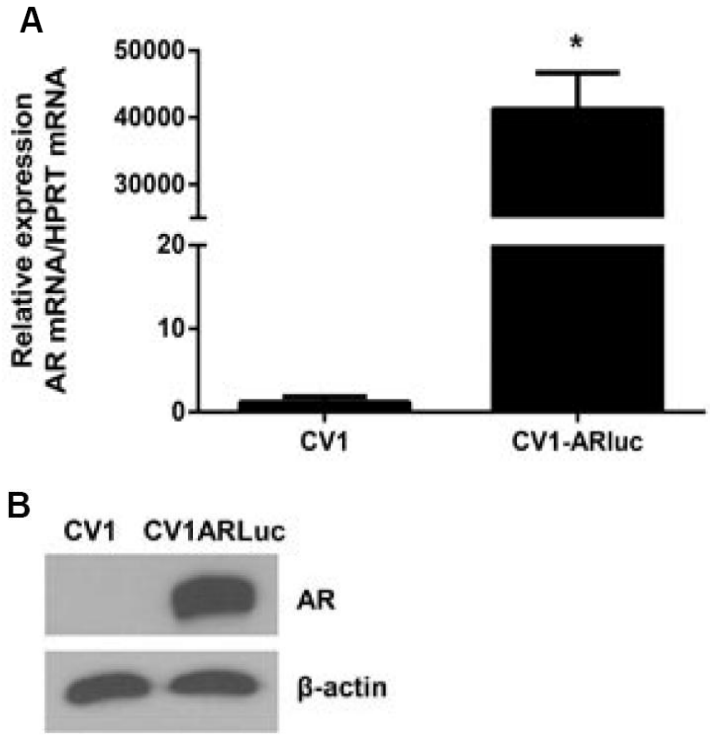


Fig. 1. **AR expression in transduced and wild type CV1 cells.** CV1 and CV1-ARluc cells were maintained in 10% FBS medium for 24 h and then lysed. (A) RT-PCR analysis of AR expression was tested in both cell lines. AR expression in CV1-ARluc was significantly higher than the CV1 cells. (B) Western analysis of AR was performed on 20 µg of total protein. β-actin was used as a loading control. The untransfected cell line did not show detectable AR, indicating the absence of this protein in this cell line. Figures are representative of three independent experiments with similar results.

We demonstrate that the CV1-ARluc cell line expresses high levels of AR, whereas AR was found to be absent in the parent CV1 cell line.

To further characterize the cell lines, we analyzed the cytoplasmic and nuclear expression of AR in CV1-ARluc by immunofluorescence (Fig. 2).

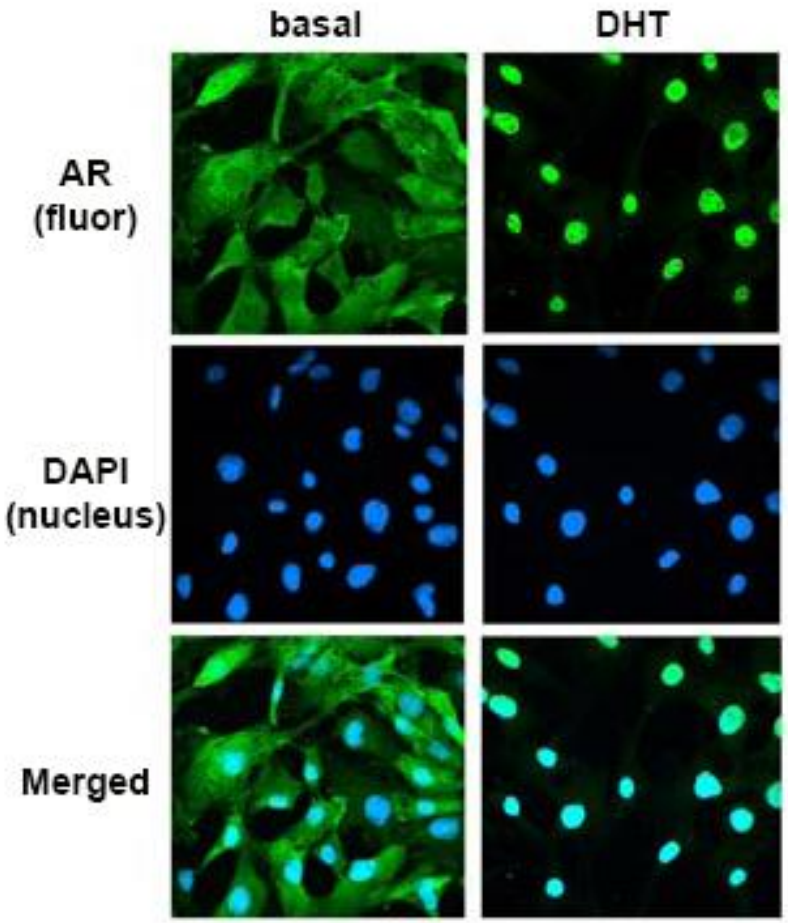


Fig. 2. **DHT binding causes AR nuclear translocation.** Fluorescence microscopy of CV1-ARluc cells stably transfected with a hAR and treated with or without 10 nM DHT for 18 h. Green fluorescence represents AR immunoreactivity and blue fluorescence is DAPI (nucleus).

Since ligand steroid receptors undergo nuclear localization, we traced the fluorescence for AR before and after treatment with DHT, a potent androgen. In the absence of the ligand, AR staining in the CV1-ARluc cells was predominantly located in the cytoplasm. Upon addition of 10 nM DHT for 18 h, 100% of the cells stained positive in the nucleus (Fig. 2). The observed translocation of AR from the cytoplasm to the nucleus in treated cells but not in untreated cells confirmed the specificity of the signal.

6.2.2 Sensitivity of CV1-ARluc cell line

As shown in Fig.3 A, the luciferase activity, after treatment with testosterone or DHT (10 nM) was clearly detectable at 3 h and increased progressively when the incubation with the respective ligands was continued up to 24 h. This result suggests that the assay can be used for steroids with androgenic activity even for short exposures.

In response to increasing concentrations of T and DHT, there was a sigmoidal increase in reporter gene expression (Fig. 3 B). The first significant response of the reporter gene expression to the androgens was detected at 0.1 nM of DHT and 0.3 nM of T (Fig. 3 B).

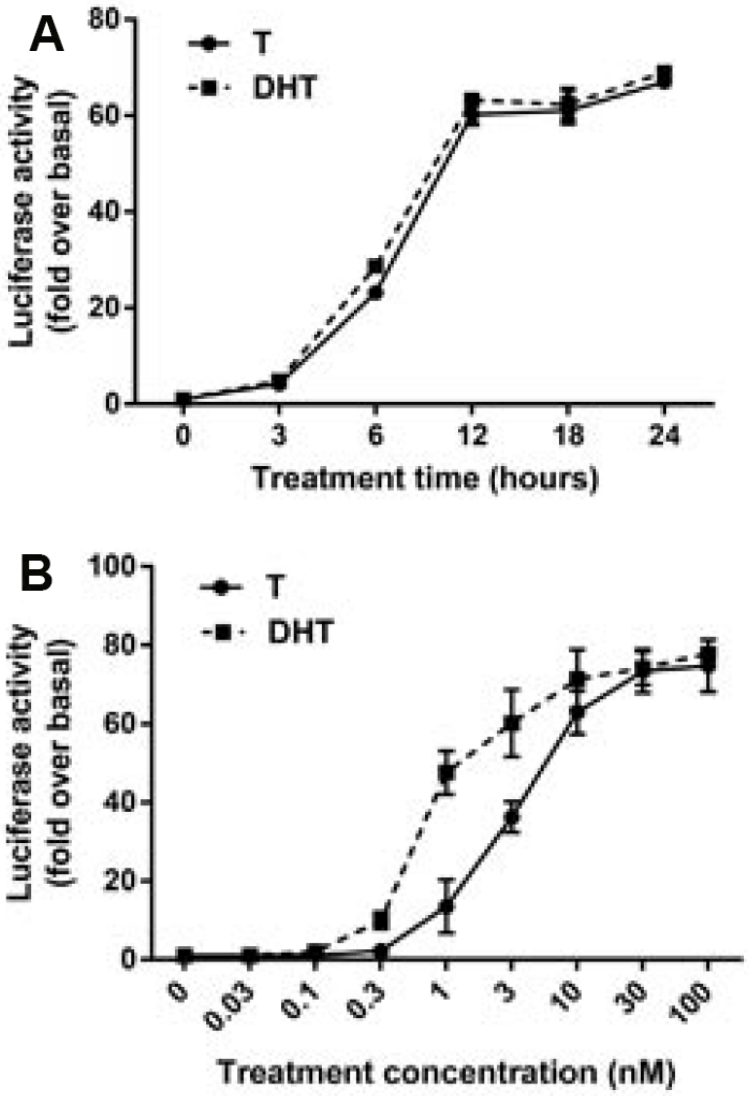


Fig.3. **Time course for AR activation and CV1-ARluc response to known androgens.** (A) The cells were incubated with testosterone and DHT 10 nM for 0-24 h. The results are expressed as fold increase over basal of luciferase activity. The results represent the mean ± S.E. of similar experiments performed in triplicate. (B) CV1-ARluc cells were incubated with increasing concentrations of two known androgens: Testosterone and DHT. The values represent the mean ± S.E. of similar experiments performed in triplicate.

The lower concentrations of steroids being able to stimulate a significant increase in luciferase expression indicate the high sensitivity of the cells.

6.2.3. Selectivity of CV1-ARluc cell line to other steroids

Since some members of the nuclear steroid receptors exhibit cross-talk activity via binding of other steroid hormones to their ligand binding domain, we examined the specificity of CV1-ARluc for androgens. This was done by incubating the CV1-ARluc cells with cortisol, progesterone and aldosterone at increasing concentrations. In addition, to insure that GR activity was not present in this model and to determine if high concentrations of cortisol regulate the AR, we incubated cells with concentrations of cortisol up to 1000 nM. As shown in Fig. 4, none of these steroids showed any significant response suggesting a high selectivity of the CV1-ARluc system towards androgenic steroids.

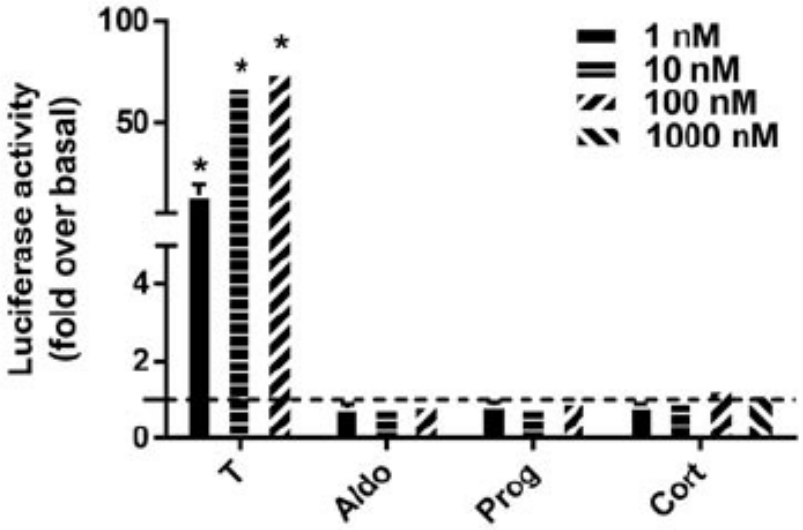


Fig. 4. **Reporter gene regulation in response to androgenic and non-androgenic steroids**. CV1-ARluc cells were incubated with 1, 10 and 100 nM of aldosterone and progesterone and 1, 10, 100 and 1000 nM of cortisol. The dotted line represents basal conditions. The data represent the mean ± S.E. of three independent experiments, each performed in triplicate (*$p < 0.05$ versus basal).

6.2.4. Treatment of CV1-ARluc cell line with different C_{19} steroids

Several studies suggested that these C_{19} steroids provide a pool of circulating precursors for peripheral conversion to more active androgens. The adrenal glands secrete a variety of C_{19} steroids including androstenedione and the 11-keto and 11-hydroxy forms of androstenedione and T. To better analyze the sensitivity of the CV1-ARluc to weaker different C_{19} steroids, the cell line was treated with T, 11-hydroxytestosterone (11OHT), 11-ketotestosterone (11KT), AD4, 11-hydroxyandrostenedione (11OHAD), 11-ketoandrostenedione (11KAD) at a constant dose of 300 nM. As showed in Fig. 5, the

luciferase activity was increased with treatment with all of the previously mentioned steroids with the exception of 11OHAD.

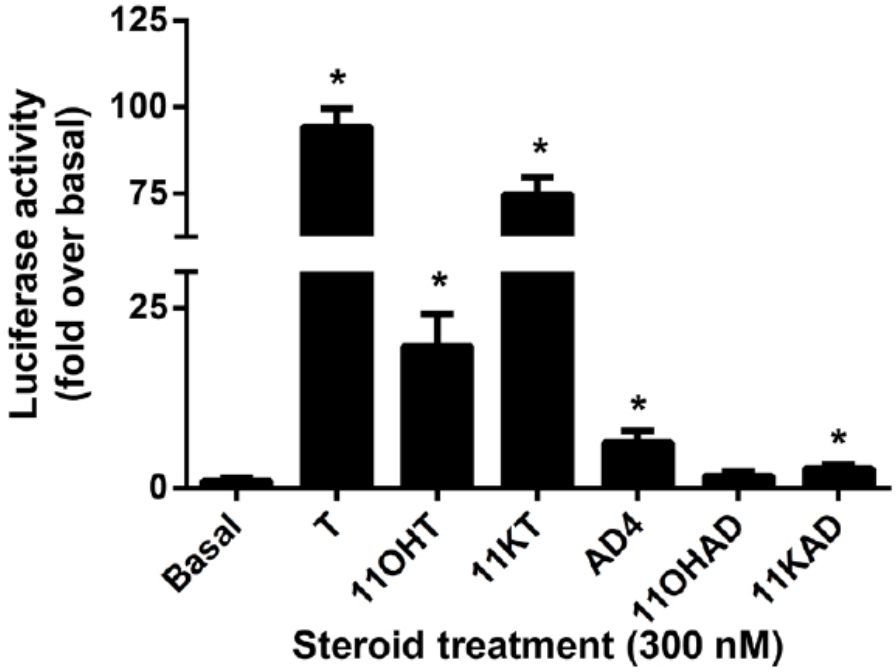


Fig. 5. **CV1-ARluc cell line treatment with different C_{19} steroids.** CV1-ARluc cells were incubated with 300 nM of T, 11OHT, 11KT, AD4, 11OHAD and 11KAD. The data represent the mean ± S.E. of three independent experiments performed in triplicate (*$p < 0.05$ versus basal).

6.2.5. Effects of a potent anti-androgens on CV1-ARluc

The androgen specificity was also demonstrated by the ability of hydroxyflutamide (OHF), a non-steroidal anti-androgen, to suppress the activity of T (Fig. 6). The effects of OHF were tested in the CV1-ARluc cells. CV1-ARluc cells were treated with increasing concentrations of OHF, in the presence and absence of the maximally stimulating concentration of T (10 nM). As shown in Fig. 6, OHF alone did not show any significant agonistic activity. However, OHF inhibited T activity in a concentration dependent manner with a significant repression seen for 1-30 µM. The response to testosterone was completely suppressed by 30 µM OHF.

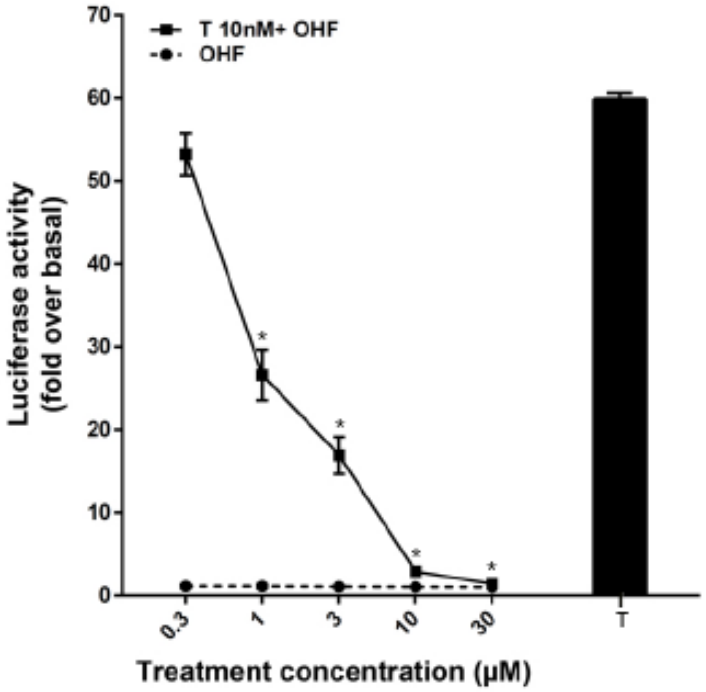


Fig. 6. **Antagonistic activity of hydroxyflutamide in CV1-ARluc.** Cells were incubated with 10 nM T alone as well as increasing concentration of OHF alone or in the presence of 10 nM testosterone. The values represent the mean ± S.E. of similar experiments performed in triplicate (*$p < 0.05$ versus 10 nM T alone).

6.*2.6 Serum androgen bioactivity*

To examine the androgen activity in human serum, the experiment was performed on extracted samples processed with solid phase extraction. In Fig. 7, a dose response curve was performed using charcoal stripped human serum prepared with different doses of T. Serum androgen bioactivity was also determined from 10 male and female serum samples between ages 20-40 years. These bioassay values indicate the androgen activity in these samples. The range value of the active androgen in female and male were equivalent to 0.9 nM and 15 nM of T, respectively. These data demonstrate that the androgen activity in serum samples can be measured with CV1-ARluc cell line.

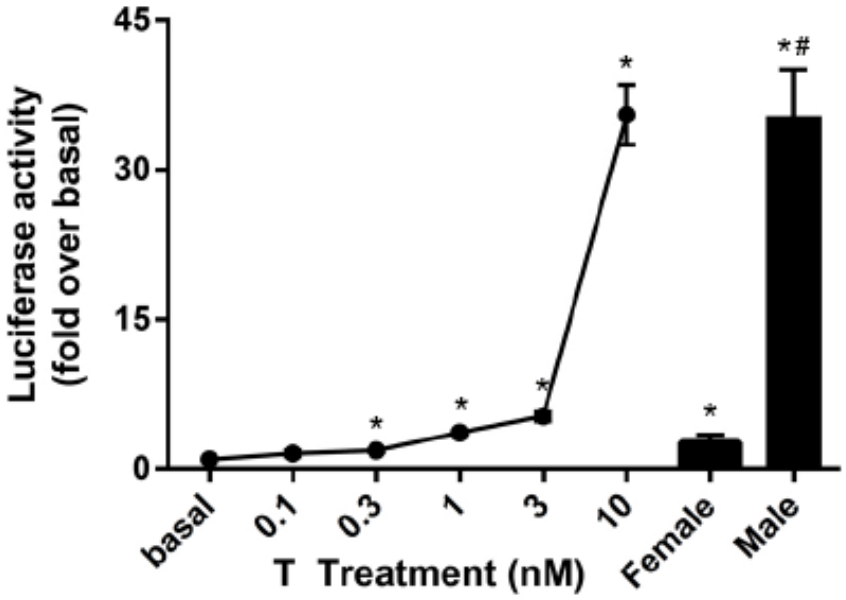


Fig. 7. **Comparison of androgen bioactivity levels in human serum samples.** Testosterone (0.3-30 nM) and human serum extract (10 male and 10 female) effects on reporter activity. Cells were treated with extracted samples for 18 h. The experiment was performed in triplicate (*$p < 0.05$ versus basal and #$p < 0.05$ versus female).

7. DISCUSSION

7.1 GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth in vitro and in vivo

In this thesis, it has been demonstrated for the first time that a selective non estrogenic ligand of GPER named G-1 is able to inhibit H295R cell growth both *in vitro* and *in vivo* in a xenograft model. Starting from these results we investigated the potential role of GPER in this event.

First, we showed GPER expression both at transcriptional and post-transcriptional level in our ACC cell model represented by H295R cells as well as in normal adrenal and ACC samples. These analyses aimed to assess only if GPER was expressed in normal and tumor adrenal and not to indicate any difference in expression levels, since overexpression of GPCR is not a common event in human diseases (Filardo et al., 2006).

Recent studies have shown that activation of GPER initiates signaling cascades that, depending on the cell type, are associated with both proliferation (Albanito et al., 2008a; Vivacqua et al., 2006b) and apoptosis (Chen et al., 2005; Chimento et al., 2013a). Ariazi et al. have highlighted the opposite effects played by GPER activation on cell proliferation of ERs negative and ERs positive breast cancer cells (Ariazi et al., 2010). Specifically, when ERs are expressed, activation of GPER leads to inhibition of cell proliferation. On the contrary, when cells are ERs negative activation of GPER leads to an increase in cell proliferation (Ariazi et al., 2010). Our work, demonstrated that micromolar concentrations of G-1 decrease H295R cell proliferation *in vitro*, significantly reduce ACC tumor volume *in vivo* and cause a marked decrease in the expression of the nuclear proliferation antigen Ki-67. Accordingly, flow cytometry analysis revealed that G-1 treatment causes changes in cellular distribution within the different phases of cell cycle. It is well established that cell cycle progression is dynamically and strictly regulated by complexes containing cyclins and cyclin dependent kinases (CDKs) (John et al., 2001). Here, we found that after G-1 treatment expression of G_1 phase cyclin CCNE was reduced, while G_2 phase cyclin CCNB1 was increased. This observation indicates that H295R cells do not bypass G_2 checkpoint. Similar data were reported for prostate cancer cells, where GPER activation by 1 μM G-1 caused cell cycle arrest at the G_2 phase (Chan et al., 2010). G_2 phase arrest was

followed by apoptotic cell death as indicated by positive staining for Annexin-V, nuclei morphological changes and appearance of DNA ladder pattern.

Apoptosis can be induced by extrinsic (Kim et al., 2006b) and intrinsic (Fadeel and Orrenius, 2005) mechanisms; the latter is strictly controlled by bcl-2 family of proteins (Cory and Adams, 2002) that consists of both pro- (Bax, Bad, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-xl) proteins able to modulate the execution phase of the cell death pathway. Bax exerts pro-apoptotic activity by allowing Cytochrome c translocation from the mitochondria to the cytosol (Antonsson et al., 2000). Cytochrome c then binds to apoptotic protease-activating factor-1 (Apaf-1) (Wang, 2001), which in turn associates with Procaspase 9 resulting in the activation of its enzymatic activity (Kuida et al., 1998), responsible for the proteolytic activation of executioner Caspase 3 (Wilson, 1998). The active Caspase 3 is then involved in the cleavage of a set of proteins including Poly-(ADP) ribose polymerase-1 (Parp-1) (Soldani and Scovassi, 2002). Bcl-2, instead, exerts its anti-apoptotic activity, at least in part, by inhibiting the translocation of Bax to the mitochondria (Wang, 2001). Changes in expression and/or activation of all the above mentioned biochemical markers of mitochondrial apoptotic pathway were observed in H295R cells in response to G-1 treatment.

MAPK family members ERK1/2 are part of GPER signaling (Lappano et al., 2013). Despite the well-defined role of ERK1/2 activation in proliferative pathways (Meloche and Pouyssegur, 2007), sustained ERK1/2 phosphorylation is involved in apoptotic events (Chen et al., 2005; Chimento et al., 2013a; Ramos, 2008). Cagnol and Chambard have summarized more than 50 publications showing a link between prolonged ERK activation and apoptosis (Cagnol and Chambard, 2010). Specifically it can be appreciated that duration of ERK activation in promoting cell death can be different depending on cell type and stimuli. G-1 caused sustained ERK1/2 activation in H295R, this event was clearly involved in the induction of apoptosis, since chemical inhibition of MEK1/2 using PD98059 abrogated G-1 ability to induce the expression of proapoptotic factor Bax. Several reports pointed out that ERK1/2 activity can be associated with upregulation of proapoptotic members of the Bcl-2 family, such as Bax (Chen et al., 2010; Tan and Chiu, 2013; Tong et al., 2011). Moreover, ERK activity has been shown to directly affect mitochondrial function (Cagnol and Chambard, 2010) by decreasing mitochondrial respiration (Nowak, 2002; Nowak et al., 2006) and mitochondrial membrane potential (Kim et al., 2003; Nowak et al., 2006), causing

mitochondrial membrane disruption and Cytochrome c release (Kim et al., 2003; Li et al., 2005; Zhang et al., 2004).

Interestingly, GPER silencing was not able to prevent G-1 induced ERK xphosphorylation, underlying the existence of alternative targets for G-1. These targets, similarly to GPER, are able to activate ERK1/2 signaling, however for a prolonged period, and clearly deserve further investigation.

Other papers evidenced inhibitory effects exerted by G-1 on the growth of different tumor cell types in a GPER independent manner (Gui et al., 2015; Holm et al., 2012; Wang et al., 2012), but a precise mechanism has not been defined. Although further studies are needed to clarify the molecular mechanisms behind G-1-dependent effects , this molecule could be a viable alternative to the current limited treatment options and therapeutic efficacy for adrenocortical cancer.

In conclusion, we demonstrated that treatment of H295R cells with G-1 reduced tumor growth *in vitro* and *in vivo* through a mechanism involving not only GPER activation. G-1 clearly causes cell-cycle arrest at the G_2 phase and apoptosis through a mechanism that requires sustained ERK1/2 activation. Our previously published results highlighting the ability of OHT, a known GPER agonist and ESR1 antagonist, to reduce ACC cell growth, together with the present findings indicating the inhibitory effects exerted by G-1, open up new perspectives for the development of therapies with molecules modulating estrogen receptors action for the treatment of ACC. To further define the molecular mechanisms activated by G-1 but that are GPER-indipendent, we used microarray technology. Microarray analysis revealed in particular a significant upregulatory effect, elicited by micromolar concentration, of EGR-1 gene. EGR-1 is a nuclear phosphoprotein that was first identified based on its early induction by mitogens and differentiation factors (Liu et al., 1998; Milbrandt, 1987). EGR1 contains a highly conserved DNA-binding domain composed of three zinc fingers that bind to the prototype target GC-rich consensus sequence GCG (G/T) GGGCG (Liu et al., 1998; O'Donovan et al., 1999). EGR-1 is induced by growth factors, cytokines and stress signals such as radiation, injury or mechanical stress (Gashler and Sukhatme, 1995; Liu et al., 1998; O'Donovan et al., 1999). Cloning of the *EGR-1* promoter has revealed the presence of response elements for various transcription factors. Specifically, the promoter contains several serum response elements (SREs), an AP-1 binding site, several cAMP regulatory elements (CREs) and Sp1 consensus sequences (Schwachtgen et al., 2000). Most often, increased transcription of EGR-1 is mediated by the MAPK

signaling pathway. While ERK1/2 mediates EGR1 expression in response to growth factors (Schwachtgen et al., 2000), a combination of ERK1/2, p38MAPK and/or JNK is required to induce EGR-1 in response to stress (Lim et al., 1998; Rolli et al., 1999). An alternative pathway for EGR-1 activation was recently discovered, in which transcription factor NF-κB mediates EGR-1 transcription in response to UV exposure of human skin. In another study, two functional nonconsensus binding sites for the tumor suppressor p53 were also identified. Binding of p53 to the *EGR-1* promoter in response to DNA damage leads to sustained expression of EGR-1 and efficient apoptosis (Yu et al., 2007). All the above mentioned factors and signaling pathways are all involved in adrenocortical cancer initiation and progression (Else et al., 2014). The paradoxes of EGR-1 function is that while it is able to mediate apoptosis in response to stress and DNA damage by regulating a tumor suppressor network, it can also promote cell proliferation in different cancer cell types by a mechanism that is not fully understood. In this latest context it is worth noting that G-1 was able to induce a growth stimulatory effect in breast cancer cells as well as in cancer associated fibroblasts by up-regulating EGR-1 gene via GPER-dependent mechanisms (Vivacqua et al., 2012). Nevertheless, the transcription factor EGR-1 is a direct regulator of multiple tumor suppressors that constitute a functional network that serve to maintain normal growth regulation and resist the emergence of transformed variants. In line with its physiological tumor suppressive role, EGR-1 could be a key factor in G-1-induced cell apoptosis in adrenocortical cancer. The microarray data suggest a deeper investigation of the molecular mechanism behind the up-regulatory effect elicited by micromolar concentration of G-1 in H295R cells. Indeed, we already performed some in vitro experiments (data not showed) on H295R that evidenced the ability of G-1 to increase EGR-1 protein content as well gene transcription, confirming the results from microarray data. These preliminary results let us to plan further experiments focusing on the role of EGR-1 in G-1-mediated apoptosis in ACC cells.

7.2 Development of a novel cell based androgen screening model

We have developed an *in vitro* assay that is simple, rapid, and quantitative with potential to screen active androgenic compounds including those in human serum. The CV1-ARluc cell line expresses an androgen-responsive luciferase reporter gene and a human AR gene. The CV1-ARluc cell line combines high stability, fast growth, high selectivity, high sensitivity and rapid response to androgens.

Cell-based assays can provide several benefits compared with immunoassay, mass spectrometry and various chromatography methods (Raivio et al., 2002). Over the past several years, there has been an expansion in the use of gas chromatography-mass and liquid chromatography tandem mass spectrometry for measurements of androgens. These methods are able to analyze multiple steroid hormones but they are not useful in defining the activity of unknown androgenic steroids. Cell-based hormone receptor assays have become an important resource for drug discovery and androgen related diseases. The cells used to develop these assays should have two specific requirements: a reporter system driven by an ARE and an abundant expression of AR. A variety of reporter genes have been used in the past years including β- lactamase (Wilkinson et al., 2008), β-galactosidase (Chatterjee et al., 2007; Gaido et al., 1997; Lee et al., 2003; Nishikawa et al., 1999; Purvis et al., 1991; Sohoni and Sumpter, 1998) and luciferase (Blankvoort et al., 2001; Eldridge et al., 2007; Hartig et al., 2002; Kim et al., 2006a; Leskinen et al., 2005; Michelini et al., 2005; Sedlak et al., 2011; Wilson et al., 2002b) reporter genes. To develop an androgen reporter line superior to the ones available, we chose not to use yeast cells for several reasons. Although yeast-based reporter models (Sohoni and Sumpter, 1998), have certain advantages (easy handling, rapid growth, inexpensive media components), they require laborious cell preparation and complex cell lysis steps. Importantly, using yeast assays to express mammalian proteins also raises concerns regarding glycosylation, phosphorylation and post translational modifications. Moreover, the estimation of androgenic bioactivity in serum with yeast-based assays has been unsuccessful. In yeast cell lines, anti-androgens have not shown any antagonistic effects, probably due the permeability problem of the cell walls.

To avoid interference with other nuclear receptors, we selected a cell line with a low background activity of C3 group nuclear receptors and with a good response to androgens when the hAR was stably introduced. We chose to use an MMTV promoter-driven luciferase gene since this approach has been shown to be successful in generation of *in vitro* and *in vivo* models for screening of estrogen compounds (Legler et al., 1999). With the present work we show that, this approach can be successfully used to generate a selective androgen reporter cell line, CV1-ARluc. The MMTV promoter is quite selective to AR as well as to GR and PR and, also, contains a number of regulatory sites that can be targeted by other steroids. Wilson et al (Wilson et al., 2002a) developed a stable cell line, MDA-kb2, a derivative of a human breast cancer cell line, containing a stably integrated MMTV-luciferase reporter. This cell line strongly responds to

glucocorticoids due to endogenous GR. This makes the cell line unsuitable as a selective screening tool. PC3 cells transfected by Kim et al (Kim et al., 2006a), show more androgen specificity obtained by transient transfection of an MMTV promoter and a human AR with high sensitivity. Other bioassays for screening of androgen compounds in human serum have been reported using transient transfection; we have used a stable transduction to develop our cell model because stable expression of AR can eliminate the variability associated with repeated transient transfection. A different cell line, using CHO cells and a stable transfection, was developed by Paris et al (Paris et al., 2002). The cell line was stably transfected with hAR but has retained glucocorticoid response due to GR. The T47D cell line was used by Blankvoort et al (Blankvoort et al., 2001) to develop a new androgen cell-based assay named AR-LUX. T47D expresses an endogenous AR and was stably transfected with a luciferase reporter gene. This assay was used to estimate the levels of some anabolic steroids in the urine of cattle. However, this cell line also responds to added estrogen and progesterone, which may reduce the specificity of the assay.

There is one additional AR bioassay that is highly selective for androgens and there is no reporter response to non-androgenic steroids (Wilkinson et al., 2008). Wilkinson et al developed an AR bioassays using the HEK293 cell line. This model is particularly applicable for high throughput screening for AR activation. However, this model makes use of a hybrid AR receptor (GAL4DBD-ARLBD) and therefore may not have all regulatory characteristics of the full length AR.

In the present study, we have developed a new cell line, named CV1-ARluc, which stably express the human AR and an androgen-driven gaussia luciferase reporter. The cell line is able to estimate levels of androgen bioactivity in human serum samples. The androgen specificity of the assay was tested by using high concentrations of different steroids such as cortisol, progesterone and aldosterone, which did not active reporter expression. The low background activity of C3 group nuclear receptors in the CV1 cell line was supported by RT-PCR data, where no GR, ER, AR, PR and MR expression was detected. The specificity of the assay was also showed by checking the inhibitory effects of a synthetic anti-androgen receptor ligand, OHF. OHF anti-androgen effects were also seen in other AR models including CHO (Roy et al., 2004) , DU-145 (Miyamoto et al., 1998) and PALM (Terouanne et al., 2000). We evaluated androgenic bioactivity in male and female serum samples. In concordance with the data previously reported using a different *in vitro* assay to detect androgen activity in human serum

samples by Roy et al (Roy et al., 2008), our assay shows an androgen bioactivity in female serum samples from 0.8 to 2 nM and from 10 to 25 nM in normal males. It can be concluded that this assay provides a valid and practical method to analyze serum samples for androgenic activity. For example this assay could be used to evaluate the physiological androgen levels during androgen-related diseases as the Polycystic ovarian syndrome.

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Development of a novel cell based androgen screening model

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ABSTRACT

The androgen receptor (AR) mediates the majority of androgen effects on target cells. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for glucocorticoid, mineralocorticoid and progesterone receptors (GR, MR and PR, respectively). As a result, many of the current AR screening models are complicated by inaccurate activation of reporters by one of these receptor pathways. Identification of more selective androgen testing systems would be beneficial for clinical, pharmacological and toxicologic screening of AR activators. The present study describes the development of a selective androgen-responsive reporter cell line that expresses AR but does not express GR, MR and PR. CV1 cells were stably transduced to express human AR and an androgen-responsive gaussia luciferase gene. Clonal populations of AR expressing cells were isolated. Quantitative RT-PCR (qPCR) and western analysis confirmed stable integration of AR in the most responsive clonal line which was named 'CV1-ARluc'. Stimulation of CV1AR-luc with androgenic ligands (testosterone and 5α-dihydrotestosterone) for 18 h caused an increase in luciferase activity in a dose-dependent manner. Other steroid hormones including aldosterone, cortisol, and progesterone did not stimulate luciferase response. The CV1-ARluc also increased luciferase activity when treated with human serum extracts. In conclusion, the CV1-ARluc cells provide a novel model system for screening of new AR agonists and antagonists and can determine the androgenic activity of human serum samples.

