PALACKY UNIVERSITY OLOMOUC

Faculty of Science

Department of Cell Biology and Genetics

CONSTRUCTION OF REPORTER CELL LINES FOR TOXICOLOGICAL AND ENVIRONMENTAL APPLICATIONS

Ph.D. Thesis

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Hereby I declare that this presented Ph.D. thesis is based on my own research carried out in the Department of Cell Biology and Genetics, Faculty of Science, Palacky University Olomouc, in the period from September 2013 to May 2017. Co-authors agree with the inclusion of published results.

Olomouc, 15th June 2017 …….

Mgr. Iveta Bartoňková

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Abstract:

Endocrine disrupting chemicals (EDCs) are substances possessing the ability to interfere with endocrine signalling by altering steroid hormone homeostasis and the transcriptional activity of nuclear receptors. Examples of EDCs are agrochemicals, food additives and contaminants, and industrial and environmental pollutants. Given the impact of EDCs on human health and living organisms in general, it is relevant and interesting to develop reliable tools for the detection of endocrine disruptors.

In the present thesis, I describe the development and characterization of human stably transfected reporter cell lines for the assessment of androgen receptor (AR; AIZ-AR cell line) and vitamin D receptor (VDR; IZ-CYP24 and IZ-VDRE cell lines) transcriptional activities. These cell lines represent unique entirely human systems derived from human cancer cell lines expressing fully functional endogenous AR and/or VDR, respectively, that were transfected with reporter plasmids containing reporter sequences from promoter regions of human AR and/or VDR-responsive genes. These cell lines remained fully functional for over 2 months in the culture (this period corresponds to more than 25 cell passages) as well as after a freeze–thaw cycle. The reliable detection of ligands of human AR and VDR, respectively, is possible as soon as after 8 hours of the incubation. The assay was sensitive enough to allow for experiments in 96-well plates. The specificity of luciferase induction was proven by applying model ligands of other nuclear and steroid receptors: no significant off-activation was observed.

In conclusion, the human stably transfected reporter cell lines AIZ-AR, IZ-CYP24 and IZ-VDRE present rapid, reliable, effective, sensitive, selective and reproducible tools for identification of ligands and activators of human AR and VDR, respectively. These cell lines can potentially be used in pharmacological, environmental and toxicological studies.

Keywords:	stable transfection, vitamin D receptor, androgen receptor, gene reporter assay, endocrine disruption
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Abstrakt:

Endokrinní disruptory (EDCs) jsou látky mající schopnost interagovat se signálními dráhami endokrinního systému, jejíž hlavním mechanismem je změna transkripční aktivity jaderných receptorů. Příkladem těchto látek jsou nejrůznější pesticidy, potravinová aditiva a kontaminanty nebo látky znečišťující životní prostředí. Vzhledem ke vlivu endokrinních disruptorů na živé organismy včetně člověka představuje vývoj spolehlivých detekčních systémů jeden z hlavních směrů jejich studia.

V předkládané disertační práci jsem se zabývala konstrukcí a charakterizací lidských stabilně transfekovaných reportérových buněčných linií určených pro stanovení transkripční aktivity androgenního receptoru (buněčná linie AIZ-AR) a vitamin D receptoru (buněčné linie IZ-CYP24 a IZ-VDRE). Tyto buněčné linie představují unikátní lidský reportérový systém odvozený od lidských nádorových buněčných linií exprimujících plně funkční androgenní receptor/vitamin D receptor, které byly transfekovány reportérovými plasmidy obsahujícími reportérové sekvence odvozené z lidských AR/VDR responsivních genů. Všechny výše zmíněné buněčné linie byly plně funkční po více než 2 měsíce v buněčné kultuře, což odpovídá více než 25 pasážím, a stejně tak i pro kryoprezervaci. Spolehlivá detekce AR/VDR ligandů je možná již po osmi hodinách po aplikaci látek. Výše uvedené stabilní buněčné linie jsou natolik citlivé, že umožňují testování v 96-jamkovém formátu. Specifita buněčných linií byla ověřena testováním modelových ligandů ostatních steroidních a jaderných

receptorů, přičemž nebyly pozorovány žádné signifikantní indukce luciferázové aktivity, které by potenciálně mohly ovlivnit interpretaci experimentálních dat.

Závěrem lze konstatovat, že lidské stabilně transfekované reportérové buněčné linie AIZ-AR, IZ-CYP24 a IZ-VDRE představují rychlý, spolehlivý, efektivní, selektivní a reprodukovatelný nástroj pro detekci ligandů a aktivátorů lidského androgenního receptoru, respektive vitamin D receptoru. Využití těchto linií spočívá ve farmakologických, environmentálních a toxikologických aplikacích.