1. Introduction

Androgens are hormones that play an essential role in the differentiation and maintenance of primary and secondary male sexual characteristics [1]. The two main human androgens are testosterone (T), which is involved in the initial virilization phases of the human male embryo, and 5α-dihydrotestosterone (DHT), which is the active hormone in most androgen target tissues [2]. T is mainly synthesized by the testicular Leydig cells, in peripheral tissues, as well as to a lesser degree in ovaries and adrenals. T is converted to DHT by 5α-reductases and also can be converted to estradiol by aromatase. DHT is the most active physiologic androgen, inducing ten-fold higher androgen receptor (AR, *NR3C4*) bioactivity than T [3,4]. In addition, other endogenously produced steroids exhibit various degrees of androgenic activity [5,6]. Several synthetic androgen-related compounds (AR agonists and antagonists) have also been developed to modulate androgen signaling in therapeutic settings [7,8].

Androgens mediate their effects through binding and activation of the AR. AR is a member of the steroid nuclear receptor superfamily [9] and acts as a ligand-dependent transcription factor [10]. Among this family, five steroid receptors are known: estrogen (ESR, *NR3A1*), progesterone (PR, *NR2C3*), androgen, mineralocorticoid (MR, *NR3C2*) and glucocorticoid (GR, *NR3C1*) receptors. AR activates a wide range of target genes that encode proteins and noncoding RNAs, including regulatory microRNA species [11].

Similar to the other steroid receptors, unbound AR is located in the cytoplasm. Upon ligand binding, AR goes through a series of

Abbreviations: AR, androgen receptor protein; ARE, androgen response element; GR, glucocorticoid receptor protein; MR, mineralocorticoid receptor protein; PR, progesterone receptor protein; T, testosterone; DHT, 5α-dihydrotestosterone; Cort, cortisol; AD4, androstenedione; Prog, progesterone; 11OHT, 11-hydroxytestosterone; 11KT, 11-ketotestosterone; 11OHAD, 11-hydroxyandrostenedione; 11KAD, 11-ketoandrostenedione.

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conformational changes, dimerization and translocation to the nucleus, which is mediated by a nuclear localization signal. Translocated AR binds to androgen response elements (ARE). These ARE are characterized by a consensus (or near consensus) sequence 5′-TGTTCT-3′, which is located in the promoter or enhancer regions of AR gene targets. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for GR, MR and PR. The similarity of the response element for AR and the other steroid receptors, and particularly the wide-spread expression of the GR, has been problematic in the development of selective receptor screening assays.

The determination of androgen levels or the discoveries of new androgenic compounds are key elements for the diagnosis of a number of diseases in children and adults. Assays that detect bioactive serum androgens in a sensitive and selective manner benefit the diagnosis and treatment of several pediatric endocrine disorders, such as precocious puberty and ambiguous genitalia. In addition, androgen bioassays provide a screening tool for androgen abuse and endocrine disruptors [12]. Over the past 10 years, several bioassays were developed using different methods [13]. One of the first assays developed relied on a chloramphenicol acetyltransferase (CAT) reporter model [14]. This system was limited by experimental variation due to the transient nature of transgene expression. A luciferase reporter bioassay, using MDA-MB453 cells, was developed by Wilson et al. [15]. The major caveat of this assay was that it responds to AR as well as to GR agonists. Other androgen-reporter cell lines were developed but most of them were transiently transfected [16–18]. Transient transfection assays [19] can provide similar information with stable assays but may not reflect endogenous levels of receptor. A stable expression of AR in the cells can eliminate the need for repetitious transient transfections, reduce the variability associated with these transient assays and moreover be utilized for high-throughput studies. Until now, a selective androgen-responsive transcriptional activation assay has not been widely available.

The aim of this study was to develop a stable cell-based *in vitro* bioassay that expresses the human AR (hAR) gene with sensitive and selective reporter readout. For this purpose, a stable cell line was made with CV1 cells stably transduced with hAR and an MMTV promoter-driven luciferase reporter gene. The resulting model is selective for androgens and does not exhibit reporter activation by other steroid receptors. In addition the model appears useful to determine circulating androgenic bioactivity in human serum samples.

2. Materials and methods

2.1. Materials

T, DHT, cortisol (Cort), progesterone (Prog), aldosterone (Aldo), androstenedione (AD4), hydroxyflutamide (OHF) and the 11-keto and 11-hydroxy forms of androstenedione and T were purchased from Sigma (Missouri, USA). Coelenterazine used for the luciferase assay was purchased from Promega (Wisconsin, USA). Penicillin, streptomycin, hygromycin, geneticin (G418) and DMEM/F12 medium were purchased from Life technologies (New York, USA).

2.2. Cell line

The CV1 monkey kidney cell line was obtained from the American Type Culture Collection (ATCC). The cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) from GE Healthcare Life Science (Utah, USA) and antibiotics including 1% penicillin/streptomycin. The cells were incubated under a humid atmosphere of 5% CO_2, at 37 °C, and the medium was changed every 3 days. CV1 cells were plated at a density of 20,000 cells/well (48 well dish) in growth medium and grown to 60% confluence after which they were treated for steroids activity.

CV1-ARluc cells were plated in a 48 wells culture plate in 500 μL of growth medium (10% FBS/DMEM-F12, G418 and Hygromycin). The cells were incubated under a humid atmosphere of 5% CO_2, at 37 °C. All treatments were performed with charcoal-stripped FBS serum to eliminate contaminating steroids.

2.3. Stable transduction

CV1 cells (20,000 cells/well) were plated about 18 h before transduction in a 48 well-dish. The lentivirus pBM14-MMTV with the *Gaussia Luciferase* gene was diluted 1:10 in DMEM/F12 medium and added to the flask with 8 μg/mL of polybrene. The flask was centrifuged at 1200 rpm for 80 min and after 4 h in a humidified 5% CO_2 incubator the cells were supplemented with 1 mL of DMEM/F12 containing 10% FBS without any antibiotics. After 48 h, the cells were selected in medium containing 1200 μg/mL of G418. The medium was changed three times a week. The obtained cells, named CV1-luc, were transduced with a lentivirus containing the hAR gene and the hygromycin selective gene. The stable transfection was performed as described above, using a multiplicity of infection (MOI) of 10 and 8 μg/mL of polybrene. 50 clones were obtained after 14 days of dual antibiotic (G418 and Hygromycin) selection. The clones were isolated using cloning rings (Sigma, Missouri, USA) and re-seeded and grown in a 48-well dish. After reaching 60% confluence, the cells were treated in DMEM/F12 containing 10% charcoal-stripped FBS and 10 nM of testosterone. After 24 h the treated cells were assayed for luciferase activity using the appropriate luminescence kit (Coelenterazine, Promega). The clone with the largest T induced luciferase activity was named CV1-ARluc and was used for further studies.

2.4. Isolation of RNA and qPCR analysis

The cells (25,000 cells/well) were grown for 24 h in 48 well culture. Total RNA was isolated from the cells previously plated using an RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quantity and purity were assessed by a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). For cDNA generation, 100 ng of total RNA was reverse transcribed using the High Capacity Kit (Applied Biosystems, Foster City, CA, USA). For qPCR, 12 ng of prepared cDNA was mixed with Fast Universal PCR Master Mix (Applied Biosystems). AR and peptidylprolyn isomerase A (PPIA) primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). PPIA was used as the housekeeping control gene.

2.5. Protein extraction and protein assay

Cells were lysed in 200 μL Mammalian Protein Extraction Reagent (Pierce Chemical Co., Illinois, USA), and the protein content was estimated by the bicinchoninic acid (BCA) protein assay using the BCA protocol (Thermo Scientific, Illinois, USA).

2.6. Western analysis

CV1 and CV1-ARluc cell lines were plated at a density of 75,000 cells/well (24 well-dish), in growth medium. Samples were lysed with lysis buffer (2% sodium dodecyl phosphate, 62.5 μM Tris, 0.04% bromophenol blue, 0.5% dithiothreitol) and heated at 95 °C for 5 min. Proteins were then loaded (20 μg) on 10% bis-Tris gel and electrophoresed for 1 h before transferring to polyvinylidene difluoride membranes. The membranes were then blocked

with 5% BSA for 1 h and incubated with primary antibody (AR, polyclonal rabbit antihuman, 1:1000 BSA, Sigma) and secondary antibody (goat anti-rabbit, 1:5000, Life Technologies). The Pierce ECL Western Blotting Substrate kit (Life technologies) was then used for signal development.

2.7. AR translocation study

CV1-ARluc cells were grown on microscope slides from Globe Scientific (previously treated with 50 μg/ mL of Poly D-iysine at room temperature for 1 h) in 100-mm plates for 24 h in growth medium. Cells were treated with DHT for 18 h and subsequently fixed with methanol at −20 °C for 20 min and washed three times with PBS. Slides were then incubated overnight with a rabbit anti-human AR antibody (Sigma) and then with a secondary goat anti-rabbit antibody (Sigma) for 1 h at room temperature. Prolong Gold mounting medium with DAPI was used to visualize the cell nucleus.

2.8. Gaussia luciferase analysis

CV1 and CV1-ARluc cells were plated at a density of 25,000 cells/well (48 well-dish), in growth medium for 24 h and then treated for indicated time points. The treated medium was collected and 25 μL was mixed with 50 μL of coelenterazine (previously diluted 1:100 in 50 mM Tris, 150 mM NaCl and water). Luminescence was then measured by FLUOstar OPTIMA microplate reader according to the manufacturer's instructions (Life Technologies).

2.9. Sera

Charcoal dextran stripped human serum was obtained by Equitech-Bio. In the present study, we analyzed human serum from 20 healthy adults (10 females and 10 males), age 20–35 years. All samples were collected under protocols approved by the Institutional Review Board (IRB) at the University of Michigan.

2.10. Extraction method

The indicated concentrations of steroids were prepared separately in DMEM/F12 medium with10% charcoal-stripped FBS, and stripped human serum using ethanol as a carrier solvent. Several extraction methods were tested and were found to have variable abilities to disrupt androgen regulation of reporter activity (data not shown). The method that exhibited androgenic activity most similar to unextracted medium was solid phase extraction using Sep-Pak Cartridges column from Waters (Chromatography division Millipore Corporation, MA, USA). The columns were activated with 4 mL of methanol and subsequently washed with 4 mL of deionized water. Standards made separately in DMEM/F12 with 10% charcoal stripped FBS and stripped human serum were dispensed at a volume of 600 μL in the columns, followed by another wash and elution with 2 mL of 100% methanol. Samples were evaporated at 37 °C using a thin stream of nitrogen gas and the dried extract was then re-suspended in 300 μL of DMEM/F12 medium with 10% charcoal stripped FBS.

3. Results

3.1. AR expression in transduced CV1 cell line

A double transduction with the lentiviral hAR and Gaussia luciferase constructs was used to obtain a CV1 cell line stably expressing the hAR and the androgen responsive Gaussia luciferase gene. A total of 48 geneticin and hygromycin resistant clones were obtained. Out of the androgen responsive clones, to the most responsive to testosterone exhibited an 80-fold induction of reporter gene activity (data not shown). All experiments were conducted using this clone, CV1-ARluc. The untransduced parent CV1 and the transduced CV1-ARluc cell lines were initially tested for human AR expression by qRT-PCR analysis and western blot (Fig. 1). We demonstrate that the CV1-ARluc cell line expresses high levels of AR, whereas AR was found to be absent in the parent CV1 cell line.

To further characterize the cell lines, we analyzed the cytoplasmic and nuclear expression of AR in CV1-ARluc by immunofluorescence (Fig. 2). Since ligand steroid receptors undergo nuclear localization, we traced the fluorescence for AR before and after treatment with DHT, a potent androgen. In the absence of the ligand, AR staining in the CV1-ARluc cells was predominantly located in the cytoplasm. Upon addition of 10 nM DHT for 18 h, 100% of the cells stained positive in the nucleus (Fig. 2). The observed translocation of AR from the cytoplasm to the nucleus in treated cells but not in untreated cells confirmed the specificity of the signal.

3.2. Sensitivity of CV1-ARluc cell line

As shown in Fig. 3A, the luciferase activity, after treatment with testosterone or DHT (10 nM) was clearly detectable at 3 h and increased progressively when the incubation with the respective ligands was continued up to 24 h. This result suggests that the assay can be used for steroids with androgenic activity even for short exposures.

In response to increasing concentrations of T and DHT, there was a sigmoidal increase in reporter gene expression (Fig. 3B). The first significant response of the reporter gene expression to the androgens was detected at 0.1 nM of DHT and 0.3 nM of T (Fig. 3B). The lower concentrations of steroids being able to stimulate a significant increase in luciferase expression indicate the high sensitivity of the cells.

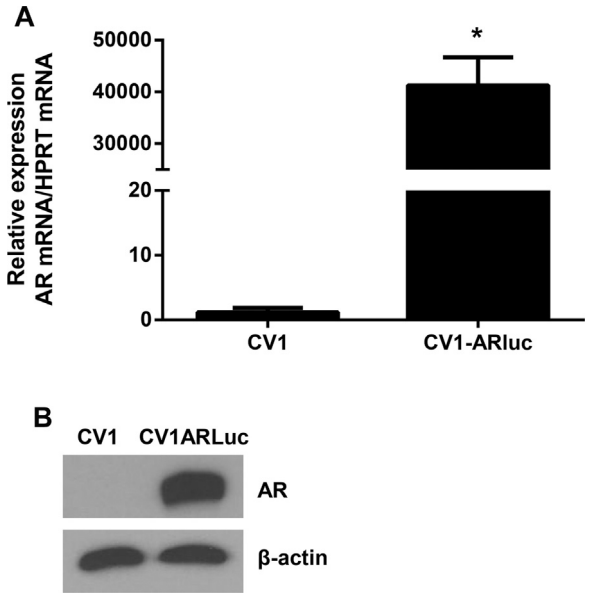

Fig. 1. AR expression in transduced and wild type CV1 cells. CV1 and CV1-ARluc cells were maintained in 10% FBS medium for 24 h and then lysed. (A) RT-PCR analysis of AR expression was tested in both cell lines. AR expression in CV1-ARluc was significantly higher than the CV1 cells. (B) Western analysis of AR was performed on 20 μg of total protein. β-actin was used as a loading control. The untransfected cell line did not show detectable AR, indicating the absence of this protein in this cell line. Figures are representative of three independent experiments with similar results.

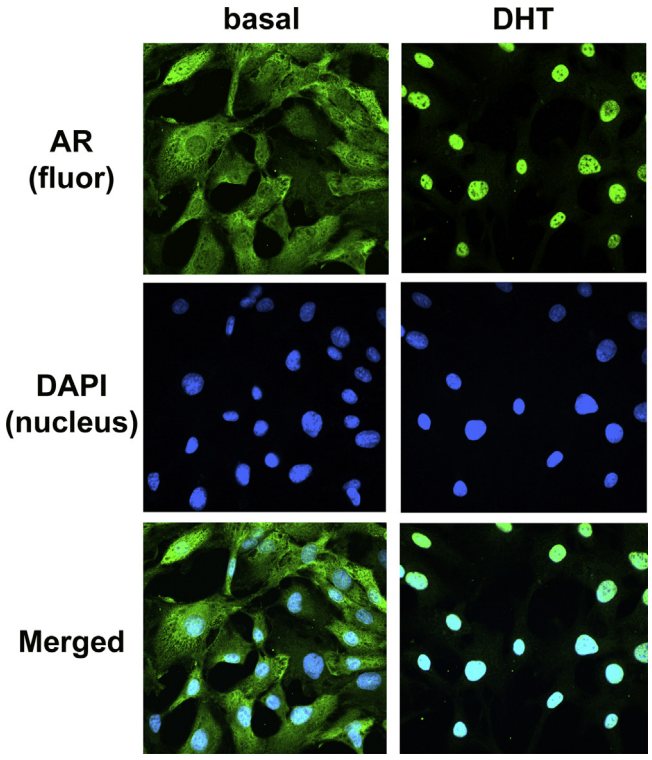

Fig. 2. DHT binding causes AR nuclear translocation. Fluorescence microscopy of CV1-ARluc cells stably transfected with a hAR and treated with or without 10 nM DHT for 18 h. Green fluorescence represents AR immunoreactivity and blue fluorescence is DAPI (nucleus).

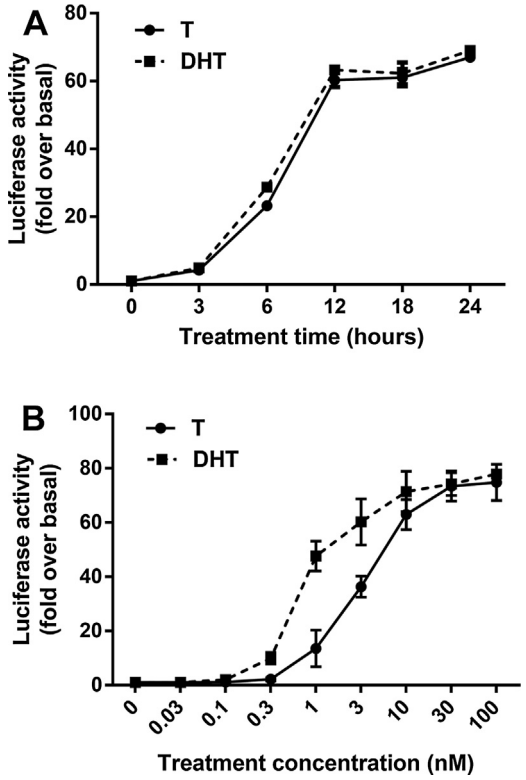

Fig. 3. Time course for AR activation and CV1-ARluc response to known androgens. (A) The cells were incubated with testosterone and DHT 10 nM for 0–24 h. The results are expressed as fold increase over basal of luciferase activity. The results represent the mean ± S.E. of similar experiments performed in triplicate. (B) CV1-ARluc cells were incubated with increasing concentrations of two known androgens: testosterone and DHT. The values represent the mean ± S.E. of similar experiments performed in triplicate.

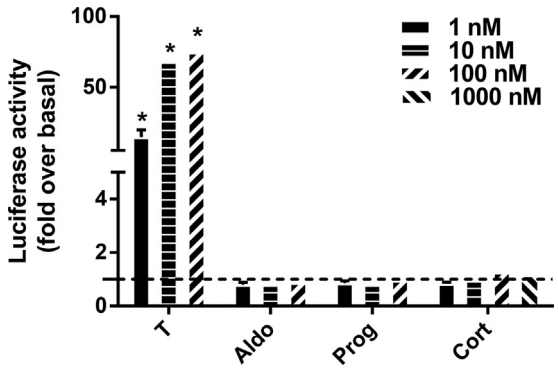

Fig. 4. Reporter gene regulation in response to androgenic and non-androgenic steroids. CV1-ARluc cells were incubated with 1–10 and 100 nM of aldosterone and progesterone and 1, 10, 100 and 1000 nM of cortisol. The dotted line represents basal conditions. The data represent the mean ± S.E. of three independent experiments, each performed in triplicate ($^*p < 0.05$ versus basal).

3.3. Selectivity of CV1-ARluc cell line to other steroids

Since some members of the nuclear steroid receptors exhibit cross-talk activity via binding of other steroid hormones to their ligand binding domain, we examined the specificity of CV1-ARluc for androgens. This was done by incubating the CV1-ARluc cells with cortisol, progesterone and aldosterone at increasing concentrations. In addition, to insure that GR activity was not present in this model and to determine if high concentrations of cortisol regulate the AR, we incubated cells with concentrations of cortisol up to 1000 nM. As shown in Fig. 4, none of these steroids showed any significant response suggesting a high selectivity of the CV1-ARluc system towards androgenic steroids.

3.4. Treatment of CV1-ARluc cell line with different C_{19} steroids

Several studies suggested that these C_{19} steroids provide a pool of circulating precursors for peripheral conversion to more active androgens. The adrenal glands secrete a variety of C19 steroids including androstenedione and the 11-keto and 11-hydroxy forms of androstenedione and T. To better analyze the sensitivity of the CV1-ARluc to weaker different C19 steroids, the cell line was treated with T, 11-hydroxytestosterone (11OHT), 11-ketotestosterone (11KT), AD4, 11-hydroxyandrostenedione (11OHAD), 11-ketoandrostenedione (11KAD) at a constant dose of 300 nM. As shown in Fig. 5, the luciferase activity was increased with

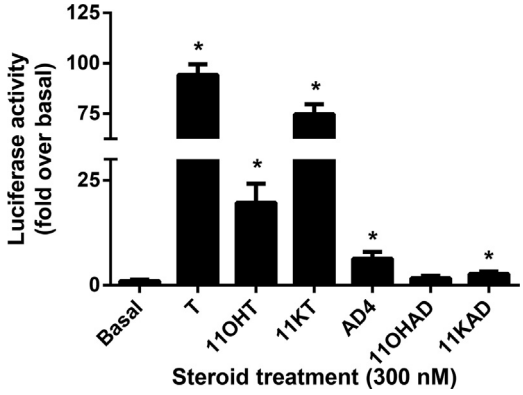

Fig. 5. CV1-ARluc cell line treatment with different C_{19} steroids. CV1-ARluc cells were incubated with 300 nM of T, 11OHT, 11KT, AD4, 11OHAD and 11KAD. The data represent the mean ± S.E. of three independent experiments performed in triplicate ($^*p < 0.05$ versus basal).

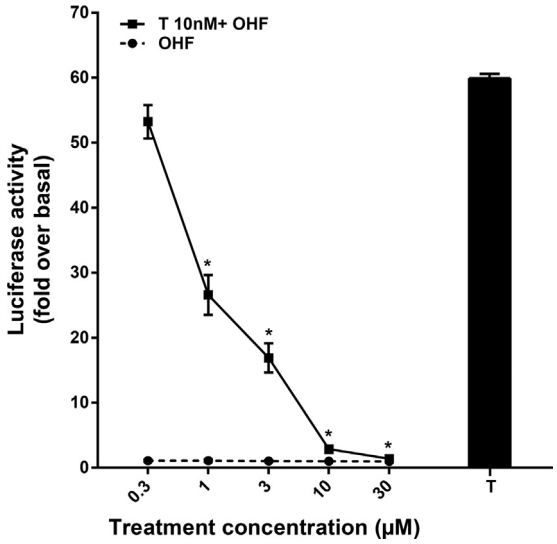

Fig. 6. Antagonistic activity of hydroxyflutamide in CV1-ARluc. Cells were incubated with 10 nM T alone as well as increasing concentration of OHF alone or in the presence of 10 nM testosterone. The values represent the mean ± S.E. of similar experiments performed in triplicate (*$p < 0.05$ versus 10 nM T alone).

treatment with all of the previously mentioned steroids with the exception of 11OHAD.

3.5. Effects of a potent anti-androgens on CV1-ARluc

The androgen specificity was also demonstrated by the ability of hydroxyflutamide (OHF), a non-steroidal anti-androgen, to suppress the activity of T (Fig. 6). The effects of OHF were tested in the CV1-ARluc cells. CV1-ARluc cells were treated with increasing concentrations of OHF, in the presence and absence of the maximally stimulating concentration of T (10 nM). As shown in Fig. 6, OHF alone did not show any significant agonistic activity. However, OHF inhibited T activity in a concentration dependent manner with a significant repression seen for 1–30 μM. The response to testosterone was completely suppressed by 30 μM OHF.

3.6. Serum androgen bioactivity

To examine the androgen activity in human serum, the experiment was performed on extracted samples processed with solid phase extraction. In Fig. 7, a dose response curve was performed using charcoal stripped human serum prepared with different doses of T. Serum androgen bioactivity was also determined from 10 male and female serum samples between ages 20 and 40 years. These bioassay values indicate the androgen activity in these samples. The range value of the active androgen in female and male were equivalent to 0.9 nM and 15 nM of T, respectively. These data demonstrate that the androgen activity in serum samples can be measured with CV1-ARluc cell line.

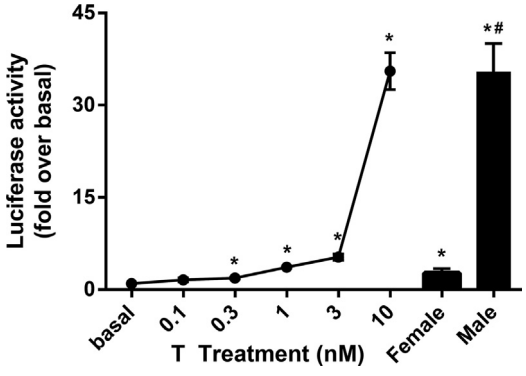

Fig. 7. Comparison of androgen bioactivity levels in human serum samples. Testosterone (0.3–30 nM) and human serum extract (10 male and 10 female) effects on reporter activity. Cells were treated with extracted samples for 18 h. The experiment was performed in triplicate (*$p < 0.05$ versus basal and #$p < 0.05$ versus female).

4. Discussion

We have developed an *in vitro* assay that is simple, rapid, and quantitative with potential to screen active androgenic compounds including those in human serum. The CV1-ARluc cell line expresses an androgen-responsive luciferase reporter gene and a human AR gene. The CV1-ARluc cell line combines high stability, fast growth, high selectivity, high sensitivity and rapid response to androgens.

Cell-based assays can provide several benefits compared with immunoassay, mass spectrometry and various chromatography methods [3]. Over the past several years, there has been an expansion in the use of gas chromatography–mass and liquid chromatography tandem mass spectrometry for measurements of androgens. These methods are able to analyze multiple steroid hormones but they are not useful in defining the activity of unknown androgenic steroids. Cell-based hormone receptor assays have become an important resource for drug discovery and androgen related diseases. The cells used to develop these assays should have two specific requirements: a reporter system driven by an ARE and an abundant expression of AR. A variety of reporter genes have been used in the past years including β-lactamase [20], β-galactosidase [21–26] and luciferase [15,18,27–32] reporter genes. To develop an androgen reporter line superior to the ones available, we chose not to use yeast cells for several reasons. Although yeast-based reporter models [22], have certain advantages (easy handling, rapid growth, inexpensive media components), they require laborious cell preparation and complex cell lysis steps. Importantly, using yeast assays to express mammalian proteins also raises concerns regarding glycosylation, phosphorylation and post translational modifications. Moreover, the estimation of androgenic bioactivity in serum with yeast-based assays has been unsuccessful. In yeast cell lines, anti-androgens have not shown any antagonistic effects, probably due the permeability problem of the cell walls.

To avoid interference with other nuclear receptors, we selected a cell line with a low background activity of C3 group nuclear receptors and with a good response to androgens when the hAR was stably introduced. We chose to use an MMTV promoter-driven luciferase gene since this approach has been shown to be successful in generation of *in vitro* and *in vivo* models for screening of estrogen compounds [33]. With the present work we show that, this approach can be successfully used to generate a selective androgen reporter cell line, CV1-ARluc. The MMTV promoter is quite selective to AR as well as to GR and PR and, also, contains a number of regulatory sites that can be targeted by other steroids. Wilson et al. [34] developed a stable cell line, MDA-kb2, a derivative of a human breast cancer cell line, containing a stably integrated MMTV-luciferase reporter. This cell line strongly responds to glucocorticoids due to endogenous GR. This makes the cell line unsuitable as a selective screening tool. PC3 cells transfected by Kim et al. [18], show more androgen specificity obtained by transient transfection of an MMTV promoter and a human AR with high sensitivity. Other bioassays for screening of androgen compounds in human serum have been reported using transient transfection; we have used a stable transduction to develop our cell model because stable expression of AR can eliminate the variability associated with repeated transient transfection. A different cell line, using CHO cells and a stable transfection, was developed by Paris et al. [4]. The cell line was

stably transfected with hAR but has retained glucocorticoid response due to GR. The T47D cell line was used by Blankvoort et al. [31] to develop a new androgen cell-based assay named AR-LUX. T47D expresses an endogenous AR and was stably transfected with a luciferase reporter gene. This assay was used to estimate the levels of some anabolic steroids in the urine of cattle. However, this cell line also responds to added estrogen and progesterone, which may reduce the specificity of the assay.

There is one additional AR bioassay that is highly selective for androgens and there is no reporter response to non-androgenic steroids [20]. Wilkinson et al. developed an AR bioassays using the HEK293 cell line. This model is particularly applicable for high throughput screening for AR activation. However, this model makes use of a hybrid AR receptor (GAL4DBD-ARLBD) and therefore may not have all regulatory characteristics of the full length AR.

In the present study, we have developed a new cell line, named CV1-ARluc, which stably express the human AR and an androgen-driven gaussia luciferase reporter. The cell line is able to estimate levels of androgen bioactivity in human serum samples. The androgen specificity of the assay was tested by using high concentrations of different steroids such as cortisol, progesterone and aldosterone, which did not active reporter expression. The low background activity of C3 group nuclear receptors in the CV1 cell line was supported by RT-PCR data, where no GR, ER, AR, PR and MR expression was detected. The specificity of the assay was also showed by checking the inhibitory effects of a synthetic anti-androgen receptor ligand, OHF. OHF anti-androgen effects were also seen in other AR models including CHO [35], DU-145 [36] and PALM [37]. We evaluated androgenic bioactivity in male and female serum samples. In concordance with the data previously reported using a different *in vitro* assay to detect androgen activity in human serum samples by Roy et al. [38], our assay shows an androgen bioactivity in female serum samples from 0.8 to 2 nM and from 10 to 25 nM in normal males. It can be concluded that this assay provides a valid and practical method to analyze serum samples for androgenic activity. The assay can be used to evaluate the physiological androgen levels during androgen-related diseases.

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Cell-Based Assays for Screening Androgen Receptor Ligands

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Abstract

The androgen receptor (AR, *NR3C4*) mediates the majority of androgen effects on target cells. The AR is activated following ligand binding that result is enhanced of target gene transcription. Several cell-based model systems have been developed that allow sensitive detection and monitoring of steroids or other compounds with AR bioactivity. Most cell-based AR reporter models use transgenic gene constructs that include an androgen response element that controls reporter gene expression. The DNA cis-regulatory elements that respond to AR share sequence similarity with cis-regulatory elements for glucocorticoid (GR, *NR3C1*), mineralocorticoid (MR, *NR3C2*), and progesterone (PGR, *NR3C3*) receptors, which has compromised AR selectivity for some models. In recent years, the sensitivity and selectivity of AR bioassays have been significantly improved through careful selection of cell models, utilization of improved reporter genes, and the use of yeast two-hybrid AR systems. This review summarizes and compares the currently available androgen-responsive cell model systems.

Keywords
- androgen receptor
- luciferase
- GFP
- in vitro bioassay
- reporter gene assays

Androgens represent a broad group of steroid hormones that mediate their effects through binding and activation of the androgen receptor (AR, *NR3C4*). The AR is expressed in a variety of human tissues, including the heart, pituitary, skeletal muscle, uterus, and thyroid, with the highest expression level observed in the prostate, adipocyte, and liver.[1] The AR can be activated by several physiologic ligands (mainly testosterone and dihydrotestosterone, DHT) that bind the AR with different affinities and bioactivity.[2,3] DHT is the most active physiologic androgen with a 10-fold higher AR bioactivity than testosterone.[4,5]

Binding of androgen to the cytosolic AR[6] results in a conformational change in the receptor that causes dissociation of heat shock proteins, transport from the cytosol into the cell nucleus, and dimerization of the androgen–AR complexes.[7,8] The AR dimer then binds to specific sequences of DNA known as the androgen response elements (ARE) that enhances transcription of AR-responsive genes.[9–18] AREs are identified by the presence of six-nucleotide half-site consensus sequences spaced by three random nucleotides in the promoter region of target genes (5′-TGTTCT-3′).[19,20] Conversely, antiandrogens, such as casodex or hydroxyflutamide, bind to the AR and in some cases enhances nuclear translocation but without transcriptional activation.[17,18] AR has a characteristic structure: two activation functions (AF1 and AF5) in the N-terminal domain (NTD), a DNA-binding domain (DBD) which contains the dimerization domain, a nuclear localization signal, a hinge region, and a carboxy-terminal ligand-binding domain (LBD) which contains a third activation function domain (AF2). All the AR regions are highly conserved except the NTD, which is important in transcriptional regulation.

AR cell–based screening models have been applied to a variety of discovery-based projects. Many focus on defining novel androgens that might play a role in human diseases of androgen excess (premature adrenarche or polycystic ovary

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syndrome).[21,22] In addition to endogenous steroid hormones, AR cell–based assays have been used to define androgenic activity in legumes, soybeans, yams, and industrial chemicals with concerns of their ability to act as endocrine disruptors and/or toxicants.[23] Finally, AR bioassays have become an alternative method for the detection of designer androgens in laboratories testing serum for sports doping.[24]

Historically, androgens have been measured as individual steroids using selective immunoassays. While these assays perform relatively well and provide a degree of high throughput, such assays can be flawed by cross-reactivity with steroids of similar structure. In addition, as there are several different steroids that can activate the AR, the immunoassay approach of measuring one steroid at a time does not provide a broad view of the circulating androgen milieu. Over the past 10 years, there has been an expansion in the use of gas chromatography–mass spectrometry (GC-MS) and liquid chromatography tandem mass spectrometry (LC-MS/MS) for measurement of natural and synthetic androgens. These methods have an improved specificity over most antibody-based immunoassays. In addition, these methods allow a broader analysis of multiple steroid hormones and may be important for disease diagnosis. However, these methods are not useful in the identification of unknown synthetic or naturally occurring androgenic steroids or other substances; for these types of studies, investigators have relied on in vitro cell-based AR bioassays.

Cell-based steroid receptor reporter assays have become an important resource for compound profiling and drug discovery because of their ability to provide quantitative and functional information within a short time span. The cells used for developing cellular AR assays have two specific requirements: abundant expression of the AR and a reporter system driven by an ARE. The principles of the reporter gene assays are quite simple and rely on AR ligand entry into the cells, binding to the cytoplasmic AR, translocation of the AR complex into the nucleus, binding to the ARE, resulting in an increase in reporter gene expression. Importantly, the activity of a ligand can be elucidated in samples without the need to have any information on chemical structure. A variety of reporter genes have been used for model development, including β-galactosidase (β-gal), luciferase, lactamase, and green fluorescent protein (GFP). In this review, we discuss the cell-based AR bioassays currently available for detection of androgenic and antiandrogenic activity (►**Fig. 1**).

The Cell-Based Androgen Assays

Yeast-Based Systems Using a β-Galactosidase Reporter

In yeast cells, steroid bioactivity of substances can be determined without the presence of any other mammalian proteins/pathways influencing the AR activity. These cells have the advantages of easy handling, fast growth, inexpensive media components, and robustness toward toxic effects of the tested chemicals or solvents.[25] These attributes make the yeast AR screen a fast and easy tool. Some disadvantages of yeast assays include laborious preassay cell preparation and complex cell lysis steps. Using yeast assays to express mammalian proteins also raises concerns regarding phosphorylation, glycosylation, folding, and posttranslational modifications.