Klíčová slova:	stabilní transfekce, vitamin D receptor, reportérová esej, endokrinní disrupce
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CONTENTS

ABBREVIATIONS

ADT	androgen deprivation therapy
AHR	arylhydrocarbon receptor
AP	alkaline phosphatase
AR	androgen receptor
AR-V	androgen receptor splice variant
ARA	androgen receptor-associated protein
ARE	androgen response element
ARR	androgen response region
ATP	adenosine triphosphate
AVR	assay variation ratio
BMD	bone mineral density
CAR	constitutive androstane receptor
CAS	CRISPR-associated genes
CAT	chloramphenicol acetyltransferase
CDK	cyclin-dependent kinase
CKII	casein kinase II
CRISPR	clustered regularly interspaced short palindromic repeats
CS-FBS	charcoal-stripped fetal bovine serum
CYP	cytochrome P450
DBD	DNA-binding domain
DBP	vitamin D-binding protein
DDT	dichlorodiphenyltrichloroethane
DEX	dexamethasone
DHT	5α-dihydrotestosterone
DMEM	Dulbecco′s Modified Eagle Medium

DMSO	dimethylsulphoxide
DRIP	vitamin D receptor interacting proteins
EC_{50}	half maximal effective concentration
ECACC	European Collection of Cell Cultures
EDC	endocrine disrupting chemical
ER	oestrogen receptor
FBS	fetal bovine serum
FGF23	fibroblast growth factor 23
GFP	green fluorescent protein
GMO	genetically modified organism
GR	glucocorticoid receptor
HRE	hormone response element
HSP	heat shock protein
HVDRR	hereditary 1,25-dihydroxyvitamin D resistant rickets
IC_{50}	half maximal inhibitory concentration
LBD	ligand-binding domain
LCA	lithocholic acid
MMTV	mouse mammary tumour virus
MR	mineralocorticoid receptor
MTT	thiazolyl blue tetrazolium bromide
NLS	nuclear localization signal
NR	nuclear receptor
$1,25(OH)_2D3$	1α,25-dihydroxyvitamin D3 (calcitriol)
PAH	polyaromatic hydrocarbon
25(OH)D3	25-hydroxyvitamin D3 (calcifediol)
PCB	polychlorinated biphenyl

PIAS4	protein inhibitor of activated STAT4
PIC	preinitiation complex
PKA	protein kinase A
PKC	protein kinase C
PR	progesterone receptor
PSA	prostate specific antigen
PTH	parathyroid hormone
PXR	pregnane X receptor
RAR	retinoic acid receptor
RE	response element
RLU	relative luciferase units
RPMI-1640	Roswell Park Memorial Institute Medium
RXR	retinoid X receptor
SD	standard deviation
SEAP	secreted alkaline phosphatase
SHBG	sex hormone-binding globulin
SW	signal window
TBP	TATA-box binding protein
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TFIIB	general transcription factor IIB
UV	ultraviolet
VDR	vitamin D receptor
VDRE	vitamin D response element

1 INTRODUCTION

Xenobiotics are compounds that are foreign to the body. They include drugs, food additives, agrochemicals, cosmetics, and environmental pollutants. A distinct class of these substances interfere with functions of nuclear receptors leading to perturbation in drug metabolism, cell signalling pathways, to the onset and progression of disease or pathological states, or even to cancerous growth. Because molecular basis of these interactions is to compromise or to disrupt the activity of hormones and endocrine signalling molecules, these xenobiotics are referred to as endocrine disruptors. These substances are often persistent in the environment (e.g. polychlorinated biphenyls, dioxins or polycyclic aromatic hydrocarbons) and they can be introduced into a food chain.

Different *in vitro* techniques have been used to evaluate the effects of xenobiotics on the transcriptional activity of nuclear receptors. Gene reporter assays were introduced in the 1980s as a rapid and reliable tool for environmental studies and drug discovery and a fundamental tool for pharmacological and toxicological research. This approach is based on a transfection of cells, either transient or stable, with a reporter vector containing a reporter gene under the control of a specific sequence termed a response element (RE). The nuclear receptor (NR) in its active (ligand-bound) form binds this RE, thus triggering the expression of the reporter gene.

In the present thesis, I describe the construction and characterization of novel stably transfected human reporter cell lines for the assessment of androgen receptor (AR; AIZ-AR cell line) and vitamin D receptor (VDR; IZ-CYP24 and IZ-VDRE cell lines) transcriptional activities. These cell lines are unique entirely human *in vitro* systems expressing endogenous AR or VDR, respectively, that were transfected with a reporter plasmid containing reporter sequences derived from the promoter region of human target genes. Therefore, these cell lines represent an optimal system for the detection of compounds that could disrupt physiological receptors-mediated functions in the human body. All cell lines remained fully functional for over 2 months in cell culture as well as after cryopreservation. Ligands of target receptors can be identified as soon as after 8 hours of treatment. The specificity of the induction of the luciferase signal was

evaluated by the applying model ligands of other NRs; no significant off-response (false positive) that could lead to the misinterpretation of experimental data was identified. The assays were sensitive enough to allow tor experimentation in 96-well plate format.