β-gal is encoded in *Escherichia coli* by the *lacZ* gene of the lac operon. The enzyme function in bacteria is to cleave lactose to form glucose and galactose. Chlorophenol red-β-D-galactopyranoside (CPRG), a chromogenic substrate, described by Seeber and Boothroyd,[26] and the synthetic compound o-nitrophenyl-β-D-galactoside (ONPG), described by Li et al,[27] are used for spectrometric detection of β-gal. Both substrates are colorless but became colored once hydrolyzed by β-gal. For the ONPG/β-gal assay, the time required for yeast exposure to the tested compounds is 6 hours. The cells are then lysed and an aliquot of the extract is mixed with the β-gal reaction substrate in a buffer containing sodium phosphate and magnesium chloride. The assay ends with the spectrophotometric measurement of the yellow reaction product (o-nitrophenol). The production of o-nitrophenol, per unit time, is proportional to the concentration of β-gal, allowing the intensity of the yellow color produced to determine the enzyme concentration.[27] The use of XGal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside) for β-gal detection requires at least 16 hours of yeast exposure to the test compounds.[28,29] XGal, a chromogenic substrate for β-gal, produces a blue color that can be detected visually over background. Using the XGal substrate, β-gal assays provide a more sensitive reporter for activity, but XGal is not as quantitative as the β-gal assay.[30]

As shown in ►**Table 1**, for most of the β-gal assays examined, as exposure times increase, EC_{50} values decrease. In 1991, Purvis et al[31] developed an androgen-inducible expression system for *Saccharomyces cerevisiae.*[31] The PGKare-lacZ (PGK promoter followed by ARE and lacZ sequence) was integrated into the *S. cerevisiae* genome at the ura3–52 locus. The resulting strain was then stably transfected with human AR (hAR) expression plasmids. The transfected cells were incubated in the presence of different concentrations of DHT and assayed for β-gal activity. EC_{50} was 1 nM for DHT treatment with a steroid exposure time of 40 hours. A similar AR assay with comparable steroid exposure time and EC_{50} was developed by Sohoni and Sumpter.[32] Based on the hypothesis that one chemical may activate multiple steroid receptors, they used two recombinant yeast strains: one containing a gene for the human estrogen receptor (also containing a plasmid carrying an estrogen-responsive element regulated lacZ reporter) and the other yeast strain expressing the hAR (also containing an ARE-regulated lacZ reporter). When an active ligand bound to either receptor, lacZ was transcribed/translated and then secreted into the medium. The medium could then be used for the chromogenic substrate CPRG. They confirmed previously reported antiandrogenic and estrogenic activity of vinclozolin and p,p′-1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE)[33,34] and they found estrogenic activity in several reported antiandrogenic compounds, namely, o,p′-1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), bisphenol A (BPA), and butyl benzyl phthalate.

Chatterjee et al[35] constructed a yeast-based AR bioassay to evaluate the androgenic activity of endocrine disruptors from

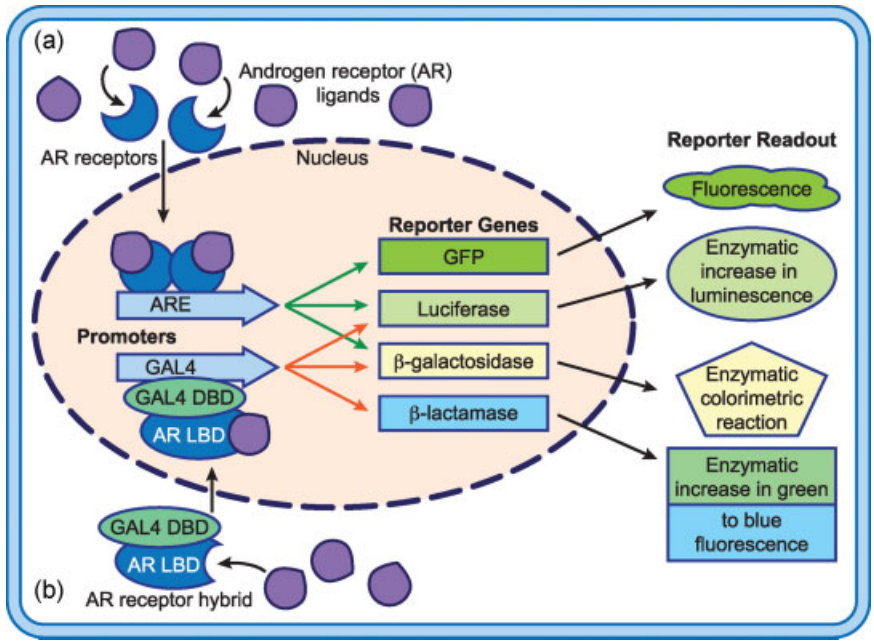

Fig. 1 Cell-based bioassays for the study of AR activity. (**a**) Models using native androgen receptor rely on ligand/antagonist effects on AR regulation of reporter gene transcription. AR binding causes translocation of the cytosolic AR into the nucleus and its binding to androgen responsive elements that drive a variety of reporters. Models are available that use GFP, luciferase, or β-galactosidase. (**b**) Models using yeast two-hybrid system for determining androgen activity and activation of reporter gene transcription. Androgen binding causes the translocation of cytosolic AR receptor hybrid (GAL4 DNA binding domain [DBD]/AR ligand binding domain [LBD]) into the nucleus and its binding and activation of GAL4 promoter-driven luciferase, β-galactosidase, or β-lactamase reporter systems.

pulp and paper mill effluents. The system consisted of hAR- and ARE-driven *lacZ* transformed in *S. cerevisiae*. Production of *lacZ* was shown to be driven by the CYC1 yeast promoter, and β-gal activity was detected using XGal. The assay detection required at least 16 hours of exposure to the tested chemicals; EC_{50} was 16 nM for testosterone and 4 nM for DHT, which was consistent with the performance of other, previously constructed, assays.[36,37]

A recently reported AR cell bioassay, more selective than those previously described, was developed by Lee et al.[38] The group developed a detection system for androgenic and antiandrogenic compounds, which was based on yeast two-hybrid protein interactions. A yeast strain, ARhLBD-ASC1, was established by cotransformation of yeast cells harboring lacZ reporter plasmid. ARhLBD-ASC1 is a dual vector expressing system containing the LexA fused hinge–ligand binding domain (hLBD) of the hAR, and B42 fused to ASC-1 that interacts with the AR-hLBD in an androgen-dependent manner. In this yeast strain, androgens, but not other hormones, stimulated β-gal activity. β-gal activity was measured as a

Table 1 Androgen receptor bioassays using the β-galactosidase reporter

AR type	Promoter	Exposure time	EC_{50} value	Assay cells	Reference
hAR[a]	ARE	40 h	1 nM DHT	*S. Cerevisiae*[b]	Purvis et al[31]
hAR[a]	ARE	40 h	1 nM DHT	*S. Cerevisiae*[b]	Sohoni and Sumpter[32]
hAR[a]	ARE	Overnight	3.5 nM DHT	*S. Cerevisiae*[b]	Gaido et al[41]
hAR[a]	ARE	16 h	4 nM DHT	*S. Cerevisiae*[b]	Chatterjee et al[35]
AR yeast two-hybrid protein-based models					
hAR-LBD	GAL4	16 h	4.8 nM DHT	EGY48[c]	Lee et al[38]
GAL4DBD-ARLBD	GAL4	4 h	10 nM DHT	Y190[c]	Nishikawa et al[39]
GAL4DBD-ARLBD	GAL4	2 h	13 nM DHT	Y187[c]	Li et al[29]

[a]Stable AR expression.
[b]Clonal cell model.
[c]Mixed cell model.

colorimetric reaction following 16 hours of incubation. This system allowed relatively high throughput and could be done in 96 well dishes.

To study the effect of endocrine disruptors on AR, Nishikawa et al[39] developed a yeast model system with short exposure time (4 hours) but relatively low sensitivity ($EC_{50\ around}$ 10 nM DHT). The major goal of the study was to develop a novel screening method to examine chemical effects on several steroid receptors. Y190 yeast cells were transformed with the pGBT9–LBD of the estrogen and ARs, GAL4-receptor DBD, and GAL4AD–coactivator fusion proteins. Because the yeast strain Y190 harbors a GAL4 binding site upstream of a lacZ reporter gene, GAL4DBD-ER binds to the regulatory region of the lacZ gene. If GAL4DBD-ER interacts with GAL4AD–coactivator, GAL4AD recruits the basal transcriptional machinery to the promoter region of the lacZ gene resulting in β-gal production. The system was adapted for other receptors by exchanging the ER portion of GAL4DBD fusion with other receptors. In addition, the models were improved by including mammalian nuclear receptor cofactors. For the development of the AR bioassay, the ER-LBD was changed to AR-LBD and the β-gal reporter responses were enhanced by adding a vector containing mammalian nuclear receptor coactivators. Based on these studies, the steroid receptor models were most effective using the following cofactors: ER–TIF2, AR–SRC1, PR–TIF2, GR–SRC1, and MR–SRC1.

Different combinations of plasmids in the yeast Y187 were used by Li et al.[29] Plasmids used were pGBT9, the AR-LBD, and pGAD424 GRIP1/FL (described by Doesburg et al[40]) or pGBT9 ERRγ and pGAD424 GRIP1/FL. This model has a low compound exposure time (2 hours) but limited sensitivity (EC_{50} around 13 nM DHT). These investigators developed the models to study endocrine disruptors in pesticides which were suspected of modulating the endocrine systems in humans. The endocrine disruptors examined for their ability to interact with the ER, AR, PR, or ERRγ included p,p′-dichlorodiphenylethane (p,p′-DDE), p,p′-dichlorodiphenyltrichloroethane (p,p′-DDT), hexachlorobenzene (HCB), and r-hexachlorocyclohexane (r-HCH). The results showed that p,p′-DDE was an ER agonist and an AR and PR antagonist (PR > AR), while p,p′-DDT was an ER agonist and AR antagonist. HCB and r-HCH were antagonists for AR and ERR, while r-HCH was a PR antagonist and a weak antagonist of ERR; the endocrine disruptor, r-HCH, was able to reverse the ERR inhibition induced by 4-hydroxytamoxifen.

Yeast-based assays assessing chemical interactions with the estrogen, androgen, and progesterone receptor (PGR) were developed by Gaido et al.[41] For the AR bioassay, the EC_{50} was around 3.5 nM DHT with an exposure time of 18 hours. The yeast contained two separate plasmids: an expression plasmid containing the CUP1 metallothionein promoter fused to the human nuclear receptor cDNA and a reporter plasmid carrying two ARE upstream of the structural gene for β-gal; this system overexpressed two proteins (RSP5 or SPT3, respectively) in yeast containing either the progesterone or the AR.

One of the earliest reporter gene assays, chloramphenicol acetyltransferase (CAT reporter), has also been used for AR bioassay development. The CAT enzyme is normally found in prokaryotes but not in eukaryotes. It transfers the acetyl group from the acetyl CoA molecule to chloramphenicol, causing its detoxification. Xu et al used this reporter system to develop an hAR reporter assay using the CV-1 cell line (African monkey kidney cell line).[42] The CV-1 cell line was transiently transfected with an ARE-driven reporter gene plasmid (pMMTV-CAT) and an hAR expression plasmid AR/pcDNA3.1. An EC_{50} of 0.39 nM was observed for DHT following an incubation period of 24 hours. Using this AR reporter model, the group investigated BPA, 4-octylphenol, 4-nonylphenol, and several pesticides for agonistic and antagonistic activities. The caveat of this system is its experimental variation due to the transient nature of transgene expression.

β-Lactamase Reporter Model

The β-lactamase (BLA) reporter system, which can be used for studying gene expression in living cells, uses the bacterial enzyme TEM-1 BLA which lacks the periplasmic secretory signal sequence. BLA is encoded by the ampicillin-resistance gene, a 29-kDa enzyme, and is active either as a monomer or when fused N or C terminally to a heterologous protein.[43] It can cleave β-lactam-containing molecules with simple kinetics and high catalytic efficiency. Overexpression of BLA does not show toxicity in eukaryotic cells. CCF2 and CCF4 (coumarin cephalosporin fluorescein), the BLA fluorescent substrates, can be detected by fluorescence resonance energy transfer (FRET). In the intact molecule, excitation of coumarin at 408 nm leads to efficient FRET to the fluorescein derivative and produces green fluorescence. Cleavage of CCF2/4 by BLA separates the two fluorophores, causing loss of FRET and excitation at 408 nm that result in blue fluorescence detectable at 460 nm. Thus, based on the change in the fluorescence emission signal, live cells expressing BLA can be distinguished by epifluorescence microscopy, fluorescent plate reader, or flow cytometry.

Wilkinson et al[44] developed a panel of steroid hormone receptor bioassays by stably engineering expression of Gal4-DBD, with specific nuclear receptor LBD, using the HEK293 cell line with stable insertion of a GAL4 promoter–driven BLA reporter. Plated cells were incubated for 16 hours with ligands or test compounds. Lactamase substrate was then added and fluorescence signal read using a fluorescent plate reader. After subtracting the average fluorescence intensity from the cell-free controls, the 460 nm/530 nm emission ratio was calculated. The response ratio corresponds to the 460 nm/530 nm emission ratio of the stimulated wells divided by the 460 nm/530 nm emission ratio of the unstimulated wells. The AR lactamase bioassay exhibits high sensitivity to DHT with an EC_{50} of 1 nM. The particular utility of this assay is its potential for high throughput screening and a high degree of selectivity for the AR.

Luciferase Reporter Model

Firefly luciferase is one of several bioluminescent reporters that have achieved broad use for molecular biology studies. Compared with the tests previously discussed, some of the luciferase AR models have higher sensitivities than those with lactamase, particularly with the mammalian cell models that can detect picomolar levels of DHT. The details, including the

sensitivity of these AR cell–based assays, are reviewed in ►**Table 2**. The most commonly used luciferase is from the firefly *Photinus pyralis*. This gene encodes a 61-kDa enzyme that oxidizes D-luciferin in the presence of oxygen, ATP, and Mg^{2+}; the fluorescent product of the reaction can be quantified by measuring the released light using a luminometer. The assay is rapid, simple, relatively inexpensive, and sensitive, and possesses a broad linear range. Cells transfected with a luciferase reporter plasmid are lysed using a detergent-containing buffer. The substrate can be mixed with the lysate; some luminometers directly inject the reagents into the lysate and the fluorescence is read at a defined time after mixing. The luciferase reporter is most often used as a read-out of gene expression to study transcriptional control mechanisms (promoter studies) or to study activity of transcription factors (as is the case for the AR models). Both yeast and mammalian cell line AR-driven luciferase reporter models have been developed.[38]

Yeast Androgen Receptor Luciferase Models

Yeast systems are inexpensive and do not contain all the mammalian enzymes, activators, and coregulators, and hence may not support maximal transcriptional activity for all receptors. However, the low costs and quick cell expansion capabilities make it a good choice for experimental goals.

Michelini et al developed a bioluminescent yeast-based bioassay for androgens.[37] The bioassay is based on *S. cerevisiae* cells, modified to express hAR, and contain ARE sequences to regulate expression of luciferase. The bioassay responds to testosterone in a concentration-dependent manner from 0.05 to 1000 nM. The EC_{50} of DHT is 10 nM. This assay is also able to respond to progesterone and 17β-estradiol, with an EC_{50} of 20 and 50 nM, respectively, apparently via an AR mechanism. An *S. cerevisiae* strain, expressing hAR, estrogen receptor α, or estrogen receptor β, with luciferase controlled by the receptors' respective hormone responsive elements, was developed by Leskinen et al.[36] These investigators describe the construction and use of a set of bioluminescent yeast strains for the detection of compounds that regulate androgen or estrogen receptor mediated hormonal signaling. The luciferase coding sequence was inserted into the vector pRS316/GPD-PGK,[45] between the GPD promoter and PGK terminator yielding pRS316luc. Sample analysis can be performed in one day and there is no requirement for cell lyses or centrifugation. Yeast cells were incubated with test

Table 2 Androgen receptor bioassays using a luciferase reporter

AR type	Promoter	Reporter	Exposure time	EC_{50} value	Assay cells	Reference
Yeast cell-based AR models						
hAR[b]	ARE	Firefly luciferase	3 h	10 nM DHT	*S. Cerevisiae*[e]	Michelini et al[37]
hAR[b]	ARE	Firefly luciferase	2.5 h	5.5 nM DHT	*S. Cerevisiae*[e]	Leskinen et al[36]
hAR[b]	ARE	Bacterial luciferase	3–4 h	9.7 nM DHT	*S. Cerevisiae*[e]	Eldridge et al[46]
Mammalian cell-based AR models						
hAR[a]	MMTV	Firefly luciferase	24 h	0.063 nM DHT	22Rv1[e]	Kim et al[50]
hAR[c]	MMTV	Firefly luciferase	24 h	0.008 nM DHT	PC3[e]	Kim et al[50]
hAR[a]	MMTV	Firefly luciferase	24 h	0.075 nM DHT	LNCaP[e]	Kim et al[50]
hAR[a]	MMTV	Firefly luciferase	48 h	∼0.2 nM DHT	MDA-MB-453[d]	Hartig et al[49]
hAR[b]	MMTV	Firefly luciferase	48 h	∼0.2 nM DHT	CV-1[d]	Hartig et al[49]
hAR[c]	MMTV	Firefly luciferase	24 h	3.6 nM DHT	CV-1[e]	Sun et al[53]
hAR[c]	MMTV	Firefly luciferase	24 h	∼0.5 nM R1881	CHO[e]	Vinggaard et al[54]
hAR[b]	MMTV	Firefly luciferase	24 h	∼0.5 nM R1881	CHO[d]	Roy et al[55]
hAR[a]	ARE	Firefly luciferase	24 h	115 nM DHT	T47D[d]	Blankvoort et al[56]
hAR[a]	MMTV	Firefly luciferase	Overnight	0.14 nM DHT	MDA-kb2[d]	Wilson et al[57]
hAR[b]	ARE	Firefly luciferase	24 h	0.13 nM DHT	U2OS[d]	Sonneveld et al[58]
hAR[b]	MMTV	Firefly luciferase	24 h	0.01 nM DHT	U2OS[e]	Sedlák et al[59]
hAR[b]	GRE	Firefly luciferase	24 h	0.01 nM DHT	U2OS[e]	Sedlák et al[59]
AR yeast two-hybrid protein models						
GAL4DBD-AR LBD	GAL4	Firefly Luciferase	24 h	0.1 nM DHT	U2OS[e]	Sedlák et al[59]

[a]Endogenous AR expression.
[b]Stable AR expression.
[c]Transient AR expression.
[d]Clonal cell model.
[e]Mixed cell model.

compounds or complex samples for 2.5 hours, resulting in an EC_{50} value of 5.5 nM DHT.

Another yeast AR bioassay, using a bacterial luciferase reporter, was developed by Eldridge et al.[46] An EC_{50} of 9.7 nM was observed for DHT using an *S. cerevisiae* strain engineered to respond to androgenic chemicals. The strain contained stable expression of the hAR and a reporter controlled by an ARE between two promoters (GPD and ADH1). Cotransformation of this plasmid with a second plasmid (pUTK404), containing the genes required for aldehyde synthesis (luxCDE) and FMN reduction (frp), yielded a bioluminescent reporter system that is responsive to a wide variety of bioactive androgens.

Mammalian Cell Androgen Receptor Luciferase Models

Mammalian cell–based bioassays have been developed in immortalized cell lines which are relatively easy to culture and maintain, and show higher sensitivity than the yeast system. However, a careful characterization of model systems is necessary. It is imperative that the parent cell line selected for the development of the bioassay does not contain steroid-metabolizing enzymes since that could give inaccurate luciferase response results. Second, parent cells containing other steroid receptors could pose a problem since the ARE consensus DNA sequence has almost 80% similarity to cis-regulatory elements of glucocorticoid (GR, *NR3C1*), mineralocorticoid (MR, *NR3C2*), and (*NR3C3*).[5,16] In these models, reporter gene expression can be activated by hormone ligands leading to false positives. Numerous mammalian cell lines, including prostate carcinoma cells (LNCaP, 22Rv1, PC3, and DU-145)[47] or other cells (HepG2, CV-1, COS-1, COS-7, and CHO), have been engineered to develop androgen reporter assays.[48,49]

Kim et al used three prostate cancer cell lines (22Rv1, PC3, and LNCaP) to develop AR-regulated reporter gene assays.[50] While 22Rv1 and LNCaP cell lines have an endogenous AR, the PC3 cell line, reported to be AR negative,[51] was transiently transfected with an hAR expression vector. Among the three cell lines that were transiently transfected with pMMTV-luc, DHT stimulated proliferation only in LNCaP cells. It is important to note that the endogenously expressed AR in LNCaP cells contains a mutation in the LBD that alters steroid binding selectivity and can lead to activation by a variety of steroids that normally do not activate AR.[52] EC_{50} values of DHT for 22Rv1, PC3/AR +, and LNCaP were 0.063, 0.008, and 0.075 nM, respectively. While the sensitivity was good, each line showed endogenous expression of GR and therefore increased luciferase reporter in response to cortisol.

Hartig et al[49] used a human breast carcinoma cell line (MDA-MB-453) and the African green monkey kidney cell line (CV-1). MDA-MB-453 cells were transduced with a luciferase reporter regulated by the MMTV. The MDA-MD-453 cell model expressed endogenous GR and AR. CV-1 cells were transduced as above with MMTV-luc and also an hAR. While the CV-1 exhibited relative selectivity for AR activation, the MDA-MB-451 transduced reporter responded to both GRs and androgens.

In 2007, Sun et al used CV-1 cells that had been transiently transfected with hAR- and MMTV-driven luciferase.[53] These investigators tested the effects of three common pyrethroids (fenvalerate, cypermethrin, permethrin) and their metabolite 3-phenoxybenzoic acid (3-PBA) for antiandrogenic and androgenic activity.[53] The assay displayed appropriate response to known AR agonists (EC_{50} 3.6 nM with DHT) as well as to AR antagonists.

A transient AR reporter assay for detection of antiandrogenic chemicals was used by Vinggaard et al.[54] Chinese hamster ovary (CHO) cells were cotransfected with vectors containing hAR and MMTV-luc by nonliposomal transfection. Cells were treated for 24 hours with the synthetic AR agonist, R1881 (10 nM), resulting in a 30- to 60-fold induction of luciferase activity. CHO cells were subsequently used to develop a stable cell line.[55] For stable line development, CHO cells were cotransfected with plasmids encoding MMTV-luc, neomycin, and hAR. After selection with neomycin and cloning, an active, responsive clone was obtained that stably expressed both the hAR and the luciferase reporter. Stimulation of the cells with androgens for 24 hours resulted in about a 15-fold stimulation of luciferase activity, with the minimum effective dose of testosterone being 0.1 nM resulting in an EC_{50} around 0.5 nM with R1881. Sixty different chemicals (pesticides or their metabolites, and common industrial chemicals) were screened with the cell line for their ability to activate or inhibit reporter as compared with a positive control. The most potent antiandrogenic compounds identified were BPA, α-hexachlorocyclohexane, vinclozolin, and 4,4-DDE.

An androgen reporter system that utilizes an endogenously expressed AR was developed by Blankvoort et al.[56] The human breast cancer cell line T47D was stably transfected with a luciferase gene under transcriptional control of the PB-ARE-2 promoter. The model system was called AR-LUX (Androgen Receptor-mediated LUciferase eXpression) and was evaluated for its responsiveness to several androgens, antiandrogens, nonandrogenic steroids, and to compounds modulating the AR itself. Following 24 hours of treatment, an EC_{50} value of 115 nM was determined for DHT. Luciferase responses were also elicited by high concentrations of the steroids progesterone, 17β-estradiol, aldosterone, and dexamethasone. The ability to selectively examine AR activation was a concern of this model.

The MDA-kb2 cell line, containing an endogenous AR, was developed by Wilson et al.[57] Cells were transformed with an androgen-responsive luciferase reporter plasmid driven by MMTV, selected with geneticin, and cloned. The active clone was chosen and the resulting line termed MDA-kb2. The MDA-kb2 has been a useful tool for studying the activation of both AR and GR because both receptors are present and both receptors can activate the MMTV promoter. Following 24 hours of treatment, an EC_{50} of 0.14 nM was determined for DHT. This model is relatively easy to use, grows well, and is stable but responds to both AR and GR agonists.

The U2OS cell line was used by Sonneveld et al.[58] These investigators developed the AR CALUX (Chemically Activated LUciferase eXpression) bioassay. It contains the hAR and a

luciferase reporter construct containing three AREs coupled to a TATA promoter. The EC_{50} of DHT was found to be 0.13 nM. The sensitivity of AR CALUX was assessed by measuring the luciferase activity induced by a series of natural steroids (DHT, testosterone, and androstenedione).

In 2011, Sedlák et al developed two panels of U2OS-based luciferase reporter cell lines using two different reporter formats.[59] In the first model, the activity of the receptor was monitored by a reporter vector containing synthetic promoter with multimerized ARE or the MMTV upstream of the luciferase gene. The second model relied on the chimeric steroid receptor, where the N-terminal part of the receptor containing AF1 and the DBD was replaced by the DBD from the yeast transcription factor Gal4. This construct was cotransfected with reporter vector containing nine copies of GAL4 response element (used even in HEK293).[60] The investigators compared the two panels using several ligands and concluded that, in general, both systems generated a similar qualitative response. Both systems (AR/GRE or AR/MMTV) and AR-LBD/9XGal4UAS showed high sensitivity to DHT with an EC_{50}, after 24-hour treatment, of 0.01 and 0.1 nM, respectively.

Green Fluorescent Reporter Model

Compared with luciferase assay, fluorescent protein assay offers cheap and faster direct detection using spectrofluorometer or fluorescence microscope. The main advantage of GFP is that it does not require enzymatic substrates for detection. In addition, the use of different fluorescent proteins enables an investigator to track the expression of two (or more) genes in the same cell (multiplexing). GFP and its genetically enhanced variations are quantitative reporters with high levels of photostability and brightness. It is an autofluorescent protein initially derived from the jellyfish *Aequorea Victoria* and can be used for a variety of biotechnological applications.[61] Most of the steroid bioassays that make use of GFP reporter were designed for the determination of estrogenic activity using yeast as the cell model.[41,62–64] The benefit of using GFP is the ability for direct quantification using either a fluorescence microscope or a luminometer. The details, including the sensitivity of these AR cell–based assays, are reviewed in ►**Table 3**.

Bovee et al[65] constructed recombinant yeast that expresses hAR and a yeast-enhanced GFP (γEGFP) as a measurable reporter protein in response to androgens. They stably integrated in the *S. cerevisiae* genome, the reporter vector, and the receptor expression vector. The γEGFP reporter gene is optimized for yeast expression under control of the CYC1 promoter which contains two ARE sequences. The hAR coding region is constitutively expressed under the control of a GDP promoter. The investigators also demonstrated that *S. cerevisiae* did not metabolize test compounds, displayed no crosstalk for nonandrogen steroids, and had a relative androgenic potency. Androgen activity can be quantified directly in a cytofluorimeter using excitation at 485 nm and measuring fluorescent emission at 530 nm. The EC_{50} value for testosterone activation of yEGFP expression was 50 nM following 24 hours of incubation.

GFP was introduced as an alternative reporter gene in the androgen assay system developed by Beck et al.[66] The hAR coding sequence was inserted into expression plasmid YEpBUbi–FLAG1, resulting in the plasmid YEpBUbiFLAG–AR, and the ERE on the reporter vector YRpE2 was substituted with an ARE, resulting in the plasmid YRpE2–ARE. The vector YRpE2-GFP was used as a backbone to create the reporter plasmid YRpE2-GFP-ARE, using GFP as a reporter gene. For evaluation of the reporter system, β-gal, as a primary reporter gene, was added. Several known AR agonistic compounds (5a-dihydrotestosterone, testosterone, androstenedione, 17a-methyltestosterone, progesterone, epitestosterone, and norgestrel) were tested to evaluate both reporter systems. The model shows an EC_{50} of 16 nM with testosterone.

In 2008, Dennis et al developed an assay for the assessment of AR transcriptional activity using destabilized enhanced

Table 3 Androgen receptor bioassays using fluorescent proteins as a reporter

AR type	Promoter	Reporter	Exposure time	EC_{50} value	Assay cells	Reference
Yeast cell AR bioassay model						
hAR[b]	ARE	yEGFP	24 h	50 nM T	*S. Cerevisiae*[f]	Bovee et al[65]
hAR[b]	ARE	GFP	24 h	16 nM T	*S. Cerevisiae*[f]	Beck et al[66]
Mammalian cell AR bioassay model						
hAR[c]	MMTV[b]	dsEGFP	24 h	0.1 nM DHT	PC-3[e]	Dennis et al[67]
Mammalian cell AR nuclear translocation model						
hAR[c,a]	NA	Nuclear AR-GFP	2 h	0.08 nM R1881[d]	HeLa[e]	Marcelli et al[68]
hAR[b,a]	NA	Nuclear AR-GFP	2 h	0.96 nM R1881[d]	HeLa[f]	Szafran et al[70]

Abbreviation: NA, not applicable.
[a]Mutated AR.
[b]Stable AR expression.
[c]Transient AR expression.
[d]EC_{50} Nuclear translocation.
[e]Mixed cell model.
[f]Clonal cell line.

GFP (dsEGFP) in PC3 cells.[67] Confocal images were collected using microscopy and the EGFP quantification was measured by the HyperCyt high-throughput flow cytometry. PC3 cells were transiently cotransfected with an expression vector for the wild-type hAR (pDsRedhAR) and an MMTV promoter EGFP (pMMTVdsEGFP). Agents with established androgenic and antiandrogenic activity were used for validation of the multifunctional AR screening assay. HyperCyt analysis requires 24-hour treatment with compounds followed by cell centrifugation. A lack of selectivity was demonstrated after treatment with R1881, DHT, E2, progesterone, bicalutamide, nilutamide, and androstenedione; all compounds induced significant increases in the percent of cells expressing dsEGFP compared with unstimulated wells. The sensitivity of the assay in response to AR was evaluated on EC_{50} of R1881 (1.34 PM) and DHT (0.1 nM).

Fluorescent Androgen Receptor Translocation Bioassay

In recent years, several cell-based models have been developed to monitor androgen activity by imaging AR nuclear translocation in response to ligands.[17,18] GFP-tagged AR is the only assay that currently allows detection with fluorescent microscopy and automated image analysis to quantify changes in AR nuclear translocation, intracellular dynamic, and solubility in response to compounds and AR mutations. AR transgenes containing GPF or its spectral variants cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) allow tracking of the dynamic events that occur following ligand binding, using real-time microscopy.

To investigate the cellular translocation of GFP-AR after treatment with agonists and antagonists, Marcelli et al[68] utilized an AR (A619Y) containing a mutation in the DBD[69] that inactivates the transcriptional activity of the receptor. A619Y is able to form distinct foci upon exposure to active compounds. The model relies on transient transfection of HeLa cells with tagged AR. This investigative group has used GFP-AR as well as CFP-AR that allows for dual examination with proteins tagged with YGF. Incubation of cells for 2 hours with ligand (R1881, Casodex, Flutamide, and Estradiol) was sufficient to allow the quantification of AR nuclear translocation (EC_{50} 0.08 of R1881). A high throughput microscopy (HTM) system was used to automate fluorescent image acquisition and analysis of AR nuclear translocation and nuclear foci formation, while the CytoShop software was utilized to quantify the translocation. The results demonstrated that agonist addition resulted in a translocation of the receptor from the cytoplasm to the nucleus where it became organized into stable foci. Interestingly, AR antagonist also caused some nuclear translocation but without the resultant focal distribution (also called hyperspeckling). Fluorescence recovery after photobleaching (FRAP) also revealed that agonist-bound GFP-AR exhibited reduced mobility relative to unliganded or antagonist-bound GFP-AR.

A different high throughput (HT) image-based assay that quantifies AR subcellular and subnuclear distribution and transcriptional reporter gene activity on a cell-by-cell basis was developed by Szafran et al.[70] This assay permitted the analysis of cell cycle–dependent changes in AR function in unsynchronized cell populations, allowing for the determination of cell cycle position with simultaneous analysis of DNA. HeLa cell lines were generated to stably express wild type (GFP-AR), mutant GFP-ART877A (LNCaP mutation),[71] or GFP-ARF764L (AIS mutation).[72] R1881, mibolerone, and DHT were tested to demonstrate the utility of the AR bioassay. All three compounds induced GFP-AR nuclear translocation in a dose-dependent manner. Using R1881, the calculated EC_{50} concentration for nuclear translocation was 0.96 nM. The AR agonists DHT and mibolerone demonstrated similar effects when compared with R1881. An automated microscope was used to capture the images, and CytoShop and Pipeline Pilot image analysis software was used to quantify.

Conclusion

There is broad interest in the use of cell-based androgen screening assays to assess androgenic activity of chemical compounds as well as to test human sera for novel androgens. Over the past 10 years, there have been significant improvements in the available AR cell–based assays which now provide rapid, inexpensive, and sensitive androgen screening tools. Improved aspects of the models include greater selectivity for AR and improved reporter gene responses. These assays provide important resource for the discovery of novel androgens that cause human diseases of androgen excess, the detection of prohibited androgenic compounds in athletes, as well as methods for defining environmental contaminants that act as AR endocrine disrupters.

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GPER agonist G-1 decreases adrenocortical carcinoma (ACC) cell growth *in vitro* and *in vivo*

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ABSTRACT

We have previously demonstrated that estrogen receptor (ER) alpha (ESR1) increases proliferation of adrenocortical carcinoma (ACC) through both an estrogen-dependent and -independent (induced by IGF-II/IGF1R pathways) manner. Then, the use of tamoxifen, a selective estrogen receptor modulator (SERM), appears effective in reducing ACC growth *in vitro* and *in vivo*. However, tamoxifen not only exerts antiestrogenic activity, but also acts as full agonist on the G protein-coupled estrogen receptor (GPER). Aim of this study was to investigate the effect of a non-steroidal GPER agonist G-1 in modulating ACC cell growth. We found that G-1 is able to exert a growth inhibitory effect on H295R cells both *in vitro* and, as xenograft model, *in vivo*. Treatment of H295R cells with G-1 induced cell cycle arrest, DNA damage and cell death by the activation of the intrinsic apoptotic mechanism. These events required sustained extracellular regulated kinase (ERK) 1/2 activation. Silencing of GPER by a specific shRNA partially reversed G-1-mediated cell growth inhibition without affecting ERK activation. These data suggest the existence of G-1 activated but GPER-independent effects that remain to be clarified. In conclusion, this study provides a rational to further study G-1 mechanism of action in order to include this drug as a treatment option to the limited therapy of ACC.