Taken together, stably transfected reporter cell lines described in this thesis present rapid, high-throughput, reliable, robust, selective and sensitive tools for the identification of ligands of human AR and VDR, with possible future use in pharmacological, environmental and toxicological studies.

2 THEORETICAL PART

2.1 The nuclear receptor superfamily

NRs are a group of intracellular proteins responsible for diverse functions in all metazoans, e.g. development, homeostasis or reproduction (Mangelsdorf *et al*., 1995; Moras *et* Gronemeyer, 1998; Escriva *et al*., 2004). Besides post-transcriptional and post-translational modifications of drug-metabolizing enzymes, NRs also present the most common way of regulation of metabolism of xenobiotics as well as metabolism of endogenous compounds (Moore *et* al., 2000; Muntane, 2009).

NRs act as sequence-specific ligand-activated transcriptional factors, thus facilitating a direct link between signalling molecules and transcription of target genes. Ligands of NRs are lipophilic compounds that can easily enter the cell directly by diffusion across the plasma membrane. These compounds include retinoic acids, steroid hormones (androgens, oestrogens, progesterone, glucocorticoids and mineralocorticoids), vitamin D, bile acids, thyroid hormones, prostaglandins and others (Mangelsdorf *et* Evans, 1995). The superfamily of NRs contains not only receptors with already known endogenous ligands (so called “nuclear hormone receptors”) but also a large group of transcriptional factors that have no endogenous ligand or it has not been discovered yet. These receptors are termed “nuclear orphan receptors” and they were identified through sequence similarity to already known receptors (Enmark *et* Gustafsson, 1996). At the end of Human Genome Project in 2001, it was found out that there are 48 NRs encoded in the human genome (Robinson-Rechavi *et al*., 2001; Germain *et al*., 2006).

Despite huge structural variability of ligands, members of nuclear receptor superfamily share common organization. From the C terminus to N terminus, NR polypeptide chains can be subdivided into five or six segments, designated as A through F (Bain *et al.*, 2007; Rastinejad *et al*., 2015). The N-terminal region (A/B domain) is the most variable region of NRs, with the length ranging from 50 to 500 amino acid residues, and it contains the ligand-independent transactivation domain AF-1, which is constitutively active. The most

evolutionary conserved domain is the DNA-binding domain (DBD; C domain), which is responsible mainly for directing the ligand-bound receptor to its target DNA sequence designated as response element (RE). It contains two highly conserved zinc-finger motifs, each with four cysteines chelating one Zn^{2+} ion. The largest domain is the ligand-binding domain (LBD; E domain) consisting of 12 α-helices. The LBD is essential for hormone recognition and specificity and selectivity of the physiological response and contains ligand-activated transactivation domain AF-2. DBD and LBD are separated by the hinge region (D domain) allowing for flexibility of the molecule. The D domain also contains nuclear localization signal (NLS), which is responsible for nuclear trafficking. The F domain in the C-terminus is extremely variable in sequence and structure, and its function has not been characterized yet; this region may not be present in all NRs.

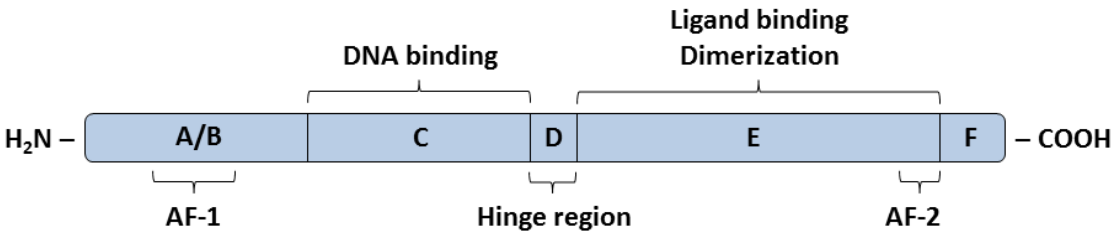

Figure 1: General structure of nuclear receptors (NRs). Adopted from Aranda *et* Pascual (2001). NRs are typically composed of several functional domains: the N-terminal A/B domain containing ligand-independent transactivation region AF-1; the most evolutionary conserved DNA-binding domain (DBD; C domain); hinge region (D domain); the largest ligand-binding domain (LBD; E domain) containing ligand-activated transactivation domain AF-2; and C-terminal F domain.

2.2 Androgen receptor

Androgen receptor (AR, NR3C4) is a member of the steroid hormone receptor family of ligand-activated transcriptional factors, along with progesterone receptor (PR), oestrogen receptor (ER), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). The main physiological function of AR is a process of male sexual determination and induction of pubertal changes (Mooradian *et al*., 1987). Any modifications in AR transcriptional activity or its

mutations can lead to pathological conditions like prostatic hyperplasia or prostate cancer (Chang *et al*., 1995).

The *AR* gene is located in chromosomal region Xq11-12 (Migeon *et al*., 1981), it is more than 90 kb long and contains eight exons encoding three major functional domains (Kuiper *et al*., 1989). The N-terminal domain is encoded by exon 1 and contains ligand-independent N-terminal activation domain AF-1. The DBD is encoded by exons 2 and 3 and LBD by exons 4 to 8. A small hinge region is located in between DBD and LBD. A NLS spans the DBD and hinge region (Gao *et al*., 2005). The amino acid sequence of AR is quite conserved among different species, with overall sequence similarity between human and rat AR of 85% (Lubahn *et al*., 1988).

The first exon of the human *AR* gene contains several regions of repetitive DNA sequences. Of these, the most important is a CAG repeat that starts at codon 58 and its length is on average 21 ± 2 repeats (La Spada *et al*., 1991). Given that CAG codes for glutamine in polypeptide chains, the polymorphism of CAG repeats results in polyglutamine tracts that differ in their length. These polyglutamine regions have been shown to inversely correlate with transcriptional activity of AR that can lead to X-linked spinal and bulbar muscular atrophy (so called Kennedy's disease) (Chamberlain *et al*., 1994) or prostate cancer (Giovannucci *et al*., 1997).

The AR protein has a molecular mass of 110 kDa (van Laar *et al*., 1989) and consists of 918 amino acid residues (Chang *et al*., 1988). The structure is similar to that of other members of the steroid hormone receptor family and shows high sequence conservation. The LBD of AR is composed of 12 helices forming a ligand-binding pocket (Matias *et al*., 2000). The binding of the ligand triggers a conformational change leading to folding of helix 12 to enclose the pocket. In contrast to other steroid receptor LBDs, the crystal structure of AR LBD is monomeric, indicating that the N-terminal domain is crucial for AR homodimerization (Sack *et al*., 2001). The DBD of AR includes 70 amino acid residues and spans exons 2 and 3. The DBD contains two zinc-finger motifs including eight cysteine residues and two Zn^{2+} ions forming a structure that binds the DNA, with the second zinc finger to be determining specificity of DNA

binding (Schoenmakers *et al*., 1999). The N-terminal domain of AR is the least conserved part of the protein and is primarily responsible for transactivation, deletions in this sequence of the AR N-terminal domain significantly reduce AR activity (Simental *et al*., 1991).

2.2.1 Molecular mechanism of AR action

In its inactive form, AR is located in the cytoplasm of the cell bound to chaperone proteins in a large hetero-complex. Many of these proteins have already been identified, including heat shock proteins HSP90/HSP70 or FKBP52 (Veldscholte *et al*., 1992). HSP90 is considered the most important chaperone protein in the AR hetero-complex and binds directly to AR LBD, thereby directly maintaining AR in a conformation able to bind the ligand (Solit *et al*., 2003).

A prototypical endogenous ligand of AR is 5α-dihydrotestosterone (DHT), which is synthesized from testosterone by enzyme 5α-reductase. The first step in the process of AR activation is the binding of the ligand to the AR hetero-complex, and this event leads to dissociation of the complex. The release of heat shock proteins results in unmasking of NLS and dimerization signal, thereby leading to the formation and translocation of the AR homodimer to the nucleus. Once translocated to the nucleus, the AR homodimer recruits a battery of co-activators and co-repressors that co-regulate the expression of AR target genes, such as ARA70 (Yeh *et* Chang, 1996), ARA55 (Fujimoto *et al*., 1999), FHL2 (Müller *et al*., 2000), and SMAD3 (Hayes *et al*., 2001). The AR-AR homodimer binds to short palindromic DNA sequences called hormone response elements (HREs) that are located in the promoter sequences of target genes. Roche *et al*. (1992) identified an AR-specific sequence (androgen response element, ARE) using a DNA-binding site selection assay, with consensus sequence 5′-GG(A/T)ACAnnnTGTTCT-3′. This consensus sequence is analogous to those for GR, MR, ER and PR, resulting in a cross-talk of AR with other members of the steroid hormone receptor family. Cleutjens *et al*. (1996) identified a 35-bp long androgen responsive sequence in the promoter region of human prostate specific antigen (*PSA*) gene, which was located

upstream of ARE and is termed androgen response region (ARR). ARR and ARE in the promoter region of *PSA* gene were found to be stimulated also by synthetic glucocorticoid dexamethasone (DEX).