INTRODUCTION

Adrenocortical carcinoma (ACC) represents a rare malignancy with a very poor prognosis. Resectability is the prime determinant of prognosis. For patients with disseminated disease, chemotherapy options are few and lack sufficient efficacy. Mitotane, a cytotoxic drug with a not well documented mechanism of action [1], is the conventional therapy. The toxicity of mitotane has been a major limit to its suitability in the treatment of ACC patients. Severe side-effects, of either the gastrointestinal or the nervous system, have been frequently reported, and many patients are not able to take the drug regularly [2, 3]. Recently, monoclonal antibodies targeting insulin-like growth factor (IGF) receptor (IGF1R) have been tested in clinical trials, however, they provided a limited effectiveness in refractory patients [4]. Rationale for targeting IGF1R comes from the observation that IGFII [5] is overexpressed in ACC. IGFII effects are mediated through its receptor IGF1R resulting in activation of the

PI3K/AKT/mTOR cascade, the RAS/MAPK and the PLC/PKC pathways [6]. We have recently demonstrated that activation of these pathways can be triggered by the estrogen receptor alpha (ESR1) [7], a gene overexpressed in ACC that mediates estrogen-dependent proliferative effects [7, 8]. Our *in vitro* experiments demonstrated that ESR1 knock down was more effective than an IGF1R antibody in controlling H295R cell proliferation [7]. Targeting ESR1 *in vivo* using tamoxifen, a selective estrogen receptor modulator (SERM), was effective in reducing H295R xenografts growth [7].

It is well known that tamoxifen and its active metabolite 4-hydroxytamoxifen (OHT), not only exert antiestrogenic activity [9], but also act as full agonist on the G protein-coupled estrogen receptor GPR30 (from the GPER gene) [10–14]. Then, can Tamoxifen effects depend on GPER activation? GPER can mediate rapid E2-induced non-genomic signaling events, including stimulation of adenylyl cyclase, mobilization of intracellular calcium (Ca^{2+}) stores and activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways [15–17]. GPER exhibits prognostic utility in endometrial [18], ovarian [19], and breast cancer [20] and can modulate growth of hormonally responsive cancer cells [10, 11, 21, 22]. Expression of GPER has been characterized in the outer zona glomerulosa (ZG) and in the medulla of the human adrenal [23], however its expression status in ACC is not known.

A non-steroidal, high-affinity GPER agonist G-1 (1-[4-(6-bromobenzo [1, 3]dioxol-5yl)-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta-[c]quinolin-8-yl]-ethanone) has been developed to dissect GPER-mediated estrogen responses from those mediated by classic estrogen receptors [24]. The biological effects triggered by G-1 appear cell type specific and dependent on the ERs expression pattern [25–29]. By using G-1, in this study we wanted to investigate the effects of GPER activation on ACC growth.

RESULTS

G-1 treatment decreases H295R cell growth *in vitro* and *in vivo*

We first examined GPER expression in human ACCs and in H295R cells. By western blot analysis (Fig. 1A) and real time RT-PCR (Fig. 1B-1C) we demonstrated that GPER is expressed in normal adrenal, in human ACCs and in H295R cells at variable levels. Effects of G-1 on cell viability and proliferation were tested using increasing concentrations (0.01-0.1-1 μM) for different times (24-48-72 h) (Fig. 1D-1E). Of the different doses tested only 1 μM caused a time-dependent reduction in H295R cell growth. Doses higher than 1uM did not show any more pronounced effect (data not shown). Knocking down of GPER gene expression, using a specific shRNA, (shGPER) was assessed by western blot analysis and revealed a substantial decrease in protein content compared to the control shRNA (insert, Fig. 1F). However, GPER silencing was able to only partially abrogate the inhibitory effects exerted by G-1 on H295R cell proliferation (Fig. 1F)

H295R cells were used to generate xenograft tumors in athymic nude mice. Twenty one days after tumor grafting all mice developed a detectable tumor and were randomized to be treated with either vehicle or G-1. G-1 administration produced a statistically significant decrease in tumor volume from day 14 post treatment (Fig. 2A). A trend of growth inhibition was observed thereafter. The drug was well tolerated without lethal toxicity or body weight loss during treatment (data not shown). Multi-slices T2-W MRI indicated larger tumor volume in vehicle treated animals compared to tumors from G-1 treated mice. Hyperintense large cystic area and haemorrahagic regions, that appear as dark areas in the tumor sections, were present in vehicle treated animals (Fig. 2B). Grafted tumors harvested after three-week treatment with G-1 showed a significant decrease in tumor weight compared to vehicle treated animals (Fig. 2C). Hematoxylin and eosin staining of xenograft tumors revealed some picnotic nuclei only in G-1 treated tumors (Fig. 2D). Ki-67 immunostaimning was significantly lower in G-1-treated tumors compared to control mice (value score control: 6, 6 ± 0, 89 (SD); value score G-1 treated cells: 3, 1 ± 0.55 * (SD) (*$p < 0.05$) (Fig. 2E).

G-1 induces H295R cell cycle arrest and cell death

Cell cycle analysis of H295R cells after 24 h of G-1 treatment demonstrated a cell cycle arrest in the G_2 phase (Fig 3A). This effect was further confirmed by a change in the expression of cyclins, after G-1 treatment (Fig. 3B). Specifically, by western analysis we observed that G-1 treatment caused a decrease in Cyclin E (CCNE), while Cyclin B1 (CCNB1), involved in the regulation of G_2 phase, was increased. CCNE and CCNB1 had similar expression pattern in protein samples extracted from xenografts tumors (Fig. 3C). Collectively these events support the idea of cells exiting G_1 but remaining stuck in G_2 phase. In agreement with the observation that inappropriate accumulation of B type cyclins is associated with the initiation of apoptotic pathways [30], we found that G-1 caused cell death by apoptosis. Cells were treated for 24 or 48 h with vehicle or G-1, incubated with an Annexin-V specific antibody and sorted by flow cytometry. As shown in Figure 3D the number of dead cells increased in a time dependent manner reaching about 40% of apoptotic cells 48 h after G-1 treatment (Fig. 3D).

G-1 causes cell nuclei morphological changes, DNA damage and apoptosis

G-1 ability to trigger apoptosis in H295R cells was further confirmed by evaluation of DNA fragmentation.

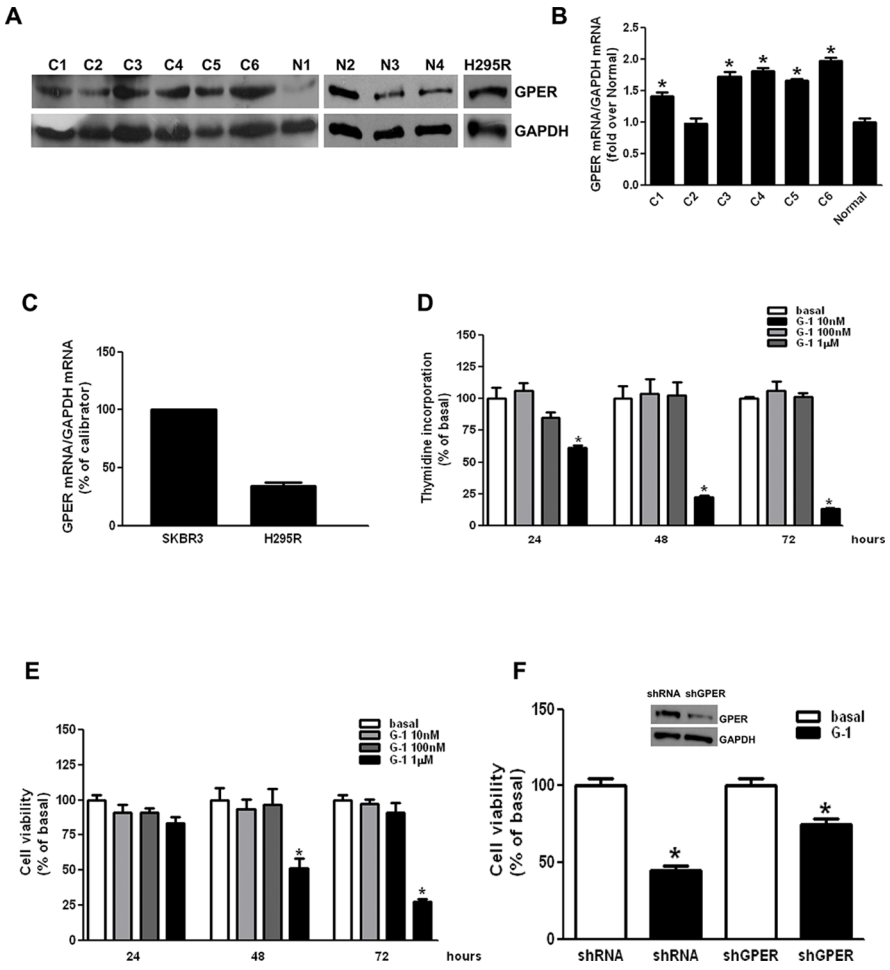


Figure 1: G-1 treatment decreases H295R cell growth *in vitro*. A. Western blot analysis of GPER was performed on 50 μg of total proteins extracted from normal adrenal, ACCs and H295R cells. GAPDH was used as a loading control. **B-C.** GPER mRNA expression in normal adrenal and ACCs (B), H295R and SKBR3 (positive control) cells (C) was analyzed by real time RT-PCR. Each sample was normalized to its GAPDH RNA content. Final results are expressed as n-fold differences of gene expression relative to calibrator. Data represent the mean + SE of values from at least three separate RNA samples; *$P < 0.05$, versus calibrator). **D-E.** H295R cells were treated with G-1 (0.01–1 μM) for different times (24, 48 and 72 h). Cell proliferation was evaluated by [^{3}H]Thymidine incorporation (D) and MTT (E) assays. Results were expressed as mean + SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*$P < 0.05$ versus basal). **F.** MTT assay was performed on H295R cells, which were previously transfected for 72 h in the presence of control vector (shRNA) or shGPER. Twenty-four hours after transfection cells were treated in 2.5% DCC-FBS medium for 48 h with G-1 (1 μM). Results were expressed as mean + SE of three independent experiments each performed in triplicate. (*$p < 0.05$ versus basal). The insert shows a Western blotting assay on H295R protein extracts evaluating the expression of GPER receptor in the presence of shRNA or of shGPER. GAPDH was used as a loading control.

TUNEL staining demonstrated the presence of increased positive cells in cells treated with G-1 (Fig. 4A). In addition, Hoechst staining evidenced that untreated H295R cells had round nuclei with regular contours; while nuclei from cells treated with G-1 appeared shrunken and irregularly shaped or degraded with condensed DNA. DNA gel electrophoresis extracted from G-1 treated H295R cells revealed a classic laddering pattern of inter-nucleosomal DNA fragmentation that was absent in control cells (Fig. 4B). This event was associated with an increase in Parp-1 cleavage (Fig. 4C). The presence of G-1 increased Bax expression while decreased Bcl-2 (Fig. 4D). Similarly, data obtained from western blot analysis of tumors samples overlap with those obtained in H295R cells (Fig. 4E). When the intrinsic apoptotic mechanism is triggered, Cytochrome c (Cyt c) is released from the mitochondria into the cytosol [31]. Therefore we fractionated G-1 treated H295R cell lysates into cytosolic and mitochondrial fractions and evaluated Cytochrome c release by western blot analysis (Fig. 4F). Cytochrome c levels increased in the cytosolic fraction of treated samples while decreased in the mitochondrial compartment. Cytochrome c release from mitochondria into the cytosol triggers caspase activation. After G-1 treatment we detected active Caspase 9 (Fig. 4G) as well as the executioner Caspase 3/7 (Fig. 4H).

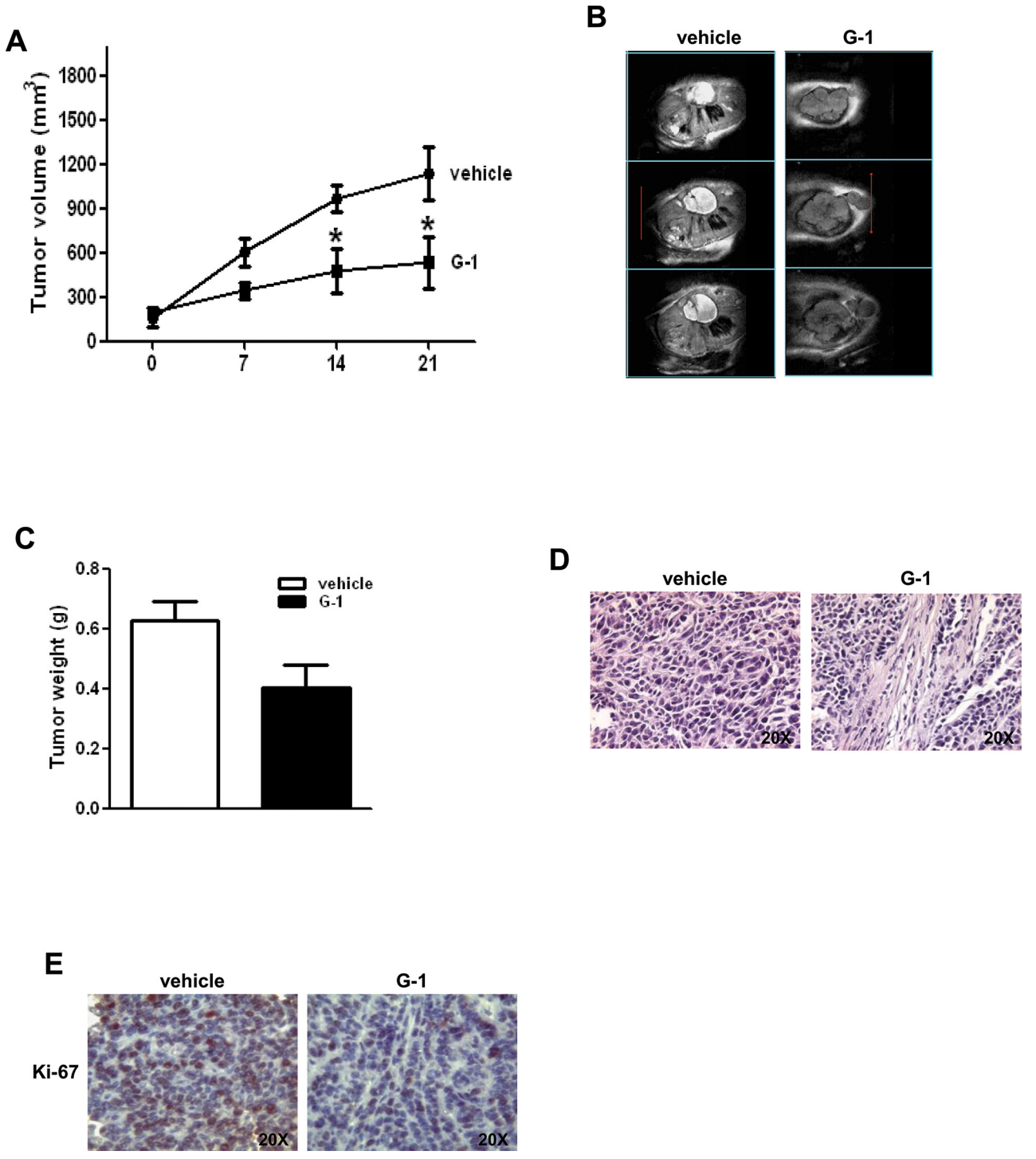
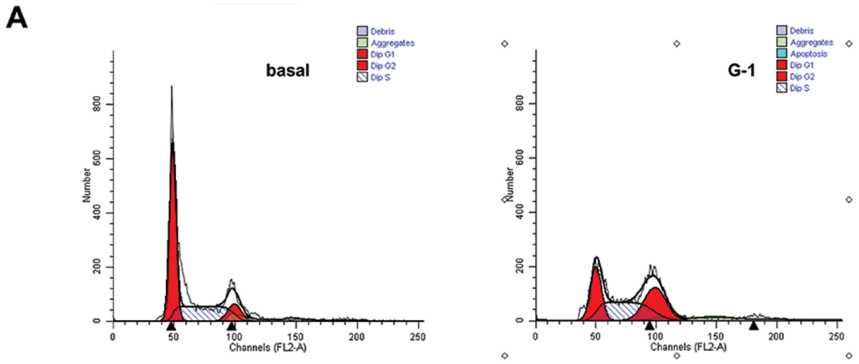
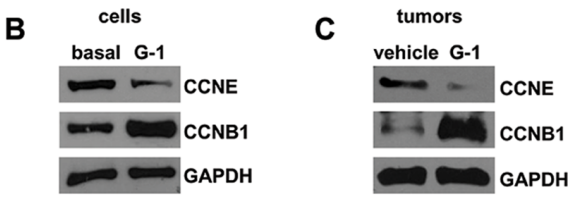


Figure 2: G-1 treatment decreases H295R cell growth *in vivo*. A. 6×10^6 H295R cells were injected subcutaneously in the flank region of immunocompromized mice and the resulting tumors were grown to an average of 200 mm^3 twenty one days after inoculation. Tumor volumes were calculated, as indicated in Materials and Methods. Values represent the mean + SE of measured tumor volume over time in the control group (filled circles, $n = 10$) and in the G-1-treated group (filled triangles, $n = 10$). Data represent pooled values from two independent experiments. (*$P < 0.05$ versus control at the same day of treatment). **B.** *In vivo* coronal T2-weighted spin-echo MR image of primary ACCs. Examples of multi-slices T2-W MRI (section thickness of 1 mm) tumors from vehicle treated mice (control tumors) show a larger volume compared to tumors from G-1 treated mice. Hyperintense large cystic area and haemorrhagic regions that appear as dark areas in the tumor sections, are present in the control tumors. **C.** After 3-week treatment tumors were harvested and weighed. Values represent the mean + SE of measured tumor weight ($n = 10$) (* $P < 0.05$ versus vehicle). **D.** Hematoxylin and eosin stained histologic images of H295R xenograft tumors. **E.** Representative pictures of Ki-67 immunohistochemical staining of H295R xenograft tumors.



	H295R cell population (%)		
Treatment	G1	S	G2/M
basal (24h)	55,08±5,52	34,38±3,44	10,54±1,05
G-1 (24h)	26,90±1,34	40,07±2,0	33,03±1,65



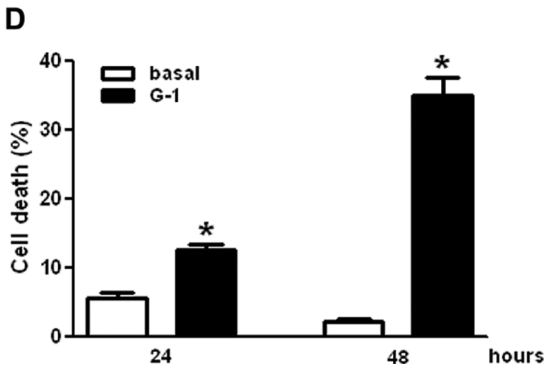


Figure 3: Effects of G-1 treatment on cell cycle distribution and on cell death. A. H295R cells were synchronized in serum-free media for 24 h and then exposed to vehicle (basal) or G-1 (1 μM) for the indicated times. The distribution of H295R cells in the cycle was determined by Flow Cytometry using Propidium Iodide stained nuclei. Table shows the distribution of H295R cell population (%) in the various phases of cell cycle. **B-C.** Western blot analyses of Cyclin E (CCNE) and Cyclin B1 (CCNB1) were performed equal amounts of total proteins extracted from H295R cells treated with G-1 (1 μM) for 24 h (B) and xenografts tumors (C) Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control. **D.** Subconfluent H295R monolayers starved for 24 h were treated for the indicated times with G-1 (1 μM). Then cells were stained with Annexin V/ FITC plus PI and examined by flow cytometer. Graph represents the percentage of cell death at the different times of treatment. (* $P < 0.05$ versus basal).

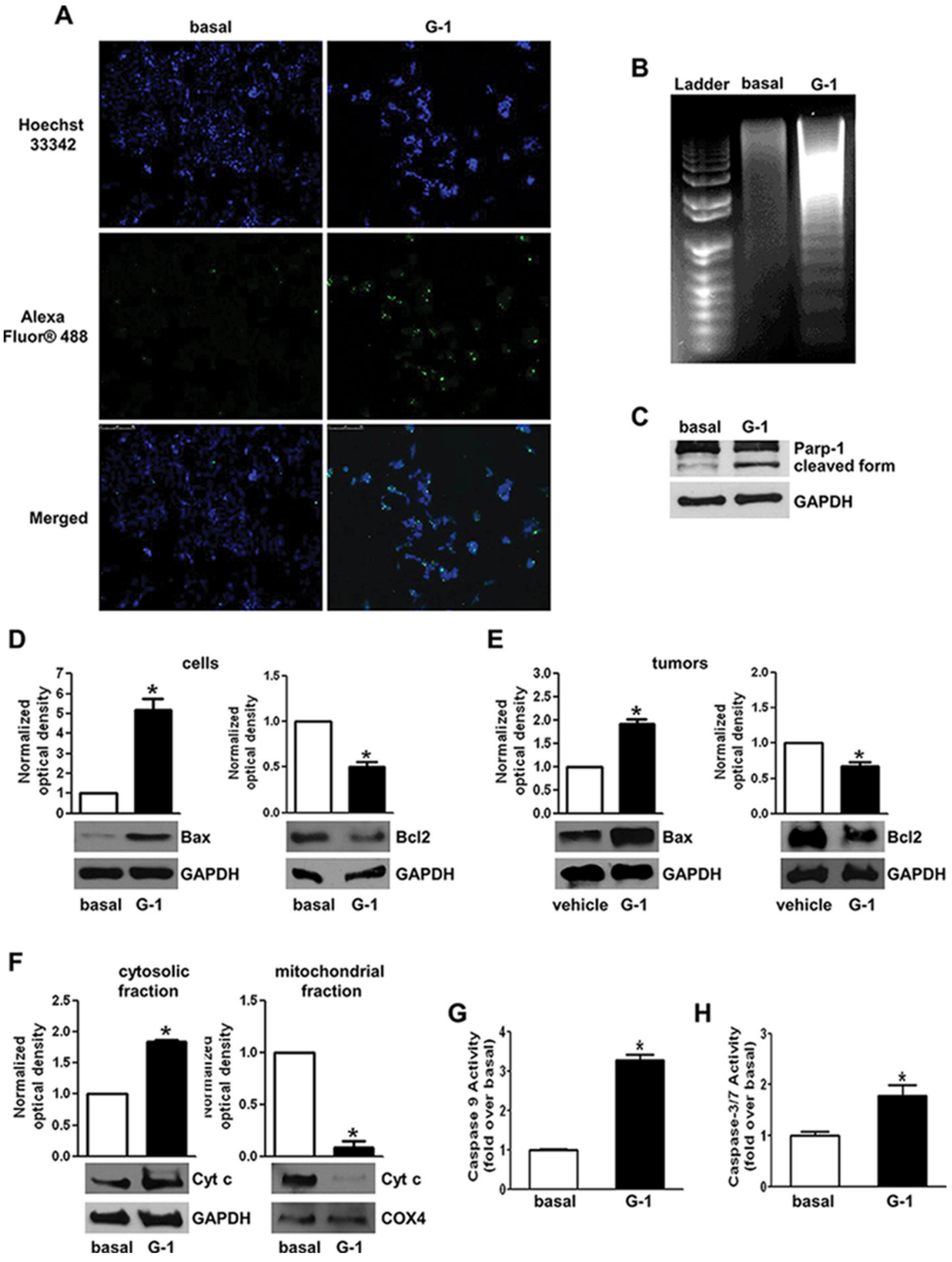


Figure 4: G-1 treatment induces apoptosis in H295R cells. A. Cells were left untreated (basal) or treated with G-1 (1 μM) for 24 h; after treatment cells were fixed with paraformaldehyde and processed for TUNEL staining. Nuclei counterstaining was performed using Hoechst 33342. Fluorescent signal was observed under a fluorescent microscope (magnification 200X). Images are from a representative experiment. **B.** After 48 h treatment DNA was extracted from cells and analyzed on a 1.5% agarose gel. Images are from a representative experiment. **C–F.** H295R cells were treated with G-1 (1 μM) for 24 h. Western blot analyses of Parp-1 (C), Bax and Bcl-2 (D). Cytochrome c (F) were performed on equal amounts of total proteins. Blots are representative of three independent experiments with similar results. Bax and Bcl-2 were analyzed on total proteins extracted from xenograft tumors (E). GAPDH was used as a loading. **G-H.** H295R cells were treated with G-1 (1 μM) for 24 h. Caspase 9 (G) and caspase 3/7 (H) activity was determined by a luminescent assay. Results were expressed as percentage of enzyme activity. Graphs represent mean + SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*$P < 0.05$ versus basal).

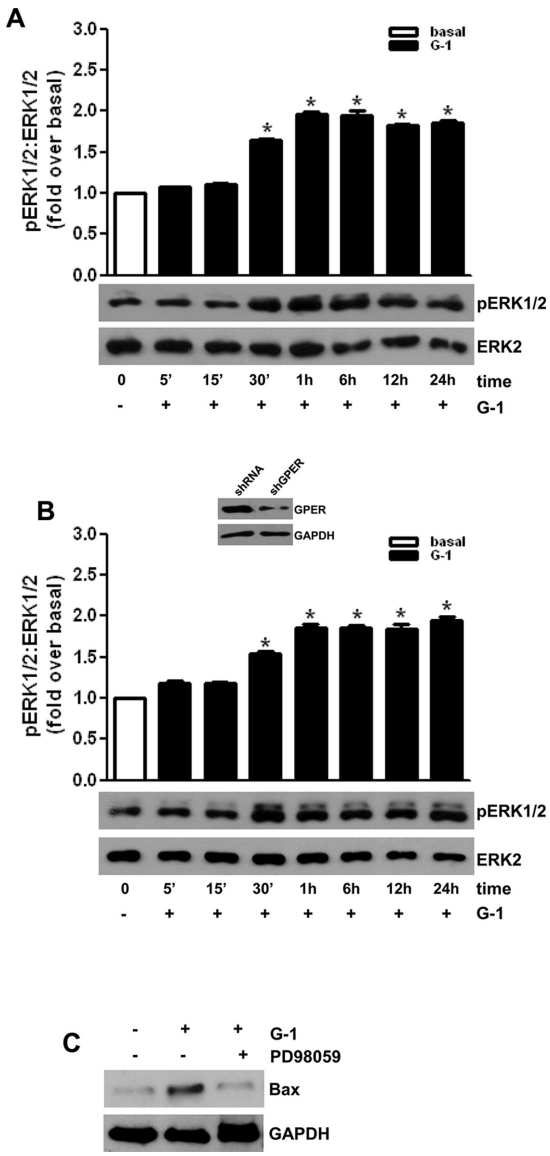


Figure 5: G-1-induced MAPK activation correlates with an increased protein expression of proapoptotic Bax. H295R cells were transfected with shRNA **A.** or shGPER **B.** for 72 h. Forty-eight hours after transfection cells were untreated (0) or treated for at the indicated time with G-1 (1 μM). Western blot analyses of pERK1/2 were performed on 10 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. The insert in (B) shows a Western blot on H295R protein extracts evaluating the expression of GPER receptor in the presence of shcontrol or of shGPER. GAPDH was used as a loading control. (A-B up panels) Graphs represent means of normalized optical densities from three experiments, bars represent SE. *$p < 0.05$ versus basal. **C.**, H295R cells were treated for 24 h with vehicle (−) or G-1 (1 μM) alone or combined with PD98059 (10 μM). Western blot analysis of Bax was performed on equal amounts of total proteins. GAPDH was used as a loading control. Blots are representative of three independent experiments with similar results.

G1 treatment causes sustained ERK1/2 phosphorylation

In order to define the molecular mechanism associated with G-1-induced apoptosis, we investigated the activation of MAPK family members extracellular signal-regulated kinase 1/2 (ERK1/2), which have been demonstrated to be involved in apoptosis if activated for a prolonged time [32]. As shown in Figure 5A, G-1 treatment activated ERK1/2 in a time-dependent manner as seen by the increased levels of their phosphorylation status. Activation started after 30-min of G-1 treatment and persisted for up to 24 h (Fig. 5A). ShGPER, that partially reversed G-1 effects on cell proliferation (Fig. 1E) did not affect ERK1/2 activation (Fig. 5B). Involvement of ERK1/2 in G-1-induced apoptosis of adrenocortical cancer cells was confirmed by the observation that MEK1 inhibitor, PD98059, prevented the up-regulatory effect exerted by G-1 on Bax expression (Fig. 5C).

DISCUSSION

Here, we demonstrated for the first time that a selective non estrogenic ligand of GPER named G-1 is able to inhibit H295R cell growth both *in vitro* and *in vivo* in a xenograft model. Starting from these results we investigated the potential role of GPER in this event.

First, we showed GPER expression both at transcriptional and post-transcriptional level in our ACC cell model represented by H295R cells as well as in normal adrenal and ACC samples. These first analyses aimed to assess only if GPER was expressed in normal and tumor adrenal and not to indicate any difference in expression levels, since overexpression of GPCR is not a common event in human diseases [20].

Recent studies have shown that activation of GPER initiates signaling cascades that, depending on the cell type, are associated with both proliferation [11, 33] and apoptosis [29, 32]. Ariazi et al. have highlighted the opposite effects played by GPER activation on cell proliferation of ERs negative and ERs positive breast cancer cells [17]. Specifically, when ERs are expressed, activation of GPER leads to inhibition of cell proliferation. On the contrary, when cells are ERs negative activation of GPER leads to an increase in cell proliferation [17]. Our work, demonstrated that micromolar concentrations of G-1 decrease H295R cell proliferation *in vitro*, significantly reduce ACC tumor volume *in vivo* and cause a marked decrease in the expression of the nuclear proliferation antigen Ki-67. Accordingly, flow cytometry analysis revealed that G-1 treatment causes changes in cellular distribution within the different phases of cell cycle. It is well established that cell cycle progression is dynamically and strictly regulated by complexes containing cyclins and cyclin dependent kinases (CDKs) [34]. Here, we found that after G-1 treatment expression of G_1 phase cyclin CCNE was reduced, while G_2 phase cyclin CCNB1 was increased. This observation indicates that H295R cells do not bypass G_2 checkpoint. Similar data were reported for prostate cancer cells, where GPER activation by 1 μM G-1 caused cell cycle arrest at the G_2 phase [35]. G_2 phase arrest was followed by apoptotic cell death as indicated by positive staining for Annexin-V, nuclei morphological changes and appearance of DNA ladder pattern.

Apoptosis can be induced by extrinsic [36] and intrinsic [37] mechanisms; the latter is strictly controlled by bcl-2 family of proteins [38] that consists of both pro- (Bax, Bad, Bak, Bid) and anti-apoptotic (Bcl-2, Bcl-xl) proteins able to modulate the execution phase of the cell death pathway. Bax exerts pro-apoptotic activity by allowing Cytochrome c translocation from the mitochondria to the cytosol [39]. Cytochrome c then binds to apoptotic protease-activating factor-1 (Apaf-1) [40], which in turn associates with Procaspase 9 resulting in the activation of its enzymatic activity [41], responsible for the proteolytic activation of executioner Caspase 3 [42]. The active Caspase 3 is then involved in the cleavage of a set of proteins including Poly-(ADP) ribose polymerase-1 (Parp-1) [43]. Bcl-2, instead, exerts its anti-apoptotic activity, at least in part, by inhibiting the translocation of Bax to the mitochondria [40]. Changes in expression and/or activation of all the above mentioned biochemical markers of mitochondrial apoptotic pathway were observed in H295R cells in response to G-1 treatment.

MAPK family members ERK1/2 are part of GPER signaling [14]. Despite the well-defined role of ERK1/2 activation in proliferative pathways [44], sustained ERK1/2 phosphorylation is involved in apoptotic events [29, 32, 45]. Cagnol and Chambard have summarized more than 50 publications showing a link between prolonged ERK activation and apoptosis [46]. Specifically it can be appreciated that duration of ERK activation in promoting cell death can be different depending on cell type and stimuli. G-1 caused sustained ERK1/2 activation in H295R, this event was clearly involved in the induction of apoptosis, since chemical inhibition of MEK1/2 using PD98059 abrogated G-1 ability to induce the expression of proapoptotic factor Bax. Several reports pointed out that ERK1/2 activity can be associated with upregulation of proapoptotic members of the Bcl-2 family, such as Bax [47–49]. Moreover, ERK activity has been shown to directly affect mitochondrial function [46] by decreasing mitochondrial respiration [50, 51] and mitochondrial membrane potential [51, 52], causing mitochondrial membrane disruption and Cytochrome c release [52–54].

Interestingly, GPER silencing was not able to prevent G-1 induced ERK phosphorylation, underlying the existence of alternative targets for G-1. These targets, similarly to GPER, are able to activate ERK1/2 signaling, however for a prolonged period, and clearly deserve further investigation.

Other papers evidenced inhibitory effects exerted by G-1 on the growth of different tumor cell types in a GPER independent manner [55–57], but a precise mechanism has not been defined. Although further studies are needed to clarify the molecular mechanisms behind G-1-dependent effects, this molecule could be a viable alternative to the current limited treatment options and therapeutic efficacy for adrenocortical cancer.

In conclusion, we demonstrated that treatment of H295R cells with G-1 reduced tumor growth *in vitro* and *in vivo* through a mechanism involving not only GPER activation. G-1 clearly causes cell-cycle arrest at the G_2 phase and apoptosis through a mechanism that requires sustained ERK1/2 activation. Our previously published results highlighting the ability of OHT, a known GPER agonist and ESR1 antagonist, to reduce ACC cell growth, together with the present findings indicating the inhibitory effects exerted by G-1, open up new perspectives for the development of therapies with molecules modulating estrogen receptors action for the treatment of ACC.

MATERIALS AND METHODS

Cell culture and tissues

H295R cells were obtained from Dr W.E. Rainey (University of Michigan at Ann Arbor, USA) [58]. Cells were cultured as previously described [9]. Cell monolayers were subcultured onto 100 mm dishes for phosphatase activity and laddering assay (8×10^6 cells/plate), 60 mm dishes for protein and RNA extraction (4×10^6 cells/plate) and 24 well culture dishes for proliferation experiments (2×10^5 cells/well) and grown for 2 days. Prior to experiments, cells were starved overnight in DMEM/F-12 medium without phenol red and containing antibiotics. Cells were treated with (±)-1-[(3a*R**, 4*S**, 9b*S**)-4-(6-Bromo-1, 3-benzodioxol-5-yl)-3a, 4, 5, 9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (G-1) (1 μM) (Tocris Bioscience, Bristol, UK) in DMEM/F-12 containing FBS-DCC 2, 5% (fetal bovine serum dextran-coated charcoal-treated). Inhibitors PD98059 (PD) (10 μM) (Calbiochem, Merck KGaA, Darmstadt, Germany) was used 1 h prior to G-1. Adrenocortical tumors, removed at surgery, and normal adrenal cortex, macroscopically dissected from adrenal glands of kidney donors, were collected at the hospital-based Divisions of the University of Padua (Italy). Tissue samples were obtained with the approval of local ethics committees and consent from patients, in accordance with the Declaration of Helsinki guidelines as revised in 1983. Diagnosis of malignancy was performed according to the histopathologic criteria proposed by Weiss et al. [59] and the modification proposed by Aubert et al. [60]. Clinical data of the six ACC patients included in this study are shown in Table 1. Patient C6 terminated mitotane treatment six months after beginning of therapy for severe gastrointestinal side effects. Patients C1 and C2 were treated with chemotherapy EAP protocol (etoposide, doxorubicin, and cisplatin) + mitotane.