Transcriptional activity of androgen receptor is also regulated by phosphorylation. Generally, AR is phosphorylated on serine, threonine and tyrosine residues. AR is already phosphorylated in its inactive state, but additional residues are phosphorylated after ligand binding (Kuiper *et* Brinkmann, 1995). Phosphorylation of serine 81 is considered the most important androgen-induced phosphorylation of AR (Gioeli *et al*., 2002). Several studies showed that S81 of AR is phosphorylated by CDK1 (Chen *et al.,* 2006), CDK5 (Hsu *et al*., 2011) and CDK9 (Gordon *et al*., 2010). It was demonstrated that the phosphorylation of S81 is necessary for nuclear localization of AR and regulation of AR transcriptional activity (Chen *et al*., 2012). Other phosphorylation sites influencing AR transcriptional activity are S213, S308, S515, S578, S650 and T850 (Koryakina *et al*., 2014).

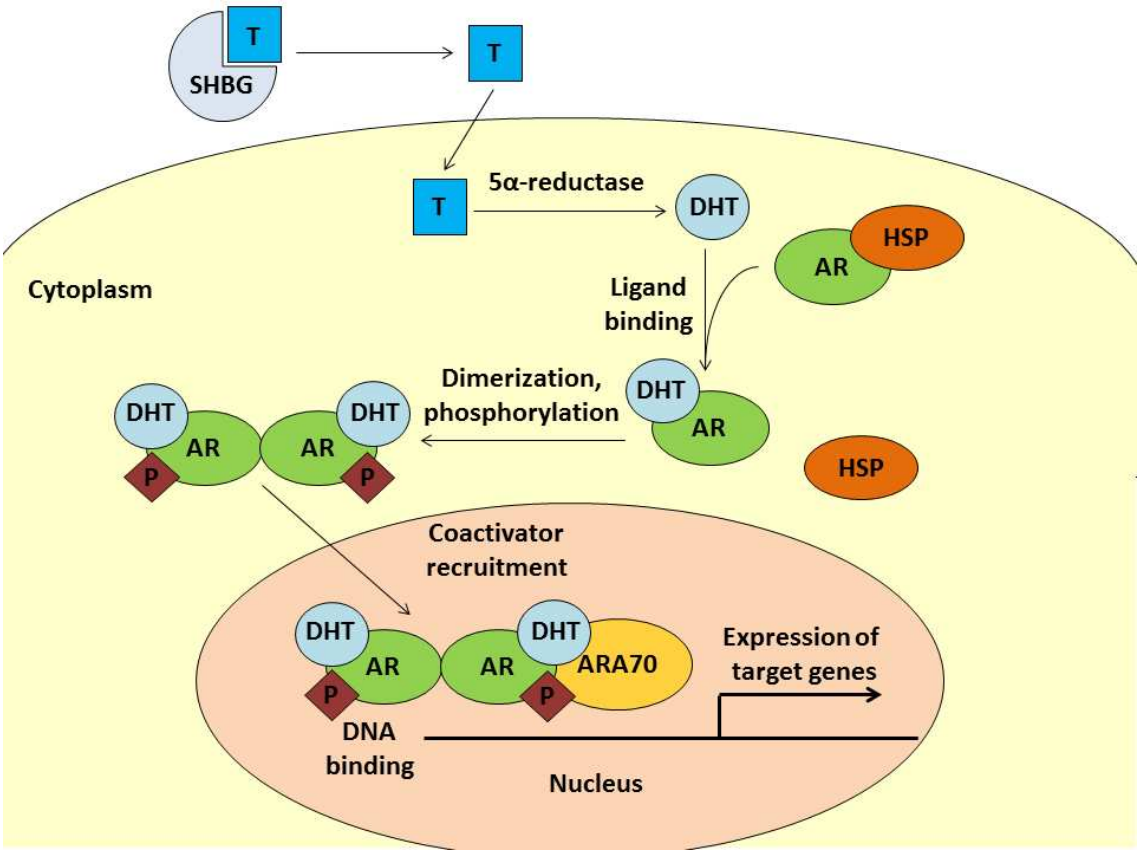

Figure 2: The molecular mechanism of AR action. Adopted from Harris *et al*. (2009). Testosterone (T) circulates in human body bound to carrier proteins, e.g. sex hormone-binding globulin (SHBG). Inside the cell, testosterone is transformed to 5α-dihydrotestosterone (DHT) by enzyme 5α-reductase. Binding of DHT to AR releases heat shock proteins from the heterocomplex and induces dimerization and phosphorylation of AR. Upon dimerization, AR translocates to the nucleus, where it binds coactivator proteins, such as androgen receptor-associated protein (ARA70), and induces the expression of AR target genes.

2.2.2 Androgen receptor and prostate cancer

Prostate cancer is currently one of the most common malignant tumours in men worldwide (Ferlay *et al*., 2015). The role of AR in the development of prostate cancer has been demonstrated. AR has been found to be expressed in both hormone-sensitive and hormone-refractory tumours (Linja *et al.*, 2001; Edwards *et al*., 2003) and was reported to drive proliferation of malignant cells by enhancing the transcription of genes involved in proliferation that are not regulated by AR under benign conditions (Memarzadeh *et al*., 2011). In most diagnosed cases, prostate cancer is androgen-dependent and responds to androgen deprivation therapy (ADT), which is intended for the reduction of

serum levels of androgens or directly for the inhibition of the AR. It has been reported that most patients with prostate cancer show a positive response to ADT. Despite this fact, many patients experience a recurrence or progression of prostate cancer after some period of time. These so-called hormone refractory tumours develop regardless of lower androgen serum levels, but they still respond to the administration of androgens indicating that they are not completely resistant to AR activators.

AR gene amplification has been found in approximately 20-30% of tumours in hormone-refractory prostate cancer (Visakorpi *et al.*, 1995; Koivisto *et al*., 1997). The increased level of functional AR leads to the augmented sensitivity to androgens and therefore to normal AR signalling even after ADT. Besides the increase in AR levels, co-regulators of AR have been found to be overexpressed in hormone-refractory prostate cancer, thereby,leading to enhancement of AR transcriptional activity and increased responsiveness to lower androgen concentrations. AR coactivators ARA70 (Gregory *et al*., 1998), SRC-1 and TIF-2 (Gregory *et al*, 2001) are often found to be overexpressed in hormone-refractory prostate tumours. Stanbrough *et al*. (2006) showed that the increased expression of prostatic enzymes converting adrenal androgens to testosterone is responsible for adaptation of prostate cancer to lower serum levels of androgens.

Over 20 different splicing variants of AR (AR-Vs) showing modified transcriptional activity were identified in prostate cancer specimens or in cell cultures (Cao *et al*., 2016). In most cases, these AR-Vs are deficient in some part of the LBD (with exception of AR45); this situation leads to changes in their transcriptional activity. Some of these proteins does not require ligand binding at all and are constitutively active, e.g. AR-V7 lacking exons 4 to 8 (Guo *et al*., 2009) or AR^{v567es} lacking exons 5 to 7 (Sun *et al*., 2010). Some AR-Vs are active depending on the cell-specific conditions, for instance AR-V1 and AR-V9 (Hu *et al*., 2011). Ahrens-Fath *et al.* found that AR45 is a variant lacking the whole exon 1, and that it inhibits wild-type AR by formation of AR45-AR heterodimers. Some variants contain mutations in their DBD. For instance, AR-V8 does not have a functional DBD; accordingly, it is not considered a transcriptional factor and is located on the plasma membrane (Yang *et al*.,

2011); AR-V3 contains only the first zinc finger motif, lacking the second one (Dehm *et al*., 2008). Splicing variants, especially those with mutated or absent LBD, can diminish the dependence of prostate cancer cells on androgens, thus prostate cancer can reoccur after ADT.

2.3 Vitamin D receptor

Vitamin D receptor (VDR, calcitriol receptor, NR1I1) is a member of the nuclear receptor superfamily of ligand-activated transcriptional factors. Its main physiological function is the control of bone and calcium homeostasis, but it also plays roles in the immune response and cancer development (Haussler *et al*., 1998). Deficiency in vitamin D is associated with increased excretion of parathyroid hormone (PTH), osteoporosis, and augmented risk of bone fractures and with more severe cases of clinical osteomalacia (Armas *et al*., 2004).

The *VDR* gene is located in chromosomal region 12q13.11 (Szpirer *et al*., 1991; Labuda *et al*., 1992). The gene size is approximately 75 kb; and the gene comprises 11 exones, of which the exons 1A, 1B and 1C are non-coding (Miyamoto *et al*., 1997). The protein is encoded by exons 2-9. Unlike in other nuclear receptors, the hinge region is encoded by three exons with an insertion in exon 5. This insertion resides near the centre of the gene and codes for residues 155-194 in the VDR protein. The *VDR* gene has been found to be highly polymorphic in humans. This polymorphism influences bone mineral density (BMD) (Morrison *et al*., 1994) and is associated with hereditary 1α,25-hydroxyvitamin D resistant rickets (HVDRR) (Hughes *et al*.,1988) and risk of osteoporosis (Wu *et al*., 2016), hyperparathyroidism (Carling *et* al., 1997) or some types of cancer (Gandini *et al*., 2014). Functional domains of the VDR protein are similar to those of other nuclear receptors, including the DBD containing two zinc finger motifs, the hinge region, the LBD and the N-terminal domain.