RNA extraction, reverse transcription and real time PCR

TRizol RNA isolation system (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from H295R, SKBR3 and ACCs. Each RNA sample was treated with DNase I (Invitrogen), and purity and integrity of the RNA were confirmed spectroscopically and by gel electrophoresis before use. One microgram of total RNA was reverse transcribed in a final volume of 30 μl using the ImProm-II Reverse transcription system kit (Promega Italia S.r.l., Milano, Italia); cDNA was diluted 1:2 in nuclease-free water, aliquoted, and stored at − 20°C. The nucleotide sequences for GPER amplification were forward, 5′-CGCTCTTCCTGCAGGTCAA-3′, and reverse, 5′-ATGTAGCGGTCGAAGCTCATC-3′ ; the nucleotide sequences for GAPDH amplification were forward, 5′-CCCACTCCTCCACCTTTGAC-3′, and reverse, 5′-TGTTGCTGTAGCCAAATTCGTT-3′. PCR reactions were performed in the iCycler iQ Detection System (Bio-Rad Laboratories S.r.l., Milano, Italia) using 0.1 μmol/L of each primer, in a total volume of 30 μl reaction mixture following the manufacturer's recommendations. SYBR Green Universal PCR Master Mix (Bio-Rad) with the dissociation protocol was used for gene amplification; negative controls contained water instead of first-strand cDNA. Each sample was normalized to its GAPDH content. The relative gene expression levels were normalized to a calibrator (normal tissue for ACC tissues or SKBR3 for H295R cells). Final results were expressed as n-fold differences in gene expression relative to GAPDH and calibrator, calculated using the ΔΔCt method as previously shown [61].

Western blot analysis

Fifty μg of protein was subjected to western blot analysis [62]. Blots were incubated overnight at 4°C with antibodies against GPER, Cyclin E (CCNE), Cyclin B1 (CCNB1), phospho-Rb, Cytochrome c, Bax, Bcl-2, Parp1, pERK1/2-ERK2 (all from Santa Cruz Biotechnology, Santa Cruz CA, USA). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and immunoreactive bands were visualized with the ECL western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ).

Table 1: Clinical data of the 6 ACC patients analyzed in this study

Sample ID	Age(years)	Gender	Stage at surgery	Syndrome	Weiss score	Size (cm)	Outcome
C1	41	M	IV	Cushing	9	16	Died, 1 year
C2	17	F	IV	Cushing	9	14	Died, 18 months
C3	43	F	III	None	4	9	Died, 8 years
C4	46	M	III	None	3	18	Remission, 7 years
C5	47	M	IV	Cushing	9	14	Died, 1 year
C6	57	M	II	SubclinicalCushing	5	14	Remission, 4 years

To assure equal loading of proteins, membranes were stripped and incubated overnight with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology).

Histopathological analysis

Tumors were fixed in 4% formalin, sectioned at 5 μm and stained with hematoxylin and eosin, as suggested by the manufacturer (Bio-Optica, Milan, Italy).

Immunohistochemical analysis

Paraffin-embedded sections, 5 mm thick, were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (seven to eight serial sections). Immunohistochemical experiments were performed as described [63], using mouse monoclonal Ki-67 primary antibody at 4°C over-night (Dako Italia Spa, Milano, Italy). Then, a biotinylated goat-anti-mouse IgG was applied for 1 h at room temperature, to form the avidin biotin-horseradish peroxidase complex (Vector Laboratories, CA, USA). Immunoreactivity was visualized by using the diaminobenzi-dine chromogen (Vector Laboratories). Counterstaining was carried out with hematoxylin (Bio-Optica, Milano, Italy). The primary antibody was replaced by normal rabbit serum in negative control sections.

Cytochrome c detection

Cells were treated for 24 h, fractioned and processed for Cytochrome c detection as previously reported [26]. Briefly, cells were harvested by centrifugation at 2500 rpm for 10 min at 4°C. Pellets were resuspended in 50 μl of sucrose buffer (250 mM sucrose; 10 mM Hepes; 10 mM KCl; 1.5 mM MgCl2; 1 mM EDTA; 1 mM EGTA) (all from Sigma-Aldrich, Milano, Italy) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF and 0.05% digitonine (Sigma-Aldrich). Cells were incubated for 20 min at 4°C and then centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants containing cytosolic protein fraction were transferred to new tubes and the resulting mitochondrial pellets were resuspended in 50 μl of lysis buffer (1% Triton X-100; 1 mM EDTA; 1 mM EGTA; 10 mM Tris-HCl, pH 7.4) (all from Sigma-Aldrich) containing 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM PMSF (Sigma-Aldrich) and then centrifuged at 13,000 rpm for 10 min at 4°C. Equal amounts of proteins were resolved by 11% SDS/polyacrylamide gel as indicated in the Western blot analysis paragraph.

Cell cycle analysis and evaluation of cell death

Subconfluent monolayers growing in 60 mm plates were depleted of serum for 24 h and treated for an additional 24 h with G-1. The cells were harvested by trypsinization and resuspended with 0.5 ml of Propidium Iodide solution (PI) (100 μg/ml) (Sigma-Aldrich) after treatment with RNase A (20 μg/ml). The DNA content was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the data acquired using CellQuest software. Cell cycle profiles were determined using ModFit LT program. Subconfluent monolayers growing in 60 mm plates were depleted of serum for 24 h and treated for 24 and 48 h with G-1. Trypsinized cells were incubated with Ligation Buffer (10 mM Hepes (pH = 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 1.8 mM CaCl2) containing Annexin-V-FITC (1:5000) (Santa Cruz) and with Propidium Iodide. Twenty minutes post-incubation at room temperature (RT) protected from light, samples were examined in a FACSCalibur cytometer (Becton Dickinson, Milano, Italy). Results were analyzed using CellQuest program.

Caspases 9 and 3/7 activity assay

H295R cells after treatments were subjected to caspases 9 and 3/7 activity measurement with Caspase-Glo 9 and 3/7 assay kits (Promega) and modified protocol. Briefly, the proluminescent substrate containing LEHD or DEVD sequences (sequences are in a single-letter amino acid code) are respectively cleaved by Caspases 9 and 3/7. After caspases cleavage, a substrate for luciferase (aminoluciferin) is released resulting in luciferase reaction luminescent signal production. Cells were trypsinized, harvested and then suspended in DMEM-F12 before being incubated with an equal volume of Caspase-Glo reagent (40 μl) at 37°C for 1 h. The luminescence of each sample was measured in a plate-reading luminometer (Gen5 2.01) with Synergy H1 Hybrid Reader.

TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assay

Cells were grown on glass coverslips, treated for 24 h and then washed with PBS and fixed in 4% formaldehyde for 15 min at room temperature. Fixed cells were washed with PBS and then soaked for 20 min with 0.25% of Triton X-100 in PBS. After two washes in deionized water, they were stained using the Click-iT® TUNEL Alexa Fluor® Imaging Assay (Invitrogen) according to the manufacturer's protocol. Co-staining with Hoechst33342 was performed to analyze the nuclear morphology of the cells after the treatment. Cell nuclei were observed and imaged under an inverted fluorescence microscope (200X magnification).

Determination of DNA fragmentation

To determine the occurrence of DNA fragmentation, total DNA was extracted from control and G-1 (1 μM) treated (48 h) cells as previously described [26]. Equal amounts of DNA were analyzed

by electrophoresis on a 2% agarose gel stained with Ethidium Bromide (Sigma-Aldrich).

Assessment of cell proliferation

[^{3}H]Thymidine incorporation assay

H295R cell proliferation after G-1 treatment was directly evaluated after a 6 h incubation with 1 μCi of [^{3}H]thymidine (Perkin- Elmer Life Sciences, Boston, MA, USA) per well as previously described [64]. Each experiment was performed in triplicate and results are expressed as percent (%) of basal.

MTT assay

The effect of G-1 on cell viability was measured using 3-[4, 5-Dimethylthiaoly]-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [7]. Briefly, cells were treated for different times as indicated in figure legends. At the end of each time point fresh MTT (Sigma-Aldrich), re-suspended in PBS, was added to each well (final concentration 0.33 mg/ml). After 30 minutes incubation, cells were lysed with 1 ml of DMSO (Sigma-Aldrich). Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer.

Gene silencing experiments

For the gene silencing experiments, cells were plated in 12 well plates (1×10^5 cells/well) for proliferation experiments or in 6 well plates (2×10^5 cells/well) for Western blot analysis; cells were transfected with control vector (shRNA) or shGPER in 2, 5% DCC-FBS medium using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendations for a total of 72 h. For proliferation experiments cells were transfected for 24 h and then treated for 48 h before performing MTT assay.

Xenograft model

Four-week-old nu/nu − Forkhead box N1^{nu} female mice were obtained from Charles River Laboratories Italia (Calco, Lecco, Italy). All animals were maintained in groups of five or less and quarantined for two weeks. Mice were kept on a 12 h/12 h light/dark regimen and allowed access to food and water *ad libitum*. H295R cells, 6×10^6, suspended in 100 μl PBS (Dulbecco's Phosphate Buffered Saline), were combined with 30 μl of Matrigel (4 mg/ml) (Becton Dickinson) and injected subcutaneously in the shoulder of each animal. Resulting tumors were measured at regular intervals using a caliper, and tumor volume was calculated as previously described [65], using the formula: $V = 0.52\ (L \times W^2)$, where L is the longest axis of the tumor and W is perpendicular to the long axis. Mice were treated 21 days after cell injection, when tumors had reached an average volume of about 200 mm^3. Animals were randomly assigned to be treated with vehicle or G-1 (Tocris Bioscience) at a concentration of 2 mg/kg/daily. Drug tolerability was assessed in tumor-bearing mice in terms of: a) lethal toxicity, i.e. any death in treated mice occurring before any death in control mice; b) body weight loss percentage = 100 − [(body weight on day x/body weight on day 1) × 100], where x represents a day during the treatment period [66, 67]. Animals were sacrificed by cervical dislocation 42 days after cell injection. All animal procedures were approved by Local Ethics Committee for Animal Research.

In vivo magnetic resonance analyses

Mice were anesthetized with 1–2% isofluorane in O2, 1 L/min (Forane, Abbott SpA, Latina, Italia) and underwent MRI/MRS study. MR analyses were performed at 4.7 T on Agilent Technologies system (Palo Alto, CA, USA). T2-weighted MRI was acquired using a spin echo sequence with the following parameters: TR/TE = 3000/70 ms, section thickness of 1.0 mm, number of acquisitions = 4, point resolution of 256 μm.

Scoring system

The immunostained slides of tumor samples were evaluated by light microscopy using the Allred Score [68] which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to < 1/10; 3 = 1/10 to < 1/3; 4 = 1/3 to 2/3; 5 = > 2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0 = none; 1 = weak; 2 = moderate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six to seven serial sections were scored in a blinded manner for each sample.

Data analysis and statistical methods

All experiments were performed at least three times. Data were expressed as mean values + standard error (SE), statistical significance between control (basal) and treated samples was analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Control and treated groups were compared using the analysis of variance (ANOVA) with Bonferroni or Dunn's post hoc testing. A comparison of individual treatments was also performed, using Student's t test. Significance was defined as $p < 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Estrogen related receptor α (ERRα) a promising target for the therapy of adrenocortical carcinoma (ACC)

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ABSTRACT

The pathogenesis of the adrenocortical cancer (ACC) involves integration of molecular signals and the interplay of different downstream pathways (i.e. IGFII/IGF1R, β-catenin, Wnt, ESR1). This tumor is characterized by limited therapeutic options and unsuccessful treatments. A useful strategy to develop an effective therapy for ACC is to identify a common downstream target of these multiple pathways. A good candidate could be the transcription factor estrogen-related receptor alpha (ERRα) because of its ability to regulate energy metabolism, mitochondrial biogenesis and signalings related to cancer progression.

In this study we tested the effect of ERRα inverse agonist, XCT790, on the proliferation of H295R adrenocortical cancer cell line. Results from *in vitro* and *in vivo* experiments showed that XCT790 reduced H295R cell growth. The inhibitory effect was associated with impaired cell cycle progression which was not followed by any apoptotic event. Instead, incomplete autophagy and cell death by a necrotic processes, as a consequence of the cell energy failure, induced by pharmacological reduction of ERRα was evidenced.

Our results indicate that therapeutic strategies targeting key factors such as ERRα that control the activity and signaling of bioenergetics processes in high-energy demanding tumors could represent an innovative/alternative therapy for the treatment of ACC.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a very rare and aggressive disease with a high risk of relapse after radical surgery. Treatment options in advanced, metastatic stages are limited, since cytotoxic chemotherapy options are poor and radiotherapy is mostly ineffective [1]. The drug mitotane (o, p′-dichlorodiphe nyldichloroethane (o, p′-DDD)) with its adrenolytic activity is the only adrenal specific drug that is currently used for ACC treatment. However, toxicity, narrow therapeutic window and side effects are the major limitation to its use as well as therapeutic success [2]. Given the high mortality and aggressiveness of ACC, more effective and specific treatment options are needed. Recently, monoclonal antibodies targeting insulin-like growth factor II (IGFII) receptor (IGF1R) have been tested in clinical trials, however they provided a limited effectiveness in refractory patients [3]. Rationale for targeting IGF1R comes from the observation that IGFII gene is overexpressed in ACC [4]. We have recently demonstrated that IGFII/IGF1R pathway can be activated by the estrogen receptor alpha (ESR1), a gene overexpressed in ACC that mediates estrogen-dependent proliferative effects [5, 6]. ESR1 knock down was more effective than an IGF1R antibody in reducing H295R cell

proliferation *in vitro* [5] and the selective estrogen receptor modulator (SERM) tamoxifen prevented the growth of H295R both *in vitro* [7] and as xenografts *in vivo* [5]. Thus, ESR1 could be a promising target to reduce ACC growth.

Indeed, a recent study [8], investigating a large cohort of advanced ACC, confirmed the presence of a large number of potentially targetable molecules involved in ACC progression. These observations confirm that ACC is an extremely heterogeneous disease and that its pathogenesis involves integration of signals and the interplay of downstream pathways. It is currently accepted that these changes are also associated with a profound reprogramming of cellular metabolism [9]. Consequently, one potential strategy to develop an effective therapy for ACC could be the identification of a common downstream target of multiple pathways capable of controlling expression and activity of various bioenergetic factors.

Estrogen Related Receptor α (ERRα) is an orphan member of the nuclear hormone receptor superfamily of transcription factors that has been identified on the basis of its high level of sequence identity to ERα and for which an endogenous ligand has yet to be defined [10]. ERRα functions downstream of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha and beta (PGC-1α and PGC-1β) and regulates the expression of genes involved in energy metabolism and mitochondrial biogenesis such as genes encoding enzymes and proteins of the tricarboxylic acid cycle, pyruvate metabolism, oxidative phosphorylation, and electron transport [11]. Research to understand how changes in cell metabolism promote tumor growth has accelerated in recent years [12]. As a consequence, research has focused on targeting metabolic dependencies of cancer cells, an approach with the potential to have a major impact on patient care. Notably, ERRα has recently been associated with dysregulated cell metabolism and cancer progression. Accordingly, increased expression of ERRα has been shown in several cancerous tissues including breast [13], ovary [14] prostate [15] and colon [16]. Several signaling pathways, also relevant to ACC development have been shown to converge upon and regulate the expression and activity of ERRα together with its coactivators such as PGC-1α and β in others tumor types [17]. Several studies have reported that ERRα inverse agonist XCT-790 [18] can induce cell growth arrest in different tumor cell lines [19, 20]. To date, few studies have investigated the role of ERRα in adrenal gland and ACC. ERRα is expressed in normal adult adrenal and regulates the expression of enzymes involved in steroidogenesis [21]. Moreover, ERRα seems to be more expressed in ACC compared to normal adrenal and adenoma [22].

The aim of this study was to establish if ERRα depletion using XCT790 can induce growth arrest in ACC cells. The data obtained support the hypothesis that ERRα could be a promising target for the treatment of adrenocortical cancer.

RESULTS

ERRα inverse agonist XCT790 decreases ERRα protein content and inhibits ACC cells proliferation *in vitro*

First, we verified that ERRα is expressed in H295R adrenocortical cancer cells. MCF-7 breast cancer cells were used as positive control [23] (Figure 1A). Moreover, we also demonstrated that in both H295R and MCF-7 cells, XCT790 treatment decreased ERRα protein levels in a dose-dependent manner (Figure 1B). The latter results confirmed the ability of XCT790 to reduce the expression of ERRα most probably by proteasome degradation [23]. Next, we evaluated the effects of different concentrations of ERRα inverse agonist XCT790 on ACC cell growth. Results from MTT assay revealed that XCT790 treatment exerted a dose- and time-dependent inhibition on H295R cell proliferation compared to vehicle-treated cells (Figure 1C). The maximum inhibitory effect on ACC cell proliferation was seen at 10 μM XCT790 that was then used for all the following experiments.

ERRα inverse agonist XCT790 inhibits ACC cells proliferation *in vivo*

We next established H295R cell xenograft tumors in immunocompromised mice to investigate the ability of XCT790 to reduce tumor growth *in vivo*. To this aim, H295R cells were injected into the intrascapular region of mice. When tumors reached an average volume of 200 mm^3, animals were randomized into two groups to be treated with either vehicle or XCT790 (2,5 mg/Kg). As shown in Figure 2A, mice treated with XCT790 displayed a significant tumor growth reduction compared to the vehicle treated control group. Accordingly, tumor reduction upon XCT790 treatment is evidenced both in terms of tumor mass (Figure 2B) and proliferation as seen in Figure 2C, showing a strong decrease in Ki67staining (value score control: 7.2 ± 0,46 (SD); value score XCT790 treated cells: 4.7 ± 0.53* (SD), *$p < 0.05$).

ERRα inverse agonist XCT790 blocks G1/S transition of ACC cells without inducing apoptosis

The observed effects of XCT790 on ACC cells proliferation led us to evaluate XCT790 action on H295R cell cycle progression.

First, by analyzing PI staining with FACSJazz flow cytometer, we investigated whether XCT790 treatment could affect the distribution of cells within the three major phases of the cycle. To this aim, H295R cells were grown for 24 h in 5% CS-FBS and then treated with either vehicle (DMSO) or 10 μM XCT790. 48 hours later, FACS analysis revealed that XCT790 treated cells accumulated

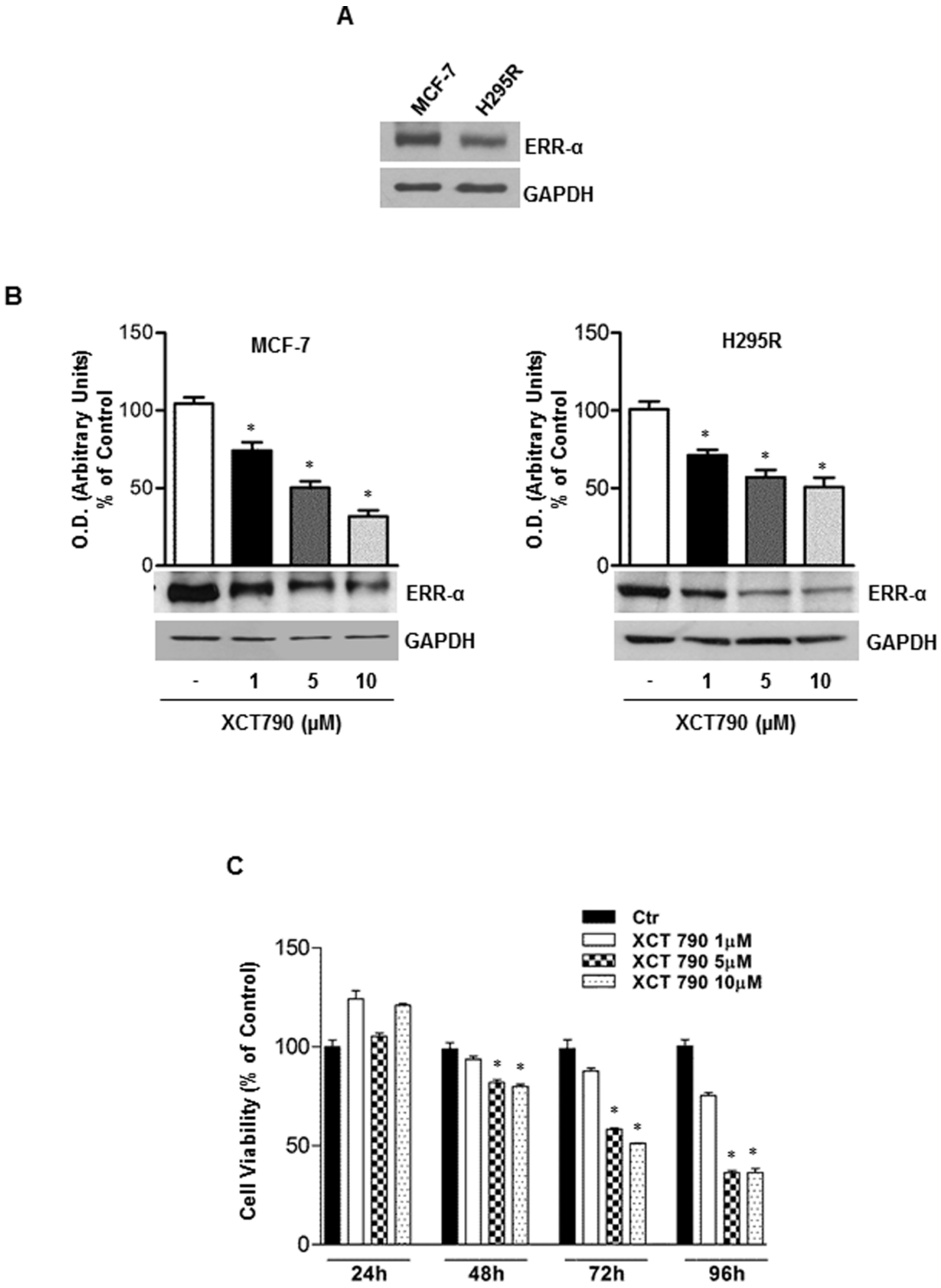

Figure 1: ERRα inverse agonist XCT790 decreases ERRα protein content and H295R cells growth *in vitro*. A. Western blot analysis of ERRα was performed on 50 μg of total proteins extracted from H295R and MCF-7 cells. Blots are representative of three independent experiments with similar results. (**B.** lower left and right panel), protein extracts from MCF-7 and H295R cells left untreated (–) or treated for 48 h with different doses of XCT790 were resolved by SDS-PAGE and subjected to immunoblot against ERRα. GAPDH served as loading control. (b, upper left and right panel), graphs represent means of ERRα optical density (O.D.) from three independent experiments with similar results normalized to GAPDH content (*$p < 0.001$ compared to untreated control sample assumed as 100). **C.** Cell viability after XCT790 treatment was measured using MTT assay. Cells were plated in triplicate in 24-well plates and were untreated (Ctr) or treated with increasing concentrations of XCT790 for the indicate times in DMEM supplemented with 2,5% Charcoal-Stripped FBS. Absorbance at 570 nm was measured on a multiwell-plate reader. Cell viability was expressed as a percentage of control, (*$p < 0.001$).

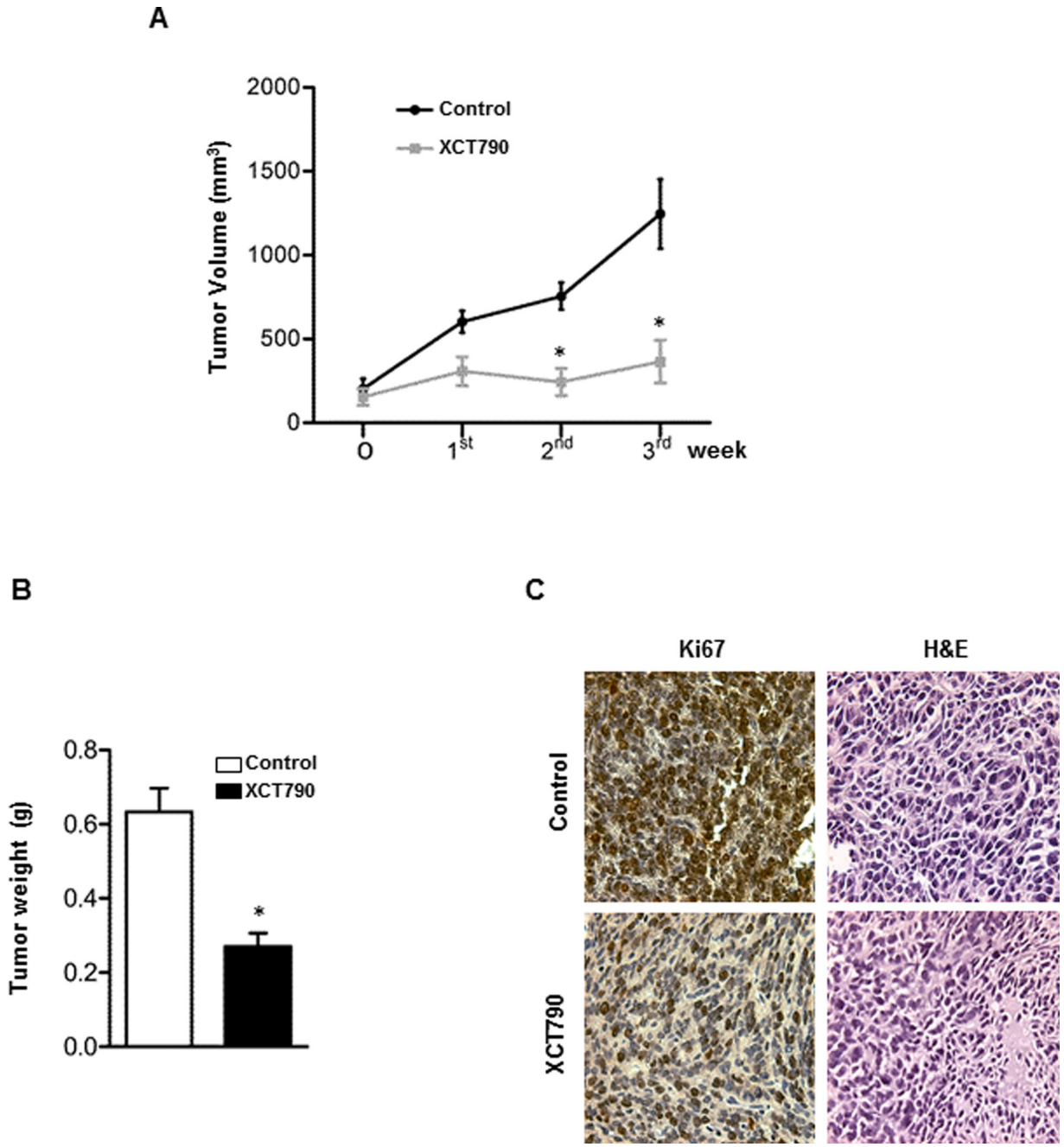

Figure 2: ERRα inverse agonist XCT790 decreases H295R cells proliferation *in vivo*. A. 6 × 10^6 H295R cells were injected subcutaneously onto the intrascapular region of immunocompromised mice and the resulting tumors were grown to an average of 200 mm^3. The animals were randomized to vehicle controls or XCT790 treatment for twenty one days. Tumor volumes were calculated, as indicated in Materials and Methods. Values represent the mean ± SE of measured tumor volume over time in the control group (filled circles, $n = 10$) and in the XCT790-treated group (filled squares, $n = 10$). **B.** After 21 days (3 weeks) tumors were harvested and weighed. Values represent the mean ± SE of measured tumour weight ($n = 10$) *$P < 0.05$ versus control at the same day of treatment. **C.** Ki67 immunohistochemical and H & E staining: histologic images of H295R explanted from xenograft tumors (magnification X 400).

in the G0/G1-phase of the cell cycle while the fraction of cells in S phase decreased compared with vehicle treated cells (Figure 3A).

In order to define the molecular mechanisms involved in XCT790-dependent cell cycle arrest, changes in levels of protein involved in cell cycle regulation were investigated by Western blotting analysis. After 48 h treatment, XCT790 reduced Cyclin D1 and Cyclin E protein content while expression levels of CDK2 and CDK4 proteins were unaffected. Consistently with the observed G1/S transition arrest of the cell cycle, Rb protein showed a hypophosphorylated status (Figure 3B–3C). As the analysis of the cell cycle revealed a minimal increase of the sub-G1 fraction (Figure 3A), a known

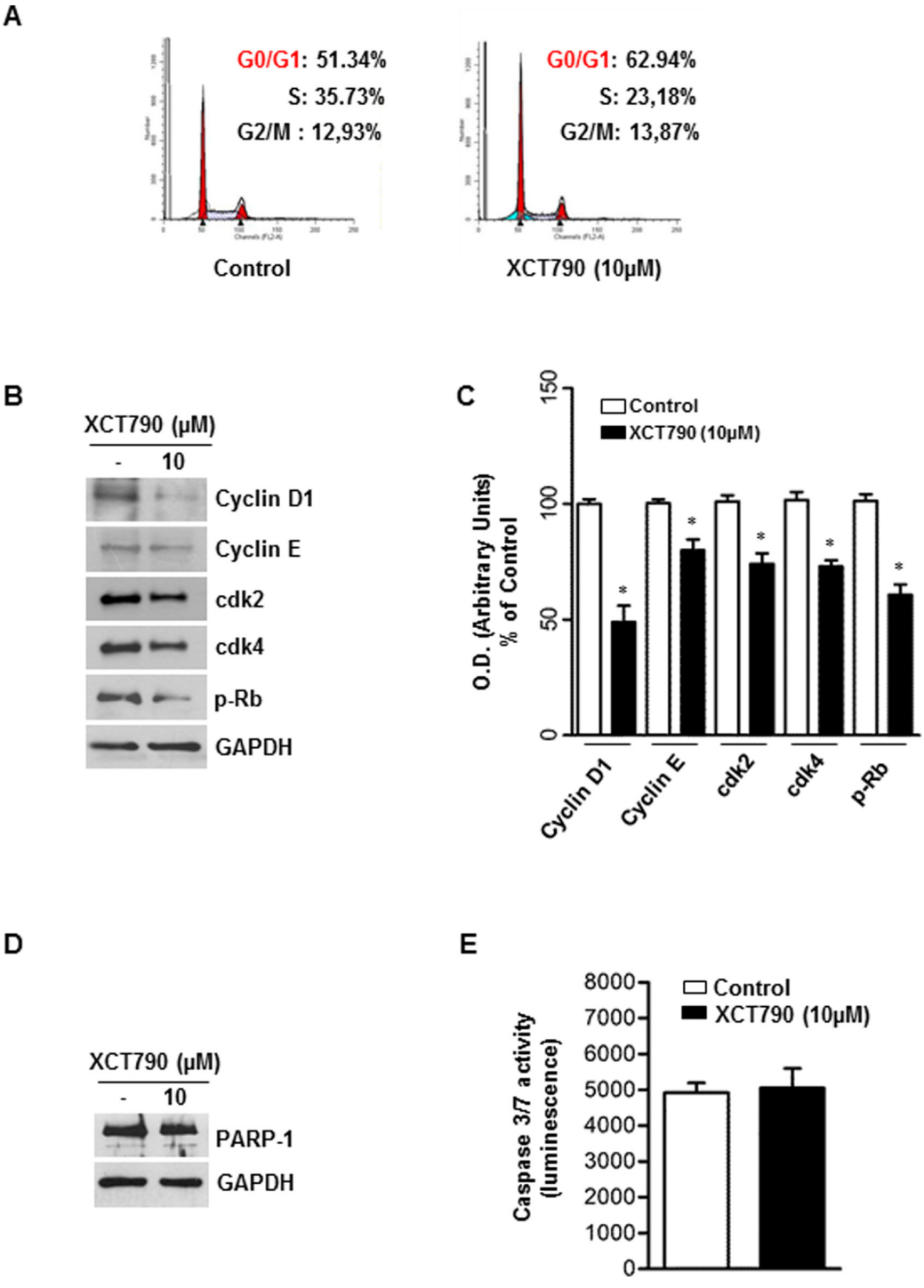

Figure 3: ERRα inverse agonist XCT790 impairs G1/S transition of ACC cells without inducing apoptosis. A. The distribution of H295R cells in the cycle was determined by Flow Cytometry using Propidium-iodide (PI) stained nuclei. The graph shows the distribution of H295R cell population (%) in the various phases of cell cycle. **B.** Total proteins from H295R cells left untreated (–) or treated with XCT790 for 48 h were resolved by SDS-PAGE and subjected to immunoblot analysis using specific antibodies against human Cyclin D1, Cyclin E, cdk2, cdk4, p-Rb. **C.** Graphs represent means of Cyclin D1, Cyclin E, cdk2, cdk4, p-Rb optical densities (O.D.) from three independent experiments with similar results normalized to GAPDH content, (*$p < 0.001$ compared to each untreated control assumed as 100); **D.** Total proteins were analyzed by Western blot for PARP-1. Blots are representative of three independent experiments with similar results. GAPDH served as loading control. **E.** Cellular caspase 3/7 activity was determined by Caspase-Glo assay system using the substrate Ac-DEVD-pNA and expressed as relative luminescence units (RLU) of treated cell to untreated control cell. Each column represents the mean ± SD of three independent experiments (*$p < 0.001$ compared to untreated control sample).

marker of apoptotic events, we next attempted to verify the presence of apoptotic features such as PARP-1cleavage and caspase 3/7 activation, all well-known biochemical markers of programmed cell death. Surprisingly, results from Western blotting analysis for PARP-1 (Figure 3D) and caspase 3/7 activity assay (Figure 3E) clearly showed that XCT790 did not activate an apoptotic pathway.

XCT-790 decreased mitochondrial mass and function in ACC cells

The activity of ERRα is highly dependent on the presence of coactivator proteins, most notably PGC-1α and PGC-1β [24], both known for their crucial role in regulating energy metabolism and mitochondrial biogenesis [24]. Moreover, it has been observed that XCT790 treatment, causing ERRα proteasome degradation, also down-regulates PGC1-α [24]. Based on these observations, we first checked if XCT790 treatment regulates PGC1-α expression in H295R cells. To this aim, ACC cells were left untreated or treated with 10 μM XCT790 for 48 h. Results from Western blotting showed (Figure 4A–4B) that XCT790 treated cells display a reduced expression of PGC1-α, with no effect on PGC-1β levels. We then asked whether reduced levels of PGC1-α would lead to reduction of mitochondrial mass. To this purpose we treated cells with MitoTracker deep red FM that stains specifically mitochondria independently of their membrane potential. Using flow cytometric analysis (Figure 4C), fluorescent imaging (Figure 4D) and fluorescent plate reader (Figure 4E), we found that XCT790 significantly decreased mitochondrial mass.