2.3.1 Vitamin D bioactivation and metabolism

There are two forms of vitamin D: vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). These two forms undergo a similar processes of bioactivation and metabolism, and both are capable of activating VDR although vitamin D2 is a less potent activator of VDR than vitamin D3 is (Armas *et al*., 2004). Because of vitamin D2 is of plant origin, whereas vitamin D3 is of human (and animal) origin, this chapter will focus only on vitamin D3 bioactivation and metabolism.

Vitamin D3 (cholecalciferol) can be delivered to the human body via two routes: it may be consumed as part of normal diet or may be formed by UV light in the skin. The exposure to sunlight leads to photolytic conversion followed by isomerization of 7-dehydrocholesterol into cholecalciferol (vitamin D3) *via* thermally labile pre-vitamin D3 (Holick, 1981). The formation of cholecalciferol in the skin is followed by its 25-hydroxylation primarily in the liver by 25-hydroxylase CYP2R1 to 25-hydroxyvitamin D3 (calcifediol; 25(OH)D3) (Cheng *et al*., 2004). The second step of metabolic activation of vitamin D3 involves 1α-hydroxylation of 25(OH)D3 by 25-hydroxyvitamin D 1α-hydroxylase (CYP27B1) resulting in the formation of the most biologically active vitamin D3 form 1α,25-dihydroxyvitamin D3 (calcitriol; 1,25$(OH)_2$D3) (Takeyama *et al*., 1997). This process occurs mainly in kidney, but under certain physiological (pregnancy) or pathological (tuberculosis, rheumatoid arthritis) conditions also in other tissues.

The catabolism of vitamin D3 is catalysed by mitochondrial 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) through 24-hydroxylation of 1α,25-dihydroxyvitamin D3 forming α,24R,25-dihydroxyvitamin D3 or 24-hydroxylation of 25-hydroxyvitamin D3 forming 24R,25-dihydroxyvitamin D3 (secalciferol), respectively (Omdahl *et al*., 2002). The catabolism of vitamin D3 proceeds via lactones to the production of calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D3), a final water-soluble inactive vitamin D metabolite, which is excreted into bile (Reddy *et* Tserng, 1989).

The metabolism of vitamin D3 is a tightly controlled process, primarily leading to a negligible serum concentration of calcitriol, which is a potent modulator of calcium levels. The first step of bioactivation, 25-hydroxylation of cholecalciferol, is poorly regulated, when serum levels of 25-hydroxyvitamin D3 increase

linearly with the vitamin D3 intake. Therefore, serum levels of 25-hydroxyvitamin D3 are used as an indicator of vitamin D status in clinical tests (Holick, 1981). On the contrary, the second step, the 1α-hydroxylation of 25(OH)D3 and catabolism of calcitriol are strictly regulated by multiple mechanisms. Parathyroid hormone (PTH) induces the activity of CYP27B1 and simultaneously represses the activity of CYP24A1 in response to hypocalcaemia (Shigematsu *et al*., 1986*;* Gao *et al*., 2002, Lechner *et al*., 2007). Nevertheless, the main mechanism of regulation of vitamin D metabolism is the direct control of CYP27B1 and CYP24A1 expression by calcitriol. Calcitriol reduces the level of CYP27B1 mRNA (Takeyama *et al*., 1997) but it induces CYP24A1 via two separate vitamin D response elements (VDREs) in the promoter region (Chen *et* DeLuca, 1995). Another important regulator is also fibroblast growth factor 23 (FGF23), which is produced in the bone after stimulation by calcitriol (Liu *et al*., 2006); this event leads to the negative feedback in vitamin D metabolism causing inhibition of CYP27B1 and induction of CYP24A1 (Shimada *et al*., 2004).