The mitochondrial citrate carrier CIC is a protein that belongs to a family of metabolites transporters embedded in the inner mitochondrial membrane [25, 26] and has been recently highlighted as important component in maintaining mitochondrial integrity and bioenergetics in normal and particularly in tumor cells [27]. We used CIC protein expression as a marker of both mitochondrial mass and function and found that XCT790 decreased mitochondrial CIC expression (Figure 4F–4G) as well as its transport activity (Figure 4H) in H295R-treated cells compared to vehicle-treated control cells.

To extend these findings, we used immunoblotting to monitor the abundance of a known reliable marker of mitochondrial mass, TOM20, in response to 10 μM XCT790 treatment. We found that XCT790 treated-H295R cells displayed a reduced expression of ERRα, as expected, concomitantly with a drastic decline of TOM20 protein expression (Figure 5A–5B). Similarly, the analysis of the expression of the mitochondrial oxidative pathway (OXPHOS) enzymes showed a substantial reduction of all the complexes (Figure 5C). In agreement with these findings, the reduction in the ATP content reveals a bioenergetics failure induced by XCT790 in treated cells (Figure 5D).

XCT790 induce cell death by necrosis in ACC cells

Very recent data revealed that low levels of CIC or its impaired expression induce mitochondrial dysfunction followed by enhanced mitochondrial turnover via autophagy/mitophagy mechanism [27]. Based on this observation and accordingly to our above reported results showing the ability of XCT790 to down-regulate CIC expression in H295R cells, we wanted to verify if autophagic features were detected in our experimental conditions. Autophagy is characterized by acidic vacuoles (AVO) formation, which can be measured by acridine orange (AO) vital staining. AO moves freely to cross biological membranes and accumulates in acidic compartment, where it is seen as bright red fluorescence [28]. As shown in Figure 6A (upper panel), AO vital staining of 48 h XCT790-treated H295R cells showed the accumulation of AVO in the cytoplasm. To quantify the accumulation of the acidific component, we performed FACS analysis of acridine orange-stained cells using FL3 mode (> 650 nm) to quantify the bright red fluorescence and FL1 mode (500–550 nm) for the green fluorescence. As shown in Figure 6A (lower panel), XCT790 treatment raised the strength of red fluorescence from 7,5% to 51%. These results corroborate the observation that XCT790, increases the formation of AVOs which suggests autophagy/mitophagy as possible mechanisms to explain the reduced mitochondrial mass. This latter event could be responsible for the inhibitory effects on cell growth elicited by XCT790 on adrenocortical cancer cells. A careful evaluation of the autophagic/mitophagic process by investigating changes in autophagic markers such as Beclin 1, LC3B, BNIP3 and Cathepsin B (Figure 6B), suggested that XCT790 treatment promotes the initial stages of the autophagic process. This is supported by the evidence of increased Beclin 1 expression and the presence of the cleaved LC3B form [29]. However, autophagy fails to terminate as indicated by decreased BNIP3, Cathepsin B and Lamp1 proteins expression [29]. Therefore, we evaluated XCT790 ability to induce H295R cells death by necrosis. To this aim, Trypan blue exclusion test was performed after 48 h of XCT790 treatment. As shown in Figure 6C, H295R displayed a significant increase in the number of positive stained cells compared to control cells indicating that membrane integrity and permeability were lost accounting for a necrotic event following a bionergetic failure triggered by ERRα depletion.

DISCUSSION

The molecular heterogeneity and complexity that characterize adrenocortical cancer biology combined with lack of an effective treatment, drive towards the discovery of new therapeutic targets. Advances in the understanding of the molecular pathogenesis of ACC have

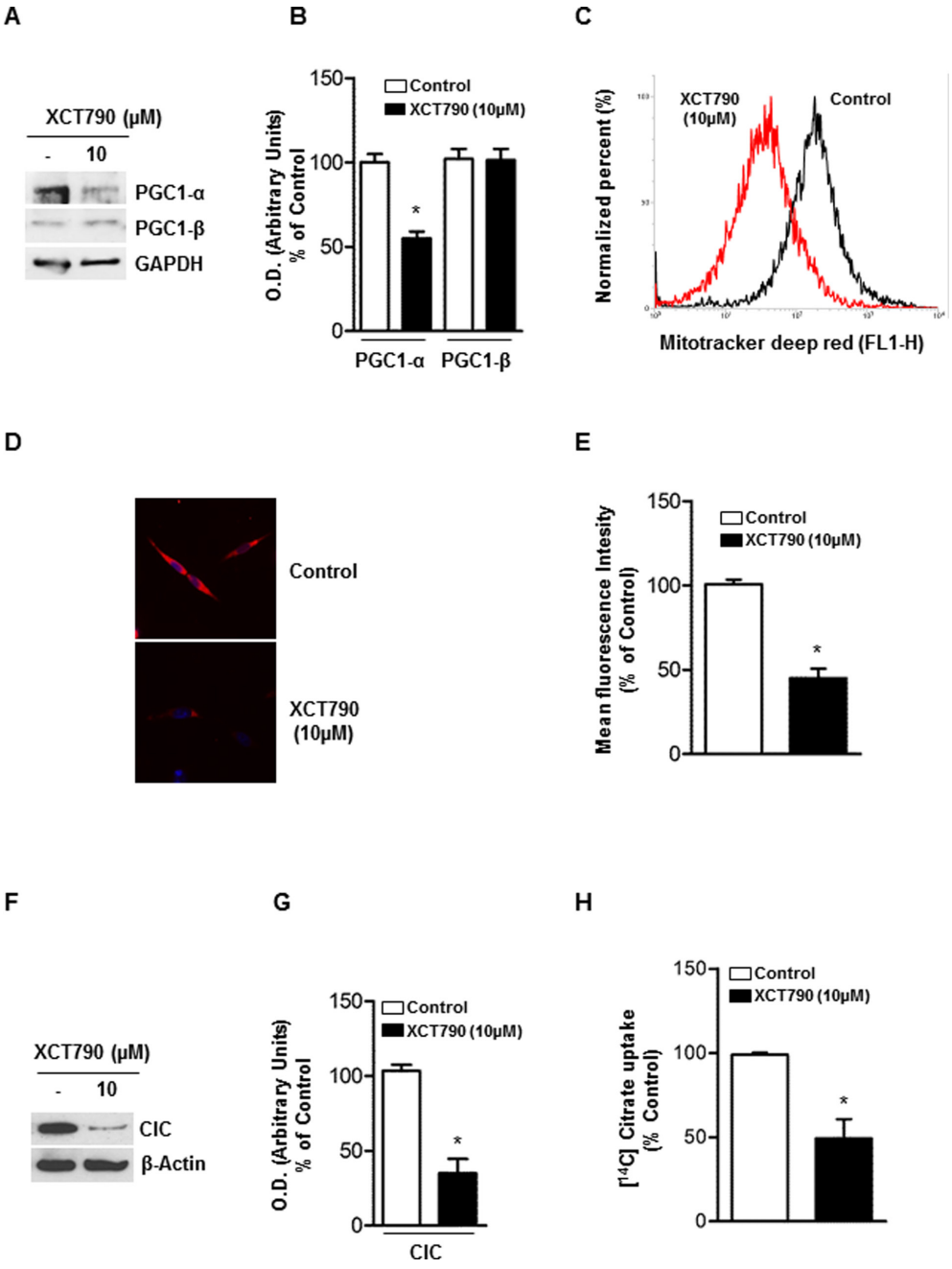

Figure 4: XCT-790 decreases mitochondrial mass and function in H295R cells. A. Total protein extracts from H295R cells, left untreated (–) or XCT790 treated in 2.5% DCC-FBS medium for 48 h were analyzed by Western blot with antibodies against PGC-1α and PGC-1β. GAPDH was used as loading control. **B.** Graphs represent means of PGC-1α and β optical densities (O.D.) from three independent experiments with similar results normalized to GAPDH content (*$p < 0.001$ compared to each untreated control sample assumed as 100). **C.** H295R cells were right untreated (control) or treated with XCT790. 48 h later, absorption of MitoTracker deep red FM was determined by FACS analysis. The uptake of MitoTracker was used as an indicator for the mitochondrial mass. **D.** Reduction in mitochondrial mass was further evaluated by fluorescence microscopy of MitoTracker-stained cells. **E.** Quantification of Mito-Tracker fluorescent signal intensity in untreated (control) or XCT790-treated H295R cells was evaluated measuring red fluorescent signal by a fluorescent plate reader (ex. 644; em. 665) *$p < 0{,}001$ compared to untreated control sample. **F.** Immunoblots for CIC expression from mitochondrial extracts in untreated (–) or XCT-790 treated H295R cells for 48 h. β-Actin served as loading control. Blots are representative of three independent experiments with similar results. **G.** Graph represent means of CIC density (O.D.) from three independent experiments with similar results normalized to β-Actin content (*$p < 0.001$ compared to untreated control sample assumed as 100). **H.** CIC activity was measured at 20 min as steady-state levels of citrate/citrate exchange. Transport was started by adding 0.5 mM [^{14}C]Citrate to proteoliposomes preloaded internally with 10 mM citrate and reconstituted with mitochondria isolated from untreated H295R cells (Control; white column) and H295R-treated cells (black column). The transport reaction was stopped at 20 minutes. Results are expressed as percentage of the control. The data represent means ± SD of at least three independent experiments.

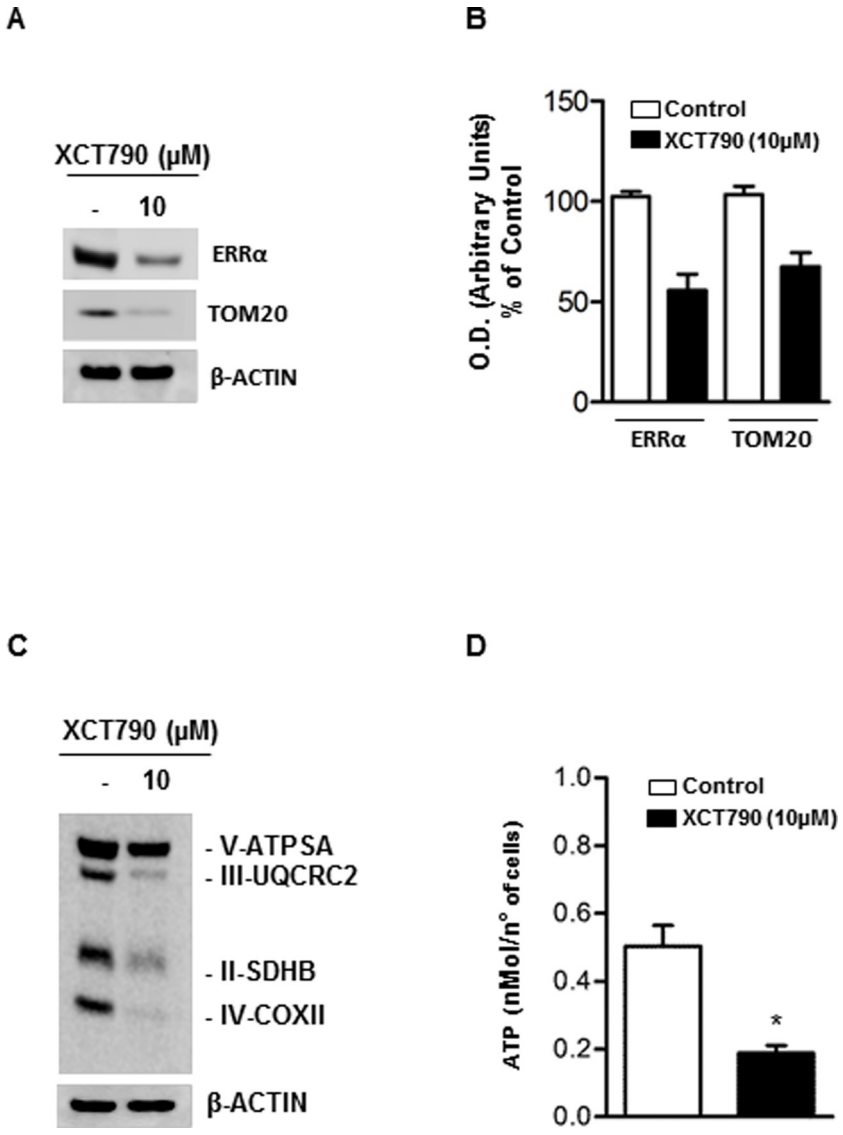

Figure 5: XCT790 decreased OXPHOS protein content and ATP concentration in H295R cells. A. Total protein extracts from H295R cells, left untreated (–) or treated for 48 h in 2.5% DCC-FBS medium with 10 μM XCT790, were analyzed by Western blot using antibodies against ERRα and TOM20. β-actin was used as loading control. **B.** Graphs represent means of ERRα and TOM20 optical densities (O.D.) from three independent experiments with similar results normalized to β-Actin content (*$p < 0.001$ compared to each untreated control sample assumed as 100). **C.** Total protein extracts from H295R cells left untreated (–) or treated for 48 h in 2.5% DCC-FBS medium with 10 μM XCT790, were analyzed by Western blot experiments using antibodies against OXPHOS subunits. β-Actin was used as loading control. Blots are representative of three independent experiments with similar results. **D.** ATP concentrations in H295R cells untreated (–) or treated with XCT790 were determined as described in Material and Methods and expressed as nmol/number of cells. Each column represents the mean ± SD of three independent experiments (*$p < 0, 001$).

been made based on studies of gene expression profiling and genetic syndromes associated with the development of ACC [30]. Results from these studies have highlighted the presence of different and important modifications such as somatic TP53 mutations, alterations at 11p15, a chromosomal locus of IGFII, H19 and cyclin-dependent kinase inhibitor 1C, β-catenin accumulation and activation of the Wnt signaling pathway and overexpression of SF-1 protein [30]. Moreover we have recently demonstrated the involvement of ESR1 in ACC cell growth regulation [5]. Genetic modifications and molecular pathways alterations have as a common purpose the survival and proliferation of the transformed phenotype. It is currently accepted that these changes are associated with a concurrent adaptation and reprogramming of cellular metabolism [31]. In this scenario adrenocortical tumors are not an exception and the metabolic receptor ERRα represents a good therapeutic target. In fact, ERRα is a common downstream target of multiple pathways and a key factor in controlling the expression and activity of various bioenergetics processes. Indeed, it has already been observed that high ERRα gene expression correlates with unfavorable clinical outcomes

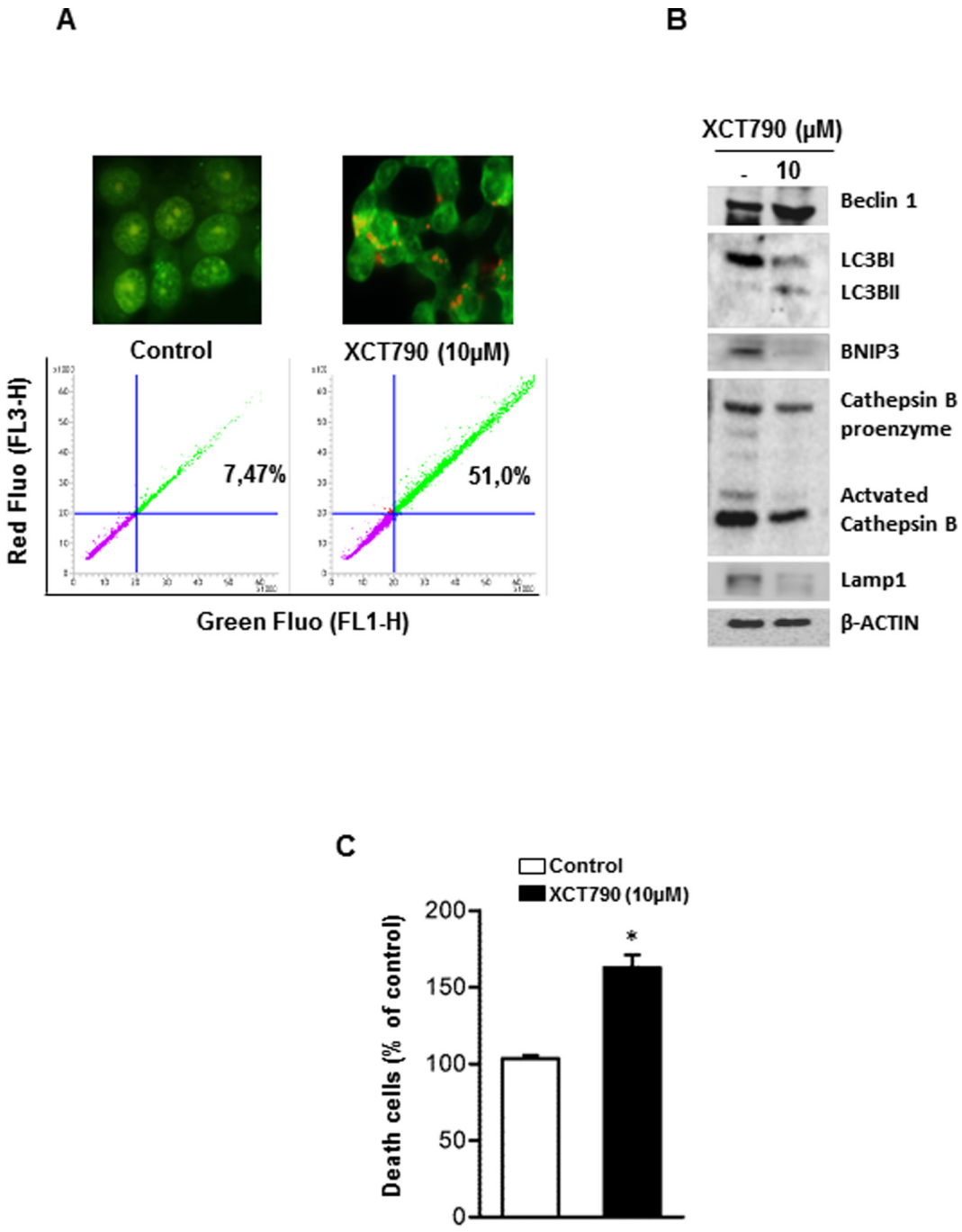

Figure 6: XCT790 induces necrosis in H295R cells. A. H295R cells were left untreated (control) or treated with XCT790 10 µM. After 48 h, cells were incubated with (1 µg/mL) acridine orange (AO) solution for 30 min at 37°C. Absorption of AO was determined by FACS analysis (lower panel). In the same experimental conditions, treated or untreated H295R cells were stained with acridine orange, mounted and immediately analyzed by fluorescent microscope (upper panel). **B.** Total protein extracts from H295R cells left untreated (–) or treated in 2.5% DCC-FBS medium with XCT790, as indicated, for 48 h were analyzed by Western blot experiments using antibodies against Beclin 1, LC3B, BNIP3, Cathepsin B, Lamp1. β-Actin was used as loading control. Blots are representative of three independent experiments with similar results. **C.** Cell death by necrosis was assessed by Trypan blue-exclusion assay in H295R cells untreated (–) or treated with XCT790. The mean ± SD of three replicates are shown. Cell death was expressed as a percentage of control, (*$p < 0.001$).

in breast [32] and ovarian cancer [14, 33] and that breast cancer cells exhibiting high ERRα activity are more sensitive to growth inhibition by an ERRα inverse agonist such as XCT790 [34]. Consistent with this findings and with very recent data reporting high ERRα expression in adrenal tumors compared to benign and normal adrenal gland [22], here we report that ERRα is expressed in H295R cells, the most valid cell model to study ACC

biology. Moreover, our data show that pharmacological down-regulation of ERRα expression impaired H295R cell proliferation *in vitro* in a dose-dependent fashion. Most importantly, the same inhibitory effect was obtained also in *in vivo* experiments using H295R cells as xenograft model. At the molecular level, the growth inhibition is associated with a G0/G1 cell cycle arrest and by the decreased levels of G1-phase markers such as Cyclin D1 and pRb while CDKs protein levels were unaffected. Noteworthy, cell cycle arrest was not followed by any apoptotic event since we were unable to detect any morphological data (data not shown) or biochemical events such caspase activation and PARP-1 cleavage.

Accumulating data provide evidence that a caspase-independent form of programed cell death such as autophagy can be at play under certain conditions [35]. Therefore we investigated whether the inhibitory effects induced by XCT790 treatment could be linked to autophagy. Our results indicated that XCT790 caused a significant increase in autophagic vesicles. Concomitantly, we observed a drastic reduction in the expression of PGC1-α protein, which plays a key role in mitochondrial biogenesis, and of mitochondrial carrier CIC. The reduction of mitochondrial mass, also confirmed by the reduction of TOM20 protein expression, is followed by a considerable and significant decrease in the ATP concentration. Despite the presence of some autophagic markers such as the up-regulation of Beclin 1 and the cleaved form of LC3 protein, the formation of autophagolysosomes seems to be incomplete as evidenced by the reduction in LAMP1 protein, known to play an important role during the final steps of autophagy process [36]. A possible explanation could be a considerable reduction in the availability of intracellular ATP, required to drive forward the active cell death mechanism including autophagy. On the other hand, we cannot exclude that the observed initial steps of autophagy are a defense cell response to keep cells alive during energy failure to counteract the reduced expression and activity of the master bioenergetic executor ERRα. Moreover, the bioenergetics crisis following treatment with ERRα inverse agonist might be responsible for the loss of plasma membrane integrity, a key signature for a necrotic cell death, allowing the significant increase in the number of Trypan blue stained cells.

However, our most significant finding is that in ACC cells ERRα depletion after XCT790 treatment clearly caused a reduction of mitochondrial function and mass leading to the activation of a number of cellular mechanisms that result in tumor cell death.

It's now well known that mitochondria with its direct involvement in bioenergetics, biosynthesis and cell signaling are mandatory for tumorigenesis. Thus, it's not surprising that many studies have begun to demonstrate that mitochondrial metabolism and signaling is potentially a successful avenue for cancer therapy. Moreover, ACC is (in most cases) characterized by steroids producing/secreting cancer cells highly dependent on functioning mitochondria to ensure steroidogenic processes. For these reasons, strategies using mitochondrial metabolism and signaling as targets should be particularly effective for ACC treatment. Moreover, our current data obtained performing *in vivo* experiments by using H295R cells as xenograft model and according to previous *in vivo* studies performed in breast [37] and leukemia [38] tumor cells also suggest that chemical depletion of ERRα may be specific for high energy demanding cells such as tumor cells without exerting any toxic effect on other tissues.

In conclusion, our study supports the hypothesis that ERRα represents a valid innovative/alternative target for the treatment of adrenocortical cancer.

MATERIALS AND METHODS

Cell culture

H295R adrenocortical cancer cells were obtained from Dr. Antonio Stigliano (University of Rome, Italy) and cultured in DMEM/F12 supplemented with 1% ITS Liquid Media Supplement, 10% fetal bovine serum (FBS), 1% glutamine, 2% penicillin/streptomycin (complete medium) MCF7 breast cancer cells were maintained in monolayer cultures DMEM/F12. supplemented with 10% FBS, 1% glutamine, 2% penicillin/streptomycin. Both cell lines were cultured at 37°C in 5% CO_2 in a humidified atmosphere. All media and supplements were from Sigma-Aldrich, Milano, Italy.

Western blot analysis

Whole cell lysate were prepared in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS and a mixture of protease inhibitors) or in ice-cold lysis buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X-100, 60 mM octylglucoside). Samples were analyzed by 11% SDS-PAGE and blotted onto a nitrocellulose membrane. Blots were incubated overnight at 4°C with anti-ERRα polyclonal antibody, anti-cyclin D1, anti-cyclin E, anti-cdk2, anti-cdk4, anti-p-Rb, anti-PARP, anti-cathepsin B, anti-LAMP1, anti-Tom20 (all from Santa Cruz Biotechnology), anti-Beclin 1 (Novus Biological), anti-LC3B antibody, anti-BNIP3 antibody, Mitoprofile Total OXPHOS Human WB Antibody Cocktail (Abcam) and then incubated with appropriate horseradish peroxidase conjugated secondary antibodies for 1 h at room temperature. The immunoreactive products were detected by the ECL Western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). GAPDH antibody (Santa Cruz Biotechnology) or anti-β-Actin antibody (Sigma-Aldrich) were used as internal control.

Cell viability assay

H295R cells were seeded in 12-well plates at a density of 1×10^5 cells per well and cultured in complete medium overnight. Before treatment culture medium was switched into in DMEM F-12 supplemented with 2.5% charcoal stripped (CS) FBS and cells were untreated or treated with different concentration of XCT790 (Tocris Bioscience, Bristol, UK) for the indicated time. DMSO (Sigma-Aldrich) was used as vehicle control. Cell viability was measured using MTT assay (Sigma-Aldrich). Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Experiments were repeated three times.

Trypan blue assay

Trypan blue stain was prepared freshly as a 0.4% solution in 0.9% sodium chloride before each experiment. After trypsinization, 20 µl cell suspension was added to 20 µl of Trypan blue solution and mixed thoroughly. Triplicate wells of dye positive cells from untreated or XCT790 treated were counted using a hemocytometer and the experiment was repeated three times.

Xenograft model

Athymic Nude- Foxn1^{nu} mouse 4–6 weeks old) from Charles River Laboratories [Calco (LC), Italy] were maintained in groups of five or less and quarantined for one week. Mice were kept on a 12 h light/dark cycle with ad libitum access to food and water.

6×10^6 H295R cells suspended in 100 µl of sterile PBS (*Dulbecco's* Phosphate Buffered Saline) and mixed with 100 µl of matrigel, were injected subcutaneously into the intrascapular region of each animal. When tumor size reached a volume of about 200 mm^3 mice were randomly divided in 2 groups. Animals were injected every other day with vehicle (soy oil) or XCT790 (2,5 mg/Kg) over a 21 day period. Tumors were measured with a caliper every two days, volumes were calculated using the formula $V = a\ b^2/2$ (*V:volume; a* is the length of the long axis, and *b* is the length of the short axis. At the end of the treatment period tumors were harvested and tumor weight and volumes were evaluated. All animal procedures were approved by the local Ethics Committee for Animal Research.

Immunohistochemical analysis

5 µm thick paraffin-embedded sections were mounted on slides precoated with poly-lysine, and then they were deparaffinized and dehydrated (seven to eight serial sections). Immuno-histochemical experiments were performed using rabbit polyclonal Ki67 primary antibody (Dako, Denmark) at 4°C over-night. Then, a biotinylated goat-anti-rabbit IgG was applied for 1 h at room temperature, followed by avidin biotin-horseradish peroxidase reaction (Vector Laboratories, CA). Immunoreactivity was visualized by using the diaminobenzidine chromogen (Sigma-Aldrich). Counterstaining was carried out with methylene-blue (Sigma-Aldrich). Hematoxylin and eosin Y staining was performed as suggested by the manufacturer (Bio-Optica, Milan, Italy).

Scoring system

The immunostained slides of tumor samples were evaluated by light microscopy using the Allred Score [39] which combines a proportion score and an intensity score. A proportion score was assigned representing the estimated proportion of positively stained tumor cells (0 = none; 1 = 1/100; 2 = 1/100 to <1/10; 3 = 1/10 to <1/3; 4 = 1/3 to 2/3; 5 = >2/3). An intensity score was assigned by the average estimated intensity of staining in positive cells (0 = none; 1 = weak; 2 = moderate; 3 = strong). Proportion score and intensity score were added to obtain a total score that ranged from 0 to 8. A minimum of 100 cells were evaluated in each slide. Six to seven serial sections were scored in a blinded manner for each sample.

Cell cycle analysis

H295R cells treated with different doses of XCT790 were fixed, treated with RNase A (20 µg/ml), stained with Propidium iodide (100 µg/ml) (Sigma-Aldrich) and analyzed by Flow Cytometry using BD FACSJazz™ Cell Sorter (Becton, Dickinson and Co) for DNA content and cell cycle status.

Caspases 3/7 activity assay

Caspases activity was measured with Caspase-Glo Assay Kit (Promega Italia SRL, Milano, Italy) following the manufacturer instruction. The luminescence of each sample was measured in a plate-reading luminometer (Gen5 2.01) with Synergy H1 Hybrid Reader. Each experiment was performed on triplicate wells per condition.

Mitochondrial mass determination

XCT790 treated or untreated H295R cells were incubated in serum free medium with 200 nM Mitotracker deep red (Invitrogen, USA) for 30 min at 37°C in the dark. After staining, cells were washed twice with cold PBS, trypsinized, centrifuged at 1200 rpm for 5 min and then resuspended in PBS. Absorption of MitoTracker deep red FM was determined by FACS analysis and by fluorescence microscopy. In the same experimental conditions, fluorescent signal intensity was also assessed using a fluorescent plate reader (ex. 644 nM; em. 665 nM).

Detection of acidic vesicular organelles (AVOs) with acridine orange

H295R cells were cultured on 6 well plates and treated in 2.5% CS-FBS with or without 10 μM XCT790. After 48 h, cells were washed with PBS and stained for 30 min at 37°C with 1 μg/mL acridine orange solution (Sigma-Aldrich). Cells were then washed three times with cold PBS and one drop of mounting solution was added. Cell were observed and imaged by an inverted fluorescence microscope (100X magnification). Accumulation of the acidic vacuoles was also determined by FACS analysis.

ATP Determination

1×10^5 cells were seeded in 96 white clear bottom multi-well plates in complete medium. Two days later, cells were treated in DMEM F-12 supplemented with 2.5% CS FBS containing 10 μM XCT790. After 48 h, ATP concentrations were determined using the CellTiter-Glo luminescent cell viability assay (Promega) following the manufacturer instruction. Results were normalized to the cell number evaluated by HOECHST staining (Sigma-Aldrich) and expressed as nMol/number of cells.

Mitochondria reconstitution and transport measurements

The transport activity was carried out as described previously [40]. Briefly, isolated mitochondria from untreated (control) or XCT790 treated H295R cells were solubilized in a buffer containing 3% Triton X, 114, 4 mg/ml cardiolipin, 10 mM Na2SO4, 0.5 mM EDTA, 5 mM PIPES pH 7. The mixture was incubated for 20 min and centrifuged at 138,000 × g for 10 min. The supernatant was incorporated into phospholipid vesicles by cyclic removal of the detergent [41]. The reconstitution mixture consisted of 0.04 mg protein solution, 10% Triton X-114, 10% phospholipids (egg lecithin from Fluka, Milan, Italy) as sonicated liposomes, 10 mM citrate, 0.85 mg/ml cardiolipin (Sigma) and 20 mM PIPES, pH 7.0. The citrate transport was measured after external substrate removal from proteoliposomes on Sephadex G-75 columns, pre-equilibrated with buffer A (50 mM NaCl and 10 mM PIPES, pH 7.0). Transport at 25°C was started by the addition of 0.5 mM [^{14}C] citrate (Amersham) to the eluted proteoliposomes and terminated by the addition of 20 mM 1,2,3-benzene-tricarboxylate. Finally, the external radioactivity was removed from the Sephadex G-75 columns, liposomes radioactivity was measured and transport activity was calculated [41].

Statistics

All experiments were performed at least three times. Data were expressed as mean values ± standard deviation (SD), statistical significance between control and treated samples was analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Control and treated groups were compared using the analysis of variance (ANOVA). A comparison of individual treatments was also performed, using Student's t test. Significance was defined as $p < 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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RESEARCH ARTICLE

Oleuropein and hydroxytyrosol activate GPER/GPR30-dependent pathways leading to apoptosis of ER-negative SKBR3 breast cancer cells

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Scope: We have previously demonstrated that oleuropein (OL) and hydroxytyrosol (HT) reduce 17β-estradiol-mediated proliferation in MCF-7 breast cancer (BC) cells without affecting the classical genomic action of estrogen receptor (ER), but activating instead the ERK1/2 pathway. Here, we hypothesized that this inhibition could be mediated by a G-protein-coupled receptor named GPER/GPR30. Using the ER-negative and GPER-positive SKBR3 BC cells as experimental model, we investigated the effects of OL and HT on GPER-mediated activation of downstream pathways.

Methods and results: Docking simulations and ligand-binding studies evidenced that OL and HT are able to bind GPER. MTT cell proliferation assays revealed that both phenols reduced SKBR3 cell growth; this effect was abolished silencing GPER. Focusing on OL and HT GPER-mediated pathways, using Western blot analysis we showed a sustained ERK1/2 activation triggering an intrinsic apoptotic pathway.

Conclusion: Showing that OL and HT work as GPER inverse agonists in ER-negative and GPER-positive SKBR3 BC cells, we provide novel insights into the potential of these two molecules as tools in the therapy of this subtype of BC.

Keywords:
Apoptosis / Breast cancer / GPER / Hydroxytyrosol / Oleuropein / SKBR3

1 Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the second leading cause of cancer death among women, accounting for 23% of the total cancer cases and 14% of the cancer deaths [1]. Moreover, epidemiological studies have shown that BC incidence is markedly higher in developed rather than in developing countries [2]. This environmental variation may account, at least in part, for differences in lifestyle habits, among which diet seems to play a crucial role [3]. BC is a heterogeneous disease with distinct clinical behavior and molecular properties. One of the important classifications of BC is based on the presence or absence of the estrogen receptor alpha (ERα). While the majority of BCs are ERα-positive, approximately 25–30% are ERα-negative [4].

Proliferation of ERα-positive BCs is driven upon binding of the receptor to 17β-estradiol (E2). The activated receptor interacts with specific sites on the chromatin, regulating expression of genes involved in cell cycle regulation [5]. Tamoxifen (Tam), a synthetic drug with chemical similarity to estrogens but antagonistic effects on the proliferation of ERα-dependent BCs is used for the therapy of ERα-positive BC patients. However, it has been demonstrated that proliferation of ERα-negative BC cells can be activated by estrogens

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Abbreviations: BC, breast cancer; **Cyt c**, Cytochrome c; **DCC-FBS**, dextran-coated charcoal-treated fetal bovine serum; **E2**, 17β-estradiol; **ER**, estrogen receptor; **ERK1/2**, extracellular regulated kinase 1/2; **GPER**, G protein-coupled estrogen receptor 1; **HT**, hydroxytyrosol; **MTT**, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; **OL**, Oleuropein; **PARP-1**, poly (ADP-ribose) polymerase-1; **shRNA**, short hairpin RNA; **Tam**, Tamoxifen; **TM**, transmembrane

upon binding to a G-protein-coupled receptor named GPR30 (referred as GPER throughout the text) [6–8]. Once stimulated, GPER triggers rapid but transient activation of extracellular regulated kinase 1/2 (ERK1/2) signaling leading to an increased cell proliferation [9–12]. Importantly, tamoxifen acts as agonist of GPER, stimulating growth of ERα-negative breast as well as endometrial cancer cell lines [7, 10].

Several epidemiological studies pointed out that food rich in bioactive components, such as vitamins, flavonoids, and polyphenols, could contribute to a low incidence of tumors. This is due to the ability of these components to show protective actions or reverse premalignant lesions [13]. In particular, phenols of natural origin such as oleuropein (OL) and hydroxytyrosol (HT) have been shown to interfere with tumor cell proliferation [14]. OL is a potent scavenger of free radicals [15] and plays an important role in the prevention of DNA damage impairing mutagenesis and carcinogenesis [16]. HT, a metabolite of OL, possesses powerful antioxidant properties [17] as well as anti-inflammatory, antiplatelet aggregation, antiatherogenic and cardioprotective, antimicrobial, antiviral, and anticancer activities [18]. It has been reported that both OL and HT are able to exert apoptotic effects in human MCF-7 BC cell line [19]. In addition, we have recently demonstrated the ability of HT and OL to inhibit MCF-7 BC cells proliferation through a mechanism that does not involve a classic ERα-mediated gene regulation but instead ERK1/2 activation [14]. The latter observation led us to hypothesize that the inhibitory effect exerted by the two phenols could be attributable to their ability to interfere with a nongenomic action of estrogen. For this reason, we decided to explore the anti-BC properties of HT and OL and the underlying molecular mechanisms, using a known ER-negative but GPER-positive BC cell line, SKBR3 cells.

In this study, we first demonstrated the ability of OL and HT to bind and activate GPER leading to a sustained activation of ERK1/2 signaling. Next, we dissected the molecular mechanism responsible for the antiproliferative effects revealing the mitochondrial apoptotic signaling as the main cell death pathway involved.

2 Materials and methods

2.1 Cell cultures and treatments

SKBR3 BC cells (an ER-negative BC cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in RPMI 1640 without phenol red supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% penicillin/streptomycin (Sigma-Aldrich, Milano, Italy) (complete medium). For experiments, cells were plated in complete medium, 24 h later treated in RPMI1640 medium with 1% DCC-FBS (dextran-coated charcoal-treated FBS) or in serum-free medium for the indicated times, with OL and HT (Sigma-Aldrich) at the indicated concentrations. Cells were treated with vehicle (DMSO) at 0.001%. Compounds were solubilized in DMSO to obtain a 1000X stock that was diluted 1:1000 in media at the time of treatment.

2.2 Molecular modeling and docking simulations

To perform the docking simulations, we used the program GOLD v.5.0.1 (the Cambridge Crystallographic Data Center, UK), a program using a genetic algorithm useful to investigate the full range of ligand conformational flexibility and a partial protein side chain flexibility. In all the simulations we used, as the protein target, the 3D atomic coordinates of the GPER molecular model built by homology as described elsewhere [20]. We identified the O atom of Phe 208 as the protein active site center on the basis of our previous docking simulations [20]. In this case, the active site atoms were considered those located within 20 Å from the centre. For each structure, 10 docking solutions were generated allowing an early termination of the process if the respective RMSDs of the three highest ranked docking solutions were within 1.5 Å of each other. The default GOLD settings were used running the simulations. In the GPER model the residues Tyr123, Gln138, Phe206, Phe208, Glu275, Phe278, and His282 were defined flexible side chains allowing their free rotation. The molecular structures of the ligands screened "in silico" were built and energy minimized with the programs InsightII and Discover3 (Biosym/MSI, San Diego, CA, USA). All the figures were drawn with the program Chimera [21].

2.3 Ligand-binding assays

In ligand-binding assay for GPER, SKBR3 cells were grown in 60 mm dishes (2×10^6 cells) for 24 h, washed twice with 1× PBS and incubated for 2 h at 4°C with 4 nM [2,4,6,7–3H]E2 (89 Ci/ mmol) (Amersham Bioscience, Glattbrugg, Switzerland) in the presence or absence of increasing concentration of nonlabeled competitors (E2, G-1, OL, and HT). Cells were then washed once with 10% trichloroacetic acid, twice with 5% trichloroacetic acid, and lysed in 1 mL of 0.1 N NaOH at 37°C for 30 min. The total suspension was added to 10 mL of Optifluor fluid and counted in a scintillation counter. Competitor binding was expressed as a percentage of maximal specific binding.

2.4 Western blot analysis

Cells were cultured in complete medium for 24 h in 60 mm dishes (1×10^6 cells) before being treated for the indicated times in 1% DCC-FBS. Methods for protein extraction and blots preparation have been previously published [22]. Blots were incubated overnight at 4°C with (a) anti-GPER polyclonal antibody (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), (b) anti-pERK antibody (1:1000) (Cell Signaling Technology, Beverly, MA, USA), (c) anti-ERK antibody

(1:1000) (Cell Signaling Technology), (d) anti-Cytochrome c (Cyt c) antibody (1:1000) (Santa Cruz Biotechnology), (e) anti-Bax antibody (1:1000) (Santa Cruz Biotechnology), (f) anti-Bcl-2 antibody (1:1000) (Santa Cruz Biotechnology), (g) anti-caspase-3 antibody (1:1000) (Santa Cruz Biotechnology), (h) anti-caspase-9 antibody (1:500) (Cell Signaling Technology), (i) anti-(PARP-1) poly (ADP-ribose) polymerase-1 antibody (1:3000) (Santa Cruz Biotechnology), (j) anti-cyclin D1 (1:1000) (Santa Cruz Biotechnology), (k) anti-p21 (1:250) (Santa Cruz Biotechnology), (l) anti-p53 (1:1000) (Santa Cruz Biotechnology). Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and immunoreactive bands were visualized with the ECL Western blotting detection system (Amersham Bioscience). To assure equal loading of proteins, membranes were stripped and incubated overnight with an anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1:3000) (Santa Cruz Biotechnology).

2.5 Assessment of cell proliferation

The effect of G-1, OL, and HT on cell viability was measured using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [23]. Briefly, cells were cultured in complete medium in 12 well plates (1×10^5 cells/well) for 24 h, then treated in serum-free medium for 72 h. Fresh MTT (Sigma), resuspended in PBS, was then added to each well (final concentration (0.33 mg/mL). After 2 h incubation, cells were lysed with 1 mL of DMSO and optical density was measured at 570 nm in a spectrophotometer.

2.6 Determination of nuclear morphological changes

Cells were cultured in complete medium for 24 h on microscope slides (1×10^5 cells), then treated in 1% DCC-FBS medium for 48 h. After treatment cells were washed with PBS and fixed in 4% formaldehyde (Sigma) for 10 min at room temperature. Fixed cells were washed with PBS and incubated with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (0.2 μg/mL) (Sigma) for 10 min in a humidified chamber, protected from light, at 37°C. Slides were washed three times with cold PBS before mounting. Cell nuclei were observed and imaged by an inverted fluorescence microscope (400× magnification) with excitation at 350 nm and emission at 460 nm. The number of apoptotic nuclei was determined in at least six randomly selected areas from three cover slips of each experimental group.

2.7 Determination of DNA fragmentation

Cells were cultured in complete medium in 100 mm dishes (1×10^6 cells) for 24 h, then treated in 1% DCC-FBS medium for 72 h. To determine the occurrence of DNA fragmentation, total DNA was extracted from control and OL (100 μM) and HT (50 μM) treated cells as previously described [24]. Equal amounts of DNA were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich).

2.8 Cyt c detection

Cells were cultured in complete medium for 24 h in 12 well plates (2×10^5 cells/well), then treated in 1% DCC-FBS medium for 24 h. Cyt c was detected by Western blot analysis in mitochondrial and cytoplasmic fractions. Cells were harvested by centrifugation at 2500 rpm for 10 min at 4°C. Pellets were resuspended in 50 μL of sucrose buffer (250 mM sucrose; 10 mM Hepes; 10 mM KCl; 1.5 mM MgCl2; 1 mM EDTA; 1 mM EGTA) (Sigma) containing 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mM PMSF, and 0.05% digitonine (Sigma). Cells were incubated for 20 min at 4°C and then centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant containing cytosolic protein fraction was transferred to new tubes and the resulting mitochondrial pellet was resuspended in 50 μL of lysis buffer (1% Triton X-100; 1 mM EDTA; 1 mM EGTA; 10 mM Tris-HCl, pH 7.4) (Sigma) containing 20 μg/mL aprotinin, 20 μg/mL leupeptin, 1 mM PMSF, and then centrifuged at 13 000 rpm for 10 min at 4°C. Equal amounts of proteins (10 μg) were resolved by 11% SDS/PAGE as indicated in the Western blot analysis paragraph.

2.9 Gene-silencing experiments

For the gene-silencing experiments, cells were plated in 12 well plates (1×10^5 cells/well) for proliferation experiments or in 6 well plates (2×10^5 cells/well) for Western blot analysis; cells were transfected with control vector (shRNA) or shGPER in 1% DCC-FBS medium using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's recommendations for a total of 72 h. For proliferation experiments cells were transfected for 24 h and then treated for 48 h before performing MTT assay.

2.10 Statistical analysis

All experiments were conducted at least three times, results were from representative experiments. Data were expressed as mean values + SE. Statistical significance between control (basal) and treated samples was analyzed with SPSS 10.0 statistical software. The unpaired Student's *t*-test was used to compare two groups. $p < 0.05$ was considered statistically significant.

3 Results

3.1 OL and HT are GPER ligands

We first evaluated the affinity of OL and HT for the ligand-binding pocket of GPER in comparison with its selective synthetic agonist G-1, using docking simulations, as described in Section 2 (Fig. 1A). GPER-binding pocket is located within a deep cleft in the protein core, surrounded by both hydrophobics and polar residues belonging to transmembrane (TM) helices III, V, VI, and VII. Using a previously tested GPER molecular model as target [20], docking simulations confirmed a good affinity of the protein for G-1 (Fig. 1A) as previously demonstrated both in silico and in vitro [25]. Subsequently, we docked OL and HT to GPER using the same settings and parameters as for G-1. Both phenols, were positioned within the GPER-binding site (Fig. 1B and C), displaying a good affinity for GPER. Particularly, OL was positioned along the protein slit, forming hydrogen bonds with residues Tyr142 (belonging to helix TM III), Gln216 (TM V), Glu275 and Asn276 (TM VI), and His307 (TM VII). A series of hydrophobic contacts with residues Val116, Leu137, Met141, Phe206, Phe208, Ile279, contribute to stabilize the complex. HT has a much smaller moiety and thus occupies a small fraction of the binding site cavity. HT is hydrogen bound to Ser134 (TM III) and Glu275 (TM VI). The aromatic

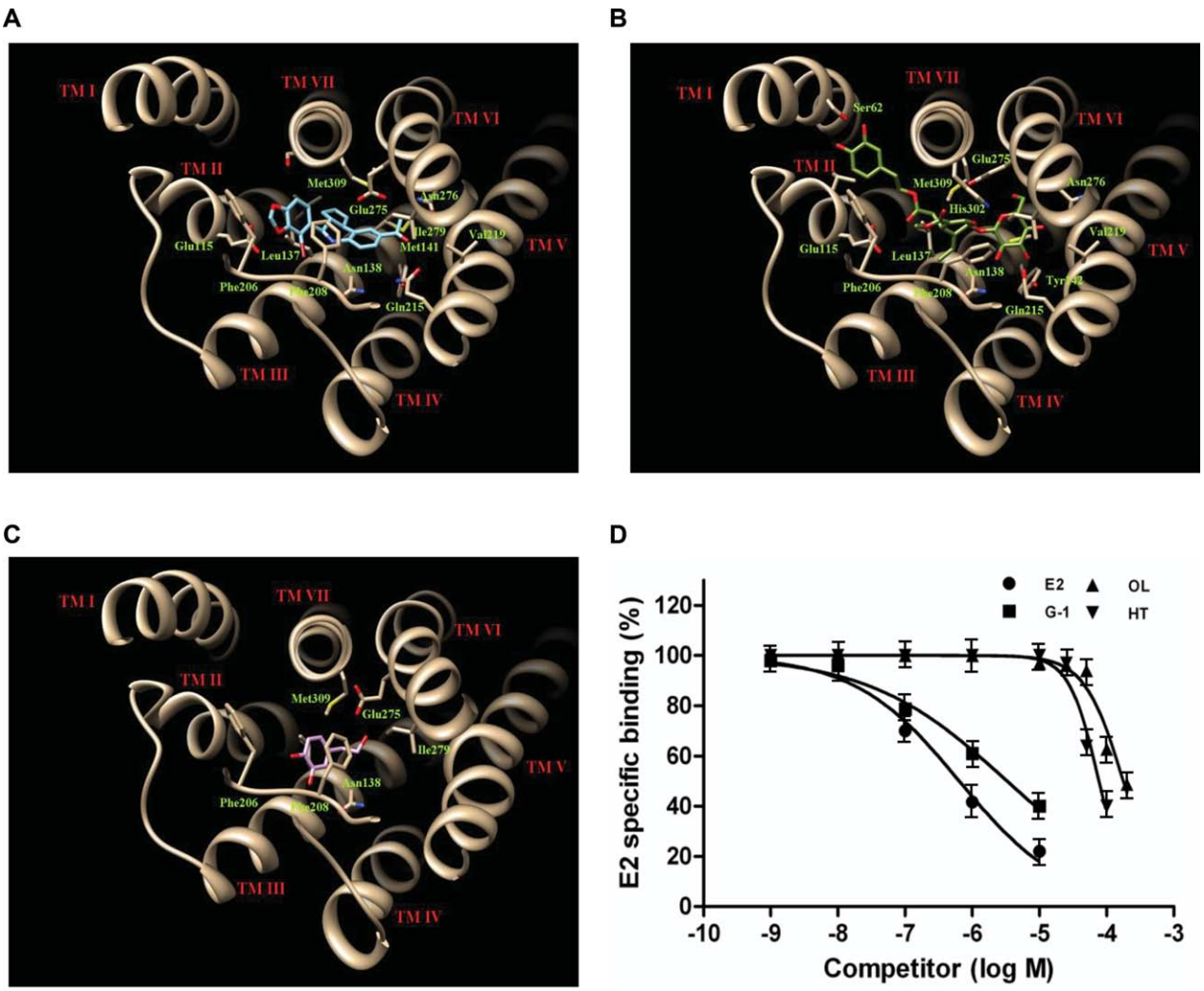
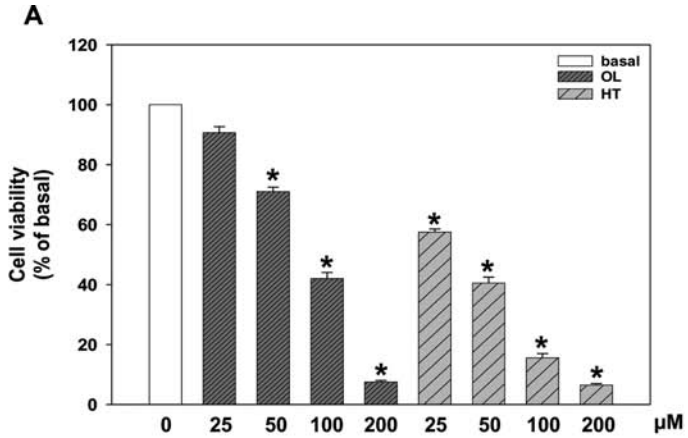
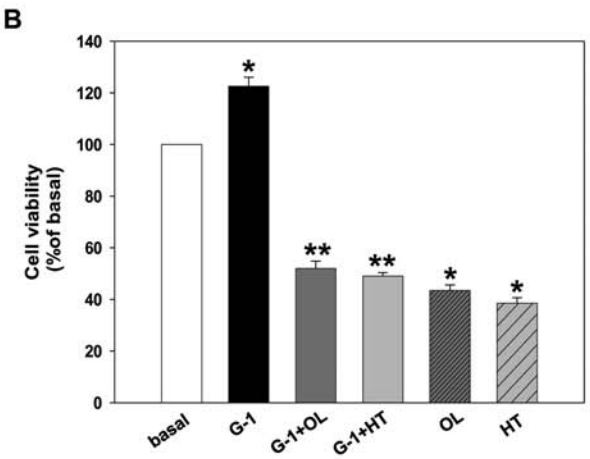
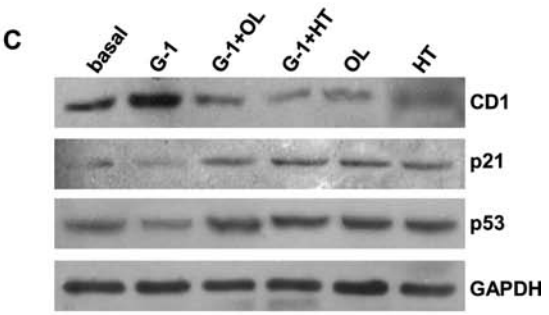
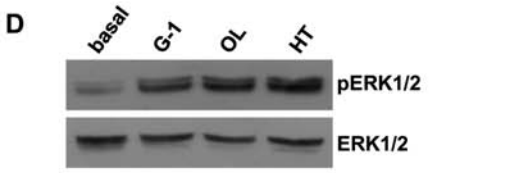


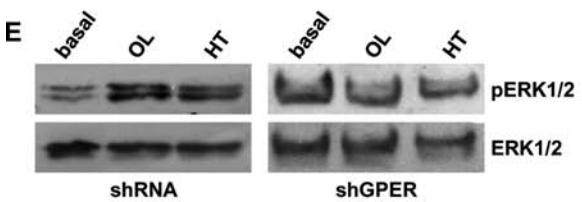
Figure 1. Evaluation of OL and HT as potential GPER ligand. (A–C). GPER docking simulations. The 3D model of GPER is schematically reported as a tan ribbon and residues involved in ligand binding are drawn as sticks. G-1 is drawn in cyan, OL and HT moieties are drawn respectively as green (B) or purple (C) sticks. (D) Ligand-binding assay in SkBr3 cells. Competition curves of increasing concentration of unlabelled E2, G-1, OL, and HT expressed as a percentage of maximum specific [^{3}H]E2 binding. Each data point represents the mean + SE of three separate experiments performed in triplicate.

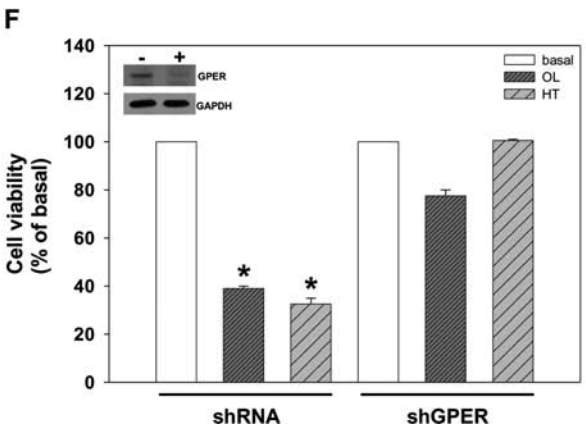












ring of HT is in a π–π stacking with Phe208 and it forms hydrophobic contacts with Leu137.

Based on the indications obtained from docking simulation we next designed experimental assays to fully evaluate the ligand-binding properties of OL and HT to GPER. To this aim, we performed ligand-binding studies using radiolabeled E2 as tracer in ER-negative but GPER-positive SKBR3 BC cells. In the same set of experiments we also included cold E2 and the selective GPER ligand G-1 as positive controls. Results illustrated in Fig. 1D showed the ability of OL and HT to displace [^{3}H]E2 demonstrating their capability to bind to GPER although with a lower binding affinity in respect to cold E2 and G-1. Taken together, results from docking simulation and ligand-binding assay suggest that OL and HT are able to bind GPER.

3.2 OL and HT cause cell growth inhibition through a GPER-mediated mechanism

In order to examine the effects of OL and HT on SKBR3 cells proliferation, cells were left untreated or treated for 72 h with different doses (0–200 μM) of OL or HT. As shown in Fig. 2A, increasing doses of both OL and HT suppressed BC cells proliferation. We thus evaluated if both OL and HT were able to influence G-1-induced BC cell proliferation. As expected, G-1 100 nM induced a significant growth stimulatory effect in SKBR3 cells that was completely inhibited by a combined treatment of G-1 with OL or HT (Fig. 2B).

Next, since the single treatment with OL or HT produced a significant cell growth reduction, we investigated if they were both able to affect the expression of proteins involved in the cell cycle regulation. As shown in Fig. 2C, SKBR3 cells treated with OL (100 μM) or HT (50 μM) showed a reduced cyclin D1 expression concomitantly with an increased p21 protein content. Furthermore, OL and HT induced an enhanced expression of the tumor suppressor p53 (Fig. 2C), suggesting a p53-dependent antiproliferative effect following treatment with either phenols.

It has been demonstrated that GPER-mediated SKBR3 cell proliferation involves rapid as well transient activation of MAPK-signaling kinases [10]. For this reason we decided to investigate the effect of OL and HT on GPER-mediated rapidly activated pathways. As expected, treatment with G-1 produced an increase in ERK1/2 phosphorylation that was prevented by the concomitant addition of OL or HT (Fig. 2D). Surprisingly, treatment with OL or HT alone showed a marked increase of ERK1/2 phosphorylation with a magnitude quite similar to G-1. To demonstrate the direct and specific GPER involvement in OL or HT-induced ERK1/2 phosphorylation, SKBR3 cells were treated with either phenol in the presence of an shRNA nonspecific (shRNA) or specific for GPER (shGPER). As illustrated in Fig. 2E, OL and HT ability to induce ERK1/2 phosphorylation was completely abrogated after knocking down GPER gene expression.

To further demonstrate the requirement of a functional GPER in OL and HT-dependent inhibitory effect on cell proliferation, we knocked down GPER expression by shRNA (insert Fig. 2F) and performed cell viability experiments. The reduction in GPER expression was able to impair completely the growth inhibitory effects exerted by HT and partially that one induced by OL (Fig. 2F).

3.3 OL and HT cause a GPER-mediated sustained ERK1/2 activation

We then investigated the time course activation of ERK1/2 in SKBR3 cells in response to OL (100 μM) or HT (50 μM). Interestingly, as showed in Fig. 3A both olive oil phenols evidenced a significant capability to stimulate ERK1/2 phosphorylation starting from 5 min and persisting until 24 h without affecting

◀ **Figure 2.** OL and HT effects on GPER-mediated cell growth inhibition. (A) SKBR3 cells were maintained in serum-free medium for 24 h and then untreated (basal) or treated in 1% DCC-FBS medium for 72 h with increasing doses (0–200 μM) of OL and HT. Cell proliferation was evaluated by MTT assay. Results were expressed as mean + SE of three independent experiments each performed in triplicate. (*$p <$ 0.001 compared with basal). (B) SKBR3 cells were maintained in serum-free medium for 24 h and then untreated (basal) or treated in 1% DCC-FBS medium with G-1 (100 nM) or OL (100 μM) and HT (50 μM) alone or in combination with G-1 for 72 h. Cell proliferation was evaluated by MTT assay. Results were expressed as mean + SE of three independent experiments each performed in triplicate. (*$p <$ 0.001 compared with basal; **$p < 0.001$ compared with G-1). (C) SKBR3 cells were maintained in serum-free medium for 24 h and then untreated (basal) or treated in 1% DCC-FBS medium with G-1 (100 nM) or OL (100 μM) and HT (50 μM) alone or in combination with G-1 for 72 h. Western blot analysis of Cyclin D1, p21, and p53 were performed on 50 μg of total proteins. Blots are representative of three independent experiments with similar results. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. (D) SKBR3 cells were maintained in serum-free medium for 72 h and then untreated (basal) or treated for 10 min with G-1 (1 μM) or OL (100 μM) and HT (50 μM). Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. (E) SKBR3 cells were transfected in serum-free medium with shRNA or shGPER for 72 h and then untreated (basal) or treated with OL (100 μM) and HT (50 μM) for 10 min. Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. (F) MTT assay was performed on SKBR3 cells, which were previously transfected for 72 h in the presence of shRNA or shGPER. Twenty-four hours after transfection cells were treated in 1% DCC-FBS medium for 48 h with OL (100 μM) and HT (50 μM). Results were expressed as mean + SE of three independent experiments each performed in triplicate. (*$p < 0.001$ compared with basal). The insert shows a Western blotting assay on SKBR3 protein extracts evaluating the expression of GPER receptor in the presence of shRNA (−) or of shGPER (+). Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control.

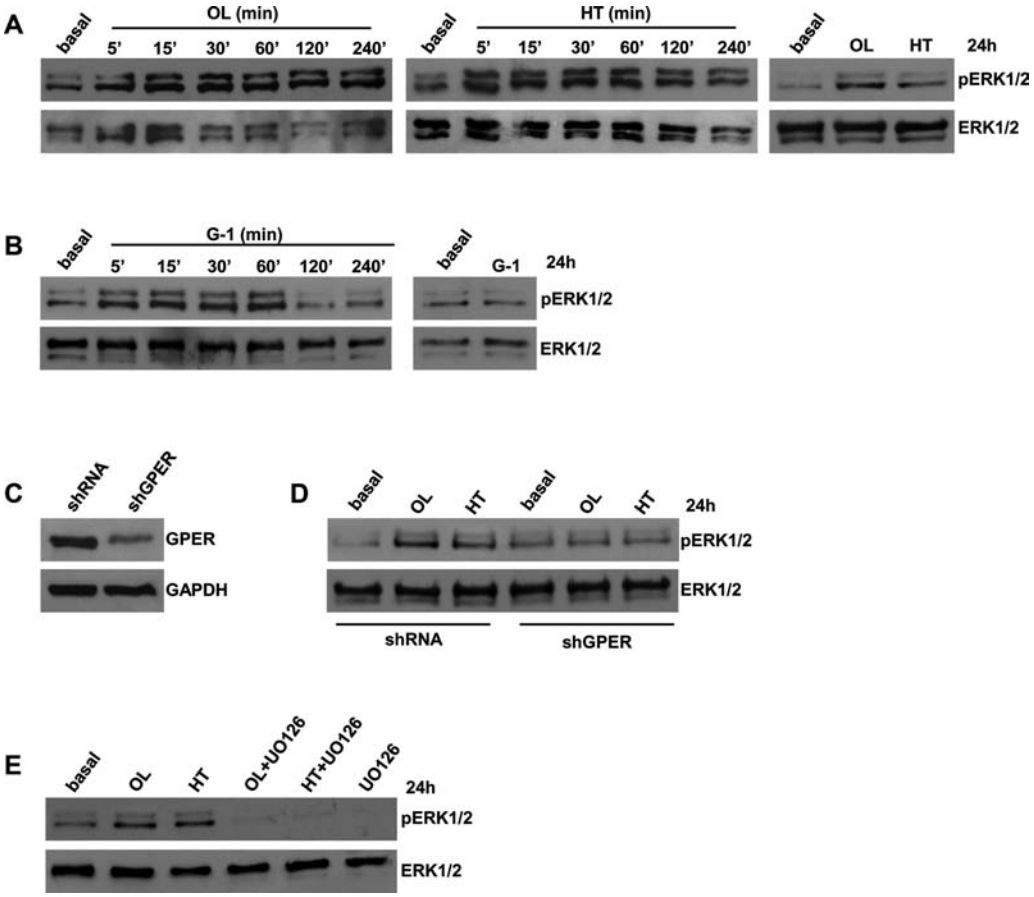


Figure 3. OL and HT effects on GPER-mediated sustained ERK1/2 activation. (A) SKBR3 cells were maintained in serum-free medium for 72 h and then untreated (basal) or treated with OL (100 μM) and HT (50 μM) for various time intervals as indicated. Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. (B) SKBR3 cells were maintained in serum-free medium for 72 h and then untreated (basal) or treated with G-1 (1 μM) for various time intervals as indicated. Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. (C) SKBR3 cells were transfected in serum-free medium with shRNA or shGPER for 72 h. Western blot analyses of GPER were performed on 30 μg of total proteins. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Blots are representative of three independent experiments with similar results. (D) SKBR3 cells were transfected in serum-free medium with shRNA or shGPER for 72 h. Forty-eight hours after transfection cells were untreated (basal) or treated for 24 h with OL (100 μM) and HT (50 μM). Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results. (E) SKBR3 cells were maintained in serum-free medium for 72 h. In the last 24 h, cells were untreated (basal) or treated for 24 h with OL (100 μM) and HT (50 μM) alone or in combination with U0126 (10 μM). Western blot analyses of pERK1/2 were performed on 20 μg of total proteins. ERK1/2 was used as a loading control. Blots are representative of three independent experiments with similar results.

ERK total content. In agreement with published data [26–28], cell treatment with G-1 caused a rapid but transient activation of ERK. Involvement of GPER in OL- and HT-mediated long term ERK1/2 phosphorylation was confirmed by knocking down GPER by shRNA. In the presence of a reduced GPER expression (Fig. 3C), long term ERK1/2 phosphorylation was no longer detectable (Fig. 3D). In addition, using U0126, a specific MEK1/2 chemical inhibitor, we confirmed that OL and HT are able to activate GPER engaging MAPK cascade activation (Fig. 3E).

3.4 OL and HT induce SKBR3 cell death through a GPER-mediated intrinsic apoptotic pathway

It has been widely demonstrated that sustained ERK1/2 activation can contribute to cell death by apoptosis [24, 29]. To verify if the inhibitory effects elicited by OL and HT on SKBR3 cell growth could be associated with apoptotic events, we first studied the main morphological features of the apoptotic process. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) staining demonstrated that untreated

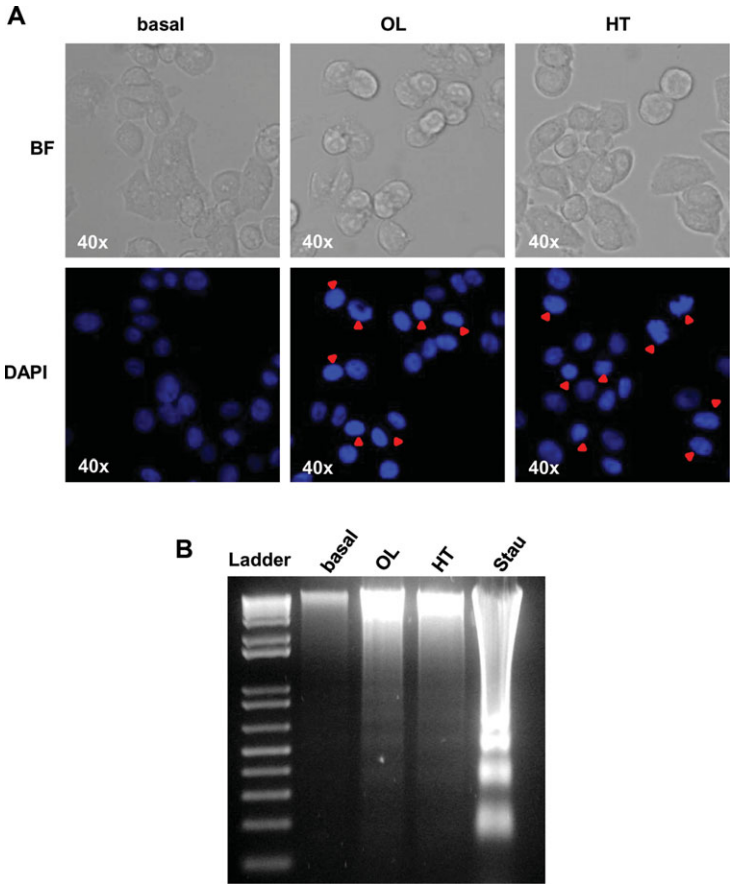


Figure 4. Effects of OL and HT on SKBR3 apoptosis and nuclei morphology. (A) SKBR3 cells were left untreated (basal) or treated in 1% DCC-FBS medium with OL (100 μM) or HT (50 μM) for 48 h. After treatment cells were fixed with paraformaldehyde, dyed with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and observed under fluorescent microscope (magnification 400×). Arrows indicate condensed nuclei. Images are from a representative experiment. (B) Cells were untreated (basal) or treated in 1% DCC-FBS medium with OL (100 μM) or HT (50 μM) for 48 h. Staurosporin (Stau) (1 μM) was used as an apoptotic-positive control. After treatment DNA was extracted from cells and analyzed on a 2% agarose gel. The image is from a representative experiment.

SKBR3 cells had round nuclei with regular contours and were large in size (Fig. 4A, basal). After OL or HT treatment, cells showed nuclei shrunken and irregularly shaped or degraded with condensed DNA (Fig 4A, OL or HT). DNA extracted from cells treated with OL or HT showed a typical 200 bp laddering indicative of DNA fragmentation (Fig. 4B).

In order to define the specific pathway involved in the apoptotic process activated by OL or HT in SKBR3 cells, we analyzed the expression of Bcl-2 family proteins to assess activation of the mitochondrial apoptotic pathway [30]. Bax and Bcl-2 protein levels were detected by Western blot analysis. Our results demonstrated that both OL and HT enhanced the expression of Bax (Fig. 5A) with a concomitant decrease in Bcl-2 content (Fig. 5B). Bax exerts pro-apoptotic activity by allowing Cyt c translocation from the mitochondria to cytosol [31] where it binds to apoptotic protease-activating factor-1 [32]. Apoptotic protease-activating factor-1 is then recruited to the amino terminus of procaspase-9 in the presence of deoxy-ATP, resulting in activation of the initiator caspase 9 [33] with subsequent proteolytic activation of executioner caspase 3 [34]. SKBR3 cells responded to OL and HT by increasing Cyt c release into the cytosol (Fig. 5C) followed by a simultaneous decrease in the mitochondrial fraction (Fig. 5D). Cyt c translocation in response to OL and HT treatment was abrogated after GPER gene silencing (Fig. 5C and D). We also observed that OL and HT induced caspase-9 (Fig. 5E) and caspase-3 (Fig. 5F) activation as revealed by Western blotting analysis showing the presence of bands corresponding to the cleaved forms of both caspases. We also detected the cleavage of PARP-1 that is considered one of the most important biochemical features of cells undergoing apoptosis (Fig. 5G) [35]. This effect resulted completely reversed by using U0126 (Fig. 5G). These data collectively explain the mechanism behind the ability of OL and HT to induce cell death in an ERα-negative BC cell line. This mechanism contemplates the activation of a GPER-mediated ERK-dependent mitochondria apoptotic pathway.

4 Discussion

The discovery of GPER as a receptor for estrogens a little over a decade ago, has helped to explain the mechanism behind the ability of cells to respond to E2 treatment activating growth and migratory processes in the absence of classic ER. The current knowledge on the function of this receptor in BC links its expression to the growth and metastasis of ERα-negative cancer [8, 36–38], to the activation of migration of ERα-positive cancer [39] and to Tam resistance [40]. The discovery of molecules able to bind and interfere with

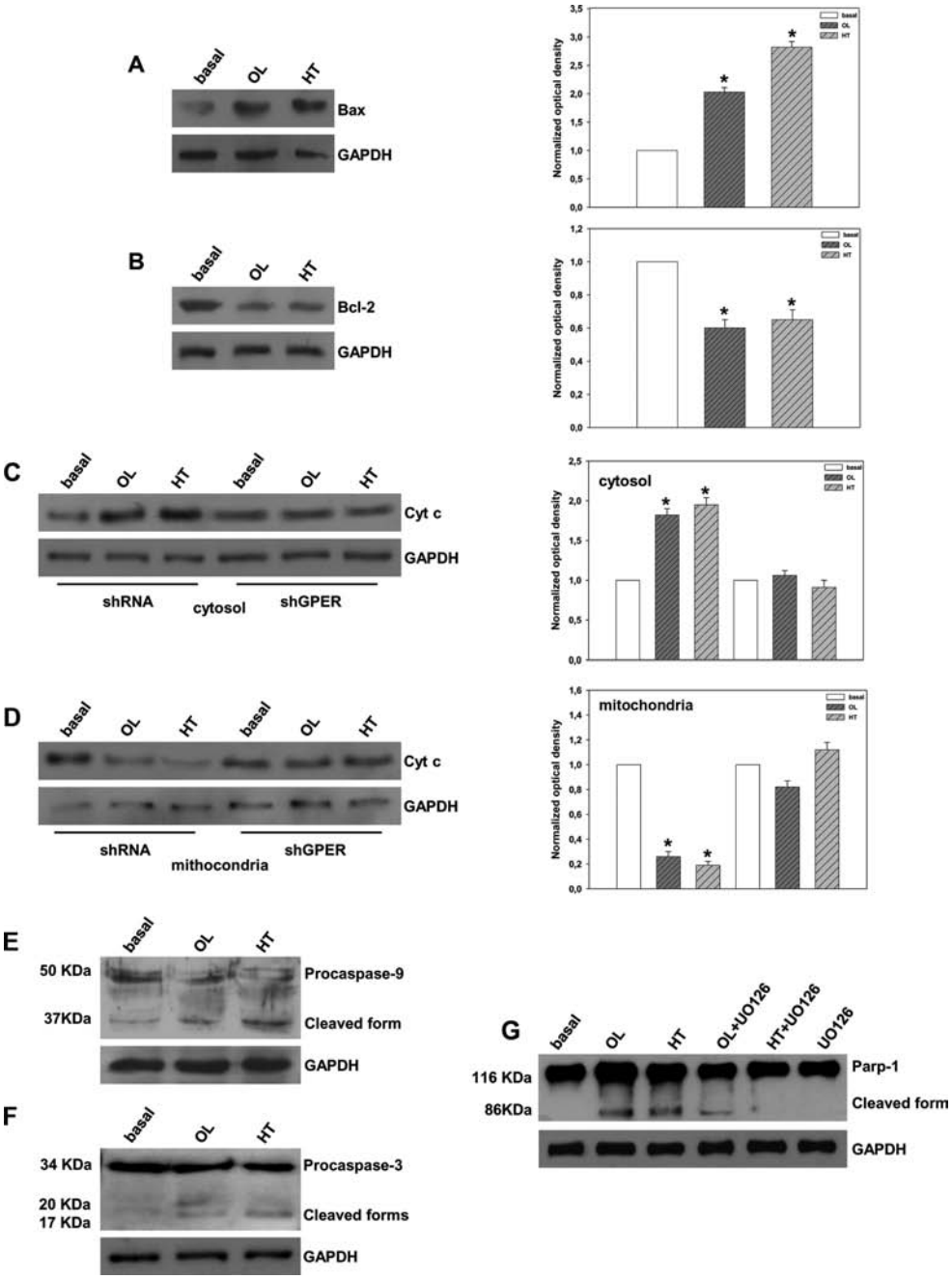


Figure 5. OL and HT effects on GPER-mediated mitochondrial apoptotic mechanism. Proteins extracted from SKBR3 cells untreated (basal) or treated in 1% DCC-FBS medium with OL (100 μM) or HT (50 μM) for 24 h were subjected to Western blot analysis for Bax (A) and Bcl-2 (B). (A and B left panels) Graphs represent means of normalized optical densities from three experiments, bars represent SEM. (A) $*p <$ 0.0001 compared with basal; (B) $*p < 0.0001$ compared with basal; (C and D) SKBR3 cells were transfected in serum-free medium with shRNA or shGPER for 72 h. Twenty four hours after transfection the medium was removed and replaced with in 1% DCC-FBS medium fresh for another 48 h; in the last 24 h, cells were untreated (basal) or treated for 24 h with OL (100 μM) and HT (50 μM). Cyt c levels in cytosolic (C) and mitochondrial (D) fractions were detected by Western blot analysis. Blots are representative of three independent experiments with similar results. (C and D left panels) Graphs represent means of normalized optical densities from three experiments, bars represent SEM. (C) $*p < 0.0001$ compared with basal shRNA; (D) $*p < 0.0001$ compared with basal shRNA. (E and F) Cells were untreated (basal) or treated in 1% DCC-FBS medium with OL (100 μM) or HT (50 μM) for 24 h. Appearance of caspase-9 (E) and caspase-3 (F) cleaved forms was determined by Western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. (G) Cells were untreated (basal) or treated for 48 h in 1% DCC-FBS medium with OL (100 μM) or HT (50 μM) alone or in combination with U0126 (10 μM); 20 μg of total proteins were analyzed by Western blot for PARP-1 cleavage. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Blots are representative of three independent experiments with similar results.

GPER-mediated signaling will then be promising therapeutics to reduce the occurrence of these events.

In the contest of Tam resistance, it was shown that long-term Tam treatment of MCF-7 cells increases the E2-stimulated upregulation of GPER and its translocation from endoplasmic reticulum to the cell surface [41]. Then, GPER converts Tam to a growth stimulator because of its ability to act as an agonist for GPER [26, 42, 43]. In BC patients GPER expression correlates significantly with EGFR and HER-2 expression, and is predictive for development of Tam resistance [40]. We are currently accumulating data that support a role for OL and HT as possible tools to reduce the growth of Tam resistant cells (our ongoing studies).

The current study focused instead on the potential application of OL and HT as therapeutics for ER-negative BC. We demonstrated that OL and HT, phenolic compounds present in olive oil, are capable of binding and activating GPER leading to apoptosis of ER-negative SKBR3 BC cells. OL and HT are then able to interfere with G-1-dependent SKBR3 proliferation as a consequence of the upregulation in cell cycle negative regulators such as p21 and p53 and a reduction in Cyclin D1 expression, one the most important proteins involved in cell cycle progression in BC [44]. To explain the mechanism behind this event, we show that after binding GPER the two phenols cause a sustained ERK1/2 activation/phosphorylation. It is not the first time that GPER activation has been shown associated with cell death through sustained activation of ERK1/2 [24, 45, 46], but other mechanisms have been evidenced as well. Activator protein 1 signaling downregulation [47], GPER-mediated protein kinase A dependent ERK inhibition [48] and GPER-mediated Ca^{++} mobilization [8] are other possible mechanisms. The effects exerted through GPER by OL or HT can be considered inverse agonistic, in fact, once OL or HT occupy GPER-binding pocket they cause long-term ERK1/2 activation different from the transient activation seen after stimulation with GPER specific ligand G-1. Indeed, the ability of GPER to activate different downstream signaling pathways according to ligand nature (e.g. G-1, E2, ICI, Tam) has been already evidenced [49] and we cannot exclude additional molecular mechanisms allowing the final apoptotic effect in response to OL, since GPER silencing did not completely abrogate OL effect on SKBR3 cell viability.

It is well known that phosphorylated ERK1/2 translocate from the cytoplasm to the nucleus to phosphorylate their nuclear targets for transcriptional regulation [50–52]. It has also been proposed that signal duration of ERK1/2 activation could dictate a cell-fate decision, with transient ERK1/2 activation closely related to cell survival and proliferation and conversely, sustained activation with nuclear accumulation of ERK1/2 transmitting antiproliferative signals [53–56] and apoptotic effects [24, 57].

In SKBR3 cells the apoptotic effect exerted by OL and HT treatment was evidenced by the upregulation of proapoptotic Bax protein followed by a decrease in anti-apoptotic Bcl-2 expression. Further, the ability of both OL and HT to activate mitochondrial apoptotic mechanism was demonstrated by their ability to induce GPER-dependent cytosolic Cyto c release. Moreover, caspase-9, caspase-3 cleavage as well as PARP-1 inactivation supported the above illustrated intrinsic apoptotic pathway.

Overall, we obtained the antiproliferative effects on SKBR3 cells only by using micromolar concentrations of OL and HT. This observation is in agreement with several in vitro studies showing antiproliferative effects of micromolar doses of HT and OL on breast and other tumor cell lines [58]. However, we must point out that in vivo OL and HT are rapidly metabolized reaching plasma and tissue concentrations on the order of nanomolar. This observation could question the role of these two olive oil derived compounds as molecules able to have a preventive effect on BC. Indeed, the role of olive oil, a major component of Mediterranean diet, in the prevention against BC is still highly debated. Some authors reported an inverse relationship [59] while others pointed out that the data are still too inconsistent [60].

Further studies, particularly in vivo, will be required to elucidate: (i) if low concentrations of OL and HT as well as their metabolites are able to activate in vivo molecular mechanisms not identified in the present work; (ii) if different phenols present in olive oil, as well as those present in foodstuff present in Mediterranean diet, could synergize leading to the achievement of those concentrations required to obtain a beneficial effect in terms of BC prevention.

Despite these limitations, the most interesting aspect that can be extrapolated from our results and those obtained by other authors [14, 58, 61] is that OL and HT are interesting molecules that could be successful employed as therapeutics adjuvant against certain tumor phenotypes, particularly BC with high histopathological variability (e.g. ER-positive/negative, GPER, and/or Her2/neu expression), different treatment responses and overall outcome. Moreover, the deepening of knowledge on OL and HT stereochemistry could be useful for the development of new pharmacological tools to enlarge the availability of chemopreventive or therapeutic drugs to fight cancer.

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Biological activity of 3-chloro-azetidin-2-one derivatives having interesting antiproliferative activity on human breast cancer cell lines

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ABSTRACT

Resveratrol (3,4′,5 tri-hydroxystilbene), a natural plant polyphenol, has gained interest as a non-toxic agent capable of inducing tumor cell death in a variety of cancer types. However, therapeutic application of these beneficial effects remains very limited due to its short biological half-life, labile properties, rapid metabolism and elimination. Different studies were undertaken to obtain synthetic analogs of resveratrol with major bioavailability and anticancer activity. We have synthesized a series 3-chloro-azetidin-2-one derivatives, in which an azetidinone nucleus connects two aromatic rings. Aim of the present study was to investigate the effects of these new 3-chloro-azetidin-2-one resveratrol derivatives on human breast cancer cell lines proliferation. Our results indicate that some azetidin-based resveratrol derivatives may become new potent alternative tools for the treatment of human breast cancer.

Breast cancer is a major cause of death in women, even if disease-free survival and overall survival of patients with breast cancer have been improved through intensive treatment.[1] Major breast cancer treatment methods consist, both separately and in combination with surgery, radiotherapy and chemotherapy. In particular, the anti-estrogen tamoxifen is widely used in the prevention and treatment of estrogen receptor positive breast cancer.[2] Inherent or acquired tumor drug resistance limits many agents that could be used to treat this disease and are often associated with severe, dose-limiting and systemic toxicities. Therefore, new agents acting on novel targets in breast cancer are currently under investigation. Interest in the pharmacological effects of bioactive compounds on cancer treatment and prevention has increased dramatically in recent years. A great number of natural agents derived from plants are studied as agents potentially useful in combined therapy for cancer patients. As well, there is a need to develop new more powerfully active drugs with reduced side effects that can substitute current pharmacological therapies.

Several studies confirmed that increasing vegetable and fruit consumption might reduce the risk of breast cancer.[3] Also, a lower incidence of breast cancer is associated with a high consumption of phytoestrogens,[4] which are biologically active plant-derived phenolic compounds that structurally mimic the mammalian estrogen, 17β-estradiol.[5]

Among many bioactive compounds, basic and preclinical researches on resveratrol, a non-flavonoid polyphenolic compound abundant in grapes, peanuts and other foods that are commonly consumed as part of human diet,[6] have shown a broad range of advantageous biological actions, including cardioprotection[7] and prolongation of lifespan in several species.[8] Resveratrol is a phytoalexin that in nature protects the plant from injury, ultraviolet (UV) irradiation, and fungal attack.[9] Resveratrol exists as cis- and transisomeric forms, with trans to cis isomerization facilitated by UV exposure. Its stilbene structure is related to the synthetic estrogen

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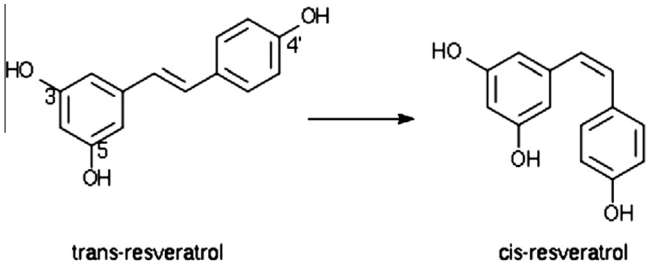


Figure 1. Chemical structure of resveratrol (RSV).

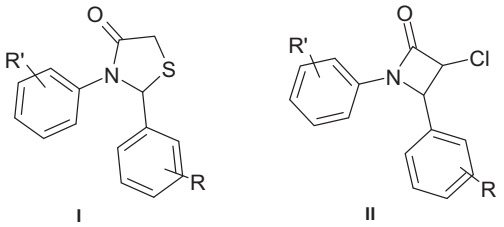


Figure 2. Structures of 2,3-thiazolidin-4-one derivatives (**I**) and of 3-chloro-azetidin-2-one derivatives (**II**).

diethylstilbestrol. Two phenol rings are linked by a styrene double bond to generate 3,4′,5-trihydroxystilbene (Fig. 1).

The biological properties of resveratrol are attributed to its ability to inhibit the oxidation of human low-density lipoprotein, while suppression of cycloxygenase-2 and inducible nitric oxide synthase activities also contribute to its anti-inflammatory and antioxidant effects.[10] Furthermore, the chemopreventive effect of resveratrol is thought to be due to inhibition of quinone reductase 2 activity, which in turn up-regulates the expression of cellular antioxidant and detoxification enzymes to improve cellular resistance to oxidative stress.[11] Resveratrol also increases the activity of SIRT (a member of the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases), resulting in improved cellular stress resistance and longevity,[8,10,12] Jang et al.[13] have suggested that it inhibits all three phases of tumor development: initiation, promotion, and progression in various cancers such as prostate,[13] colon,[14] endometrial,[15] hepatocarcinoma[16] and breast cancer.[17,18] Resveratrol contains strong anti-proliferative properties in many cultured cancer cell lines, and acts both by arresting cell cycle and by inducing apoptosis,[19–23] but the apoptosis inducing effects of resveratrol seemed diverse on different tumor cells.[19–21] Resveratrol has been shown to interfere with signal transduction pathways, to modulate cell cycle regulating proteins and to induce apoptosis in multiple cancer cell lines with various mechanisms, including through a p53-dependent pathway and PKC/Akt pathway.[24]

However, therapeutic application of these beneficial effects of resveratrol remains very limited due to its short biological half-life, labile properties, and rapid metabolism and elimination.[10] In fact, in human and rodent, three metabolic pathways have been identified, that is, sulfate and glucuronic acid conjugation of the phenolic groups and hydrogenation of the aliphatic double bond, the latter likely produced by the intestinal microflora.[25] Extremely rapid sulfate conjugation by the intestine/liver appears to be the rate-limiting step in resveratrol's bioavailability.[26] Analysis of recent literature reveals an increasing number of formulations under study, which reflects the major interest in developing pharmaceutical forms able to improve resveratrol bioavailability as a step towards applying its therapeutic potential in vivo.[26]

Several studies were undertaken to obtain resveratrol synthetic derivatives with potent anticancer activity, enhanced structural rigidity and major bioavailability.[27] Structure-activity studies have revealed crucial elements of the parental components that are required for specific effects. To give one example, the 4-hydroxy group in the trans conformation on the 4- and 4′-positions of the stilbenic backbone and the methoxy groups added to the trihydroxystilbene scaffold of resveratrol have been identified as crucial for antiproliferative[28] or cytotoxic[29] resveratrol effects, respectively. In addition, the structural replacement of the stilbene moiety of resveratrol has been found to be a promising strategy for thegeneration of synthetic analogues with improved pharmacokinetic parameters. Mayhoub et al.[30] described a series of trans and cis 2,3-thiadiazol analogs of resveratrol using as design strategy the replacement of the alkene linker between the two aromatic rings with a heterocyclic system. Starting from this approach, recently we have synthesized 2,3-thiazolidin-4-one resveratrol analogues that have increased structural rigidity and potent activity (I Fig. 2) fixing the cis-conformation of resveratrol, hypothesizing that also using cis stilbene template could have active derivatives. We demonstrated that some derivatives displayed stronger antiproliferative effects than resveratrol in human breast cancer cells.[27]

Table 1
Bioaccessibility (%) of **4a–f** compounds

Sample	Bioaccessibility (%)
Resveratrol	31 ± 0.9
4a	79 ± 0.8
4b	78 ± 0.9
4c	83 ± 1.1
4d	87 ± 0.7
4e	80 ± 1.0
4f	70 ± 0.9

In the present work, we prepared a new series of 1,4-diaryl-3-chloro-azetidin-2-one derivatives in which a azetidin-2-one nucleus connects two aromatic rings (II Fig. 2); also these new compounds have structural rigidity, major bioavailability and antiproliferative activity.

The designed compounds (**4a**–f, Table 1)[31] were prepared by the following Staudinger reaction.[32] Cycloaddition of imines (**3**) with 2-chloro-acetylchloride in the presence of triethylamine afforded beta-lactams **4a–f** in high yields (47–93%). The imines were obtained by condensation of aldehydes (**1**) and amines (**2**) in toluene (Scheme 1).

The bioavailability of the new synthesized 3-chloro-azetidin-2-one resveratrol derivatives was measured by dialysis tubing procedure,[33,34] a quick and low cost method that represents a good model to evaluate the bioavailability of different kinds of molecules.

In most cases, the oral route represents the most convenient one for drug administration and, thus, it is important to choose an appropriate model to investigate absorption and bioavailability of therapeutic agents in the gastrointestinal system.

Bioavailability was defined as the percentage of tested compounds recovered in the bioavailable fraction, after in vitro digestion, in relation to the original non-digested samples.

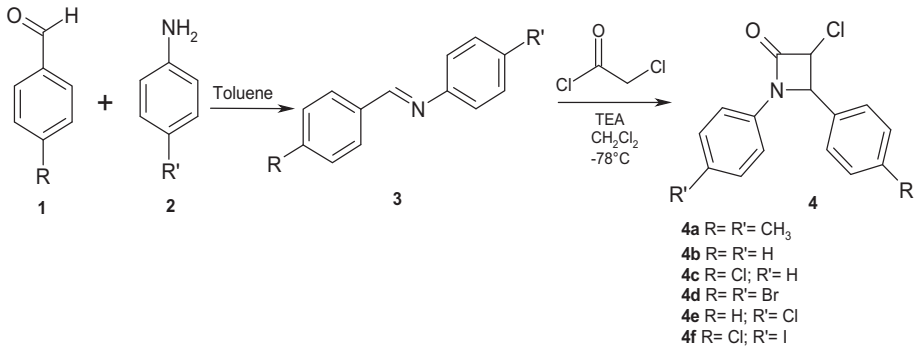
This value can be calculated by the following equation (1):

(bioavailable content/total content) × 100 (**1**).

The obtained results, after six hours, were reported in Table 1.

As it is possible to note, all the synthesized compounds showed a higher bioavailability compared to resveratrol, which was more than 2 times less bioavailable, and the best results were observed for **4d** with about 87% of the initial content recovered in the bioavailable fraction.

The higher bioavailability of these novel 3-chloro-azetidin-2-one resveratrol derivatives could be due to their modified struc-



Scheme 1. Synthesis of 3-chloro-azetidin-2-one derivatives (**4**).

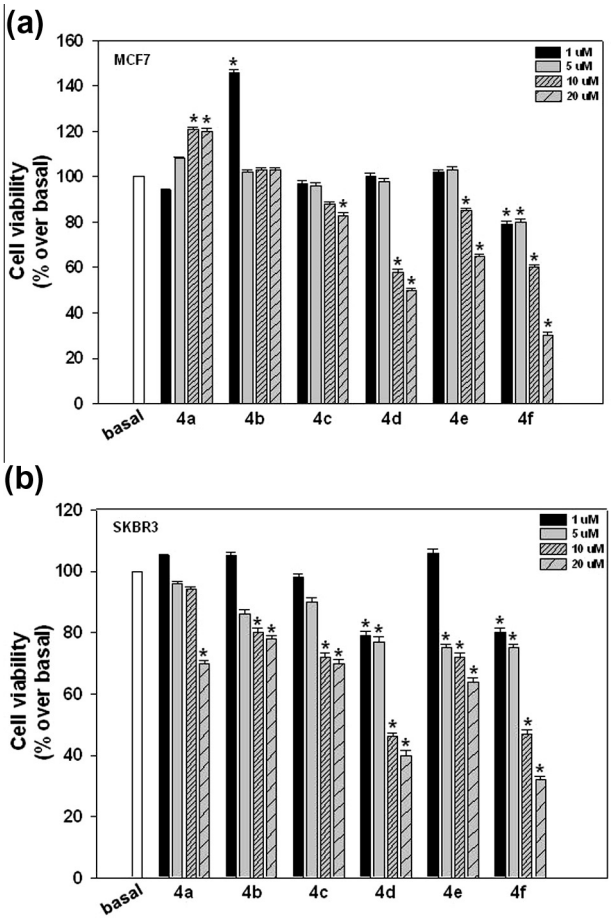


Figure 3. Effects of different doses of 3-chloro-azetidin-2-one derivatives on MCF-7 (a) and SKBR3 (b) cell proliferation. Cells were treated for 72 h with the indicated concentrations of compounds **4a–f**. Cell viability was evaluated by MTT assay. Statistically significant differences are indicated. Columns, mean of three independent experiments each performed with triplicate samples expressed as percent of basal, bars, SD; (*P <0.01 compared with basal).

tures making these molecules more lipophilic compared to resveratrol.

Starting from bioavailability results of **4a–f** compounds, we evaluated its effects on cell proliferation against estrogen receptor positive (ER+) MCF-7 and estrogen receptor negative (ER−) SKBR3 human breast cancer cell line (Fig. 3) at different concentration (1, 5, 10, 20 μM) using the MTT assay.[35] Therefore, we treated for 72 h MCF-7 and SKBR3 cells with each compounds and also with RSV in order to compare the antiproliferative effects of the chemicals used to this well-known anticancer agent.[35–41]

Table 2
IC_{50} of resveratrol and its derivatives **4d** and **4f** for MCF7 and SKBR3 cells on cell viability

Compounds	MCF7		SKBR3	
	IC_{50} (μM)	95% Confidence interval	IC_{50} (μM)	95% Confidence interval
4d	16.72	13.42–20.84	11.09	8.37–14.68
4f	11.77	8.93–15.52	9.51	7.69–11.75
RSV	28.38	25.71–31.33	41.22	34.21–49.67

As showed in Figure 3, in MCF7 cells (Fig. 3a) **4d–f** compounds, determined a clear dose-dependent inhibitory effect on cell growth. Higher doses of **4a** compound elicited an stimulation while only low dose of **4b** compound favored it. However, **4c** compound decreased cell viability only at 20 μM concentration.

Among all tested compounds in SKBR3 cells (Fig. 3b), higher concentrations of **4b**, **4c**, **4e** derivatives elicited a negative inhibitory effect. Remarkably, a 72 h treatment with **4a** compound (20 μM) was able to prevent SkBr3 cell growth. It should be pointed out that **4d** and **4f** compounds inhibited the proliferation in a dose dependent manner in both estrogen dependent MCF-7 and in estrogen independent SKBR3 cell lines suggesting that these compounds could be potentially active in different breast cancer subtypes.

As showed in Figure 3, **4d**, **4f** compounds are more active, as also evidenced by its half-maximal inhibitory concentration (IC50) values (Table 2) that are much more relevant respect to IC50 value of RSV.

In fact, it is evident that the dose at which RSV shows antiproliferative effects on MCF7 and SKBR3 cells (Supplementary data Fig. S1), is relatively higher (>20 μM) (Table 2)[42] compared to that of the tested compounds.

On the basis of the aforementioned promising results obtained in two different model systems of breast cancer cells, the capability of **4d** and **4f** compounds to elicit strong repressive effects on breast cancer cell growth, could be determined by presence, as electron-attractor atoms, of two halogens on aromatic rings.

In addition, a control experiment using 3T3 mouse embryonic fibroblast cells has been performed; no effects on cell viability was obtained using all azetidin-based resveratrol derivatives of 5 μM from to 20 μM after 72 h of treatment (Supplementary data Fig. S2), suggesting that these compounds have specific inhibitory effect on breast cancer cells.

In conclusion, considering the widespread chemopreventive and chemotherapeutic applications of resveratrol, a strong demand exists to search for other pharmacologically active resveratrol analogs with enhanced bioavailability, potency and selectivity. For such reason, a series of new 3-chloro-azetidin-2-one derivatives

has been synthesized and evaluated for their growth regulatory effects in MCF7 and SkBr3 human breast cancer cells. Among these compounds, that showed moderate to high antitumor activity and displayed a more in vitro bioavailability respect to resveratrol, the strongest antiproliferative activity against human breast cancer cells tested was displayed especially by **4d** and **4f**. Hence, the capability of these compounds to have greater bioavailability than resveratrol and to elicit selective inhibitory effects on breast cancer cell growth could be taken into account towards novel pharmacological approaches in breast cancer therapy. Experiments useful to investigate the molecular mechanism involved and to evaluate in vivo bioavailability and chemotherapeutic potential of compounds are in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.054.

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31. *Experimental section:* Reagents, starting material and solvents were purchased from Sigma–Aldrich (Milano, Italy) and used as received. Analytical TLC was performed on plates coated with a 0.25 mm layer of silica gel 60 F254 Merck and preparative TLC on 20 × 20 cm glass plates coated with a 2 mm layer of silica gel PF254 Merck. Silica gel 60 (300–400 mesh, Merck) was used for flash chromatography. Melting points were measured with a Köfler apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker 300 spectrometer operating at 300 and 75 MHz, respectively. Chemical shifts are reported in δ values (ppm) relative to internal Me4Si and J values are reported in Hz. Mass spectra were obtained using a ESI mass spectrometer: Finnigan LCQ Advantage max (Thermo Finnigan; San Jose, CA, USA).
*General procedure for the synthesis of imine (**3**).* To a solution of the arylamine (10 mmol) in toluene (40 mL) was added aryl-aldehyde (12 mmol) and the mixture was refluxed overnight using a Dean–Stark water separator (monitored by TLC). When the reaction was over, toluene was evaporated under reduced pressure, and the crude product was used as such for the next reaction.
*General Procedure for the synthesis of β-lactam (**4**).* A solution consisting of 2-chloro-acetylchloride (1.5 mmol) in dichloromethane (10 mL) was added drop wise to a stirred solution containing imine (1 mmol) and distilled triethylamine (3 mmol) in dry dichloromethane (10 mL) at −78 °C. The reaction mixture was then stirred overnight at room temperature, washed with saturated sodium bicarbonate solution (10 mL), dilute hydrochloric acid (10%, 10 mL), brine (10 mL), dried with anhydrous sodium sulfate, and evaporated to obtain the crude product. The pure product (48–68%) was then isolated via column chromatography over silica gel using ethyl acetate:hexanes (1:4) as the solvent.
*3-Chloro-1,4-di-p-tolylazetidin-2-one (**4a**):* Yellow solid. Yield 51%. Mp 103–104 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.29 (d, 6H), 5.27 (d, J = 6 Hz, 1H), 5.39 (d, J = 6 Hz, 1H), 7.07 (m, 2H), 7.22–7.26 (m, 4H), 7.28 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 21.3, 62.0, 68.1, 125.3, 129.2, 133.4, 136.4, 136.5, 136.8, 140.5, 162.2. ESI m/z calcd for 285.77; found: 286.04. Anal. Calcd for $C_{17}H_{16}ClNO$: C, 71.45; H, 5.64. Found: C, 71.48; H, 5.62.
*3-Chloro-1,4-diphenylazetidin-2-one (**4b**).* White solid. Yield 61%. Mp 189–190 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz,1H), 5.43 (d, J = 6 Hz, 1H), 7.13 (m, 2H), 7.26–7.40 (m, 6H), 7.43 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1,117.7, 126.7, 126.9, 128.0, 128.5, 129.9, 139.5, 143.5,162.2. ESI m/z calcd for $C_{15}H_{12}ClNO$: 257.71; found: 257.95. Anal. Calcd for $C_{15}H_{12}ClNO$: C, 69.91; H, 4.69. Found: C, 69.94; H, 4.71.
*3-Chloro-4-(4-chlorophenyl)-1-phenylazetidin-2-one (**4c**).* White solid. Yield 48%. Mp 178–179 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.33 (d, J = 12 Hz, 1H), 5.41 (d, J = 6 Hz, 1H), 7.14 (m, 2H), 7.26–7.31 (m, 5H), 7.40 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1, 117.7, 127.2, 128.6, 128.0, 128.9, 132.3, 139.5, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{11}Cl_2NO$: 292.16; found: 292.08. Anal. Calcd for $C_{15}H_{11}Cl_2NO$: C, 61.67; H, 3.79. Found: C, 61.70; H, 3.81.
*1,4-Bis(4-bromophenyl)-3-chloroazetidin-2-one (**4d**).* White solid. Yield 59%. Mp 163–164 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz, 1H), 5.39 (d, J = 6 Hz, 1H), 7.17–7.20 (m, 4H), 7.44 (m, 2H), 7.56 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$):62.0, 68.1, 121.1, 122.3, 127.2, 131.4, 131.8, 136.7, 138.5, 142.5, 162.2. ESI m/z calcd for $C_{15}H_{10}Br_2ClNO$: 415.51; found: 415.03. Anal. Calcd for $C_{15}H_{10}Br_2ClNO$: C, 43.36; H, 2.43. Found: C, 43.34; H, 2.40.
*3-Chloro-1-(4-chlorophenyl)-4-phenylazetidin-2-one (**4e**).* White solid. Yield 65%. Mp 156–157 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 3 Hz, 1H), 5.41(d, J = 6 Hz, 1H), 7.31–7.44 (m, 7H), 7.54 (m, 2H).^{13}C NMR (75 MHz, $CDCl_3$): 62.0, 68.1, 86.1, 123.2, 127.2, 128.6, 132.3, 137.8, 138.4, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{11}Cl_2NO$: 292.17; found: 292.04. Anal. Calcd

for $C_{15}H_{11}Cl_2NO$: C, 61.67; H, 3.79. Found: C, 61.70; H, 3.77.

3-Chloro-4-(4-chlorophenyl)-1-(4-iodophenyl)azetidin-2-one (**4f**). Yellow solid. Yield 68%. Mp 191–193 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.30 (d, J = 6 Hz, 1H), 5.40 (d, J = 6 Hz,1H), 7.06 (m, 2H), 7.25 (m, 2H), 7.40 (m, 2H), 7.60 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$):62.0, 68.1, 86.1, 123.2, 127.2, 128.6, 132.3, 137.8, 138.4, 141.6, 162.2. ESI m/z calcd for $C_{15}H_{10}Cl_2INO$: 418.06; found: 418.10. Anal. Calcd for $C_{15}H_{10}Cl_2INO$: C, 43.10; H, 2.41. Found: C, 43.08; H, 2.43.

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33. *In vitro bioavailability studies:* In vitro bioavailability studies in simulated gastric and intestinal fluids were carried out by performing a slight modified version of the dialysis tubing procedure.[34]

The dialysis tubing method is characterized by two consecutive enzymatic digestions: pepsin and pancreatin digestion, respectively. These steps are described as follows.

Pepsin Digestion. 100 μL of each sample (**4a**–**f**, 10 mM in DMSO) were mixed with 1.0 mL of a 0.85 N HCl solution containing 24,000 U of porcine pepsin per mL and 3 mL of a sodium cholate solution (2% w/v in distilled water). The obtained mixture was introduced into a dialysis bag (Spectrum Laboratories Inc., MWCO: 12–14,000 Dalton, USA) which was then carefully closed and immersed inside a flask containing 10 mL of a 0.85 N HCl solution (pH 1.0). The flask was then incubated into a shaking water bath at 37 °C to simulate the human body conditions of temperature for 2 h.

Pancreatin Digestion. At the end of the 2 h pepsin digestion, the dialysis bag was opened and 11 mg of amylase, 11 mg of esterase and 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 22.60 mg porcine pancreatin/mL were added to the peptic digesta. After the digesta and enzyme solution were well-mixed, the dialysis bag was sealed on each end with clamps and placed into a flask with 10 mL of buffer solution at pH 7.0. The flask was incubated into the shaking water bath at 37 °C for further 4 h.

In the aim to evaluate the bioavailability of the different samples, 2 mL of the medium were withdrawn from the flask for sample analysis at the time points of two and six hours, after pepsin and pancreatin digestions respectively. The concentration of the samples was determined by UV/VIS spectroscopy (UV/VIS spectrometer V-530, Jasco—U.S.) and the percentages were calculated by using the equations obtained from the calibration curves of five standard solutions, for each tested compound, at pH 1.0 and 7.0, respectively.

For this purpose, all the prepared standard solutions were analysed by UV–Vis spectrophotometer and the correlation coefficient (R2), slope and intercept of the regression equations obtained by the method of least square were calculated at pH 1.0 and 7.0, respectively. Each experiment was performed in triplicate.

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35. *Cell culture and treatments.* MCF-7 breast cancer cells (a ER positive breast cancer cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained as previously described.[36] SKBR3 breast cancer cells (a ER negative breast cancer cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained in RPMI1640 without phenol red supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (Sigma–Aldrich, Milano, Italy) (complete medium).[37] 3T3 mouse embryonic fibroblast cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in DMEM with phenol red supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin/streptomycin (Sigma–Aldrich, Milano, Italy) (complete medium). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were screened periodically for Mycoplasma contamination. All 3-chloro-azetidin-2-one derivatives compounds were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, Missouri, USA) at a concentration of 10 mM and diluted in DMEM/F12 (for MCF-7 cells), in DMEM (for 3T3 cells) or in RPMI (for SKBR3 cells) medium supplemented with 1% DCC-FBS (dextran-coated charcoal-treated newborn calf serum serum) to obtain the working concentration.

Assessment of cell viability. MCF-7, SKBR3 and 3T3 cells were seeded on twenty-four well plates (0.2×10^5 cells/well) and grown for 48 h in complete medium. Before being treated, cells were starved in DMEM/F12 (for MCF-7 cells), in DMEM (for 3T3 cells) or in RPMI (for SKBR3 cells) serum free medium for 24 h to the purpose of cell cycle synchronization. The effect of the different doses of 3-chloro-azetidin-2-one derivatives was measured using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT) assay as previously described.[27,36–42] Seventy two hours after treatments, fresh MTT (Sigma), re-suspended in PBS, was added to each well (final concentration (0.33 mg/mL). After 3 h incubation, cells were lysed with 1 mL of DMSO. Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Statistical analyses. All experiments were conducted at least three times and the results were from representative experiments. Data were expressed as mean values ± standard deviation (SD), and the statistical significance between control (basal) and treated samples was analyzed using the GraphPad Prism 5 software program. The unpaired Student's t-test was used to compare two groups. P <0.05 was considered statistically significant.

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