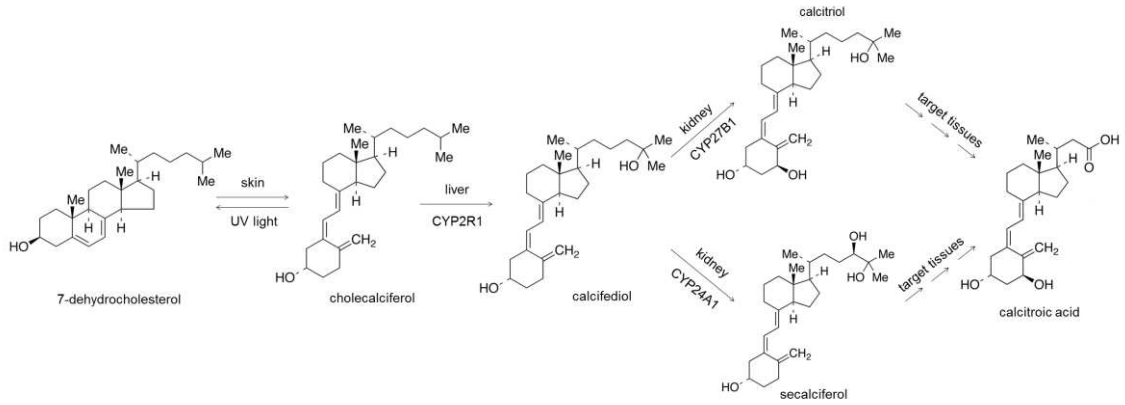

Figure 3: A schematic overview of vitamin D3 metabolism. Vitamin D3 (cholecalciferol) is formed from 7-dehydrocholesterol in the skin by UV irradiation. Its biological activation involves 25-hydroxylation by CYP2R1, which is followed by 1α-hydroxylation by CYP27B1. CYP24A1 mediates the first step of vitamin D3 catabolism, which ends in the formation of water-soluble calcitroic acid.

2.3.2 The mechanism of VDR action

The active metabolite of vitamin D3, calcitriol, circulates in the blood stream bound to vitamin D- binding protein (DBP) or albumin, leaving less than 1% of the free form, which can enter cells and bind to VDR (Bikle *et al*., 1985). Once inside the cell, calcitriol triggers the machinery initiating the expression of VDR target genes.

Unliganded VDR resides partially in the nucleus and partially in the cytoplasm (Walters *et al.*, 1986; Barsony *et al*., 1997). It forms protein-protein complexes with HSP70 and HSP90 (Craig *et al*., 1999). Upon ligand binding, cytoplasmic VDRs rapidly translocates to the nucleus where it forms heterodimers with retinoid X receptor (RXR). The selective binding of VDR to RXR is mediated by three regions of VDR and induces the change of the VDR conformation to its active form. The VDR-RXR heterodimer binds specific DNA sequences designated as vitamin D response elements (VDREs) in promoter sequences of VDR target genes. The consensus VDRE can be described as a direct hexanucleotide repeat of the sequence AGGTCA separated by 3 nucleotides (DR-3 motif). The VDR-RXR heterodimer binds to these elements in a specific orientation that guides VDR to the 3′ half-site proximal to the transcription initiation site (Haussler *et al*., 1997). Besides the VDR-RXR heterodimers, VDR homodimers were also identified and proposed to interact with DR-6 motifs, e.g. in the promoter of the human fibronectin gene (Polly *et al*., 1996). These DR-6 motifs were described as RXR-independent (Carlberg *et al*., 1993) and calcitriol treatment was found to destabilize VDR homodimers (Cheskis *et* Freedman, 1994).

Besides the RXR, VDR recruits other proteins in the process of transcription of target genes. The first group of these proteins include the factors generally participating in the formation of the pre-initiation complex (PIC) for transcription. VDR directly binds TFIIB via a specific interaction with VDR ligand-binding domain (Blanco *et al.*, 1995; MacDonald *et al*., 1995). Interaction of VDR with members of a group of TBP-associated factors has also been described. TAF(II)28, TAF(II)55 and TAF(II)135 have been shown to bind AF-2 and modulate (either potentiate or repress) the activity of VDR in a cell type-specific

manner (May *et al*., 1996; Mengus *et al*., 1997; Lavigne *et al*., 1999). Human VDR interacts with members of SRC/p160 family SRC-1 and GRIP-1/TIF-2 (Hong *et al*., 1997; Masuyama *et al*., 1997). Rachez *et al*. (1998) identified a novel coactivator complex called DRIP (vitamin D receptor-interacting proteins). The DRIP complex is not related to the SRC/p160 family and its interaction with VDR is strictly ligand- and AF-2-dependent.

VDR signalling is regulated also by ligand-independent mechanisms, such as phosphorylation. VDR is phosphorylated on serine residues S51, S182 and S208. Serine 51 is selectively phosphorylated by protein kinase C-β (PKC-β) (Hsieh *et al*., 1991) and mutation analyses revealed that it is necessary for VDR transactivation (Hsieh *et al*., 1993). Phosphorylation of the S182 residue is mediated by cAMP-dependent protein kinase A (PKA) and attenuates heterodimerization of VDR with RXR, and consequently decreases the expression of VDR target genes (Hsieh *et al*., 2004). Casein kinase II (CKII) was found to be responsible for the phosphorylation of S208 of human VDR that is important for interaction of ligand-bound VDR with its coactivators (Jurutka *et al*., 1996, Arriagada *et al*., 2007). Aside from the phosphorylation sites, human VDR has been found to be sumoylated on lysine residue K91 facilitated by protein inhibitor of activated STAT4 (PIAS4), with cell-specific effects on VDR transcriptional activity (Zenata *et* Vrzal, 2017).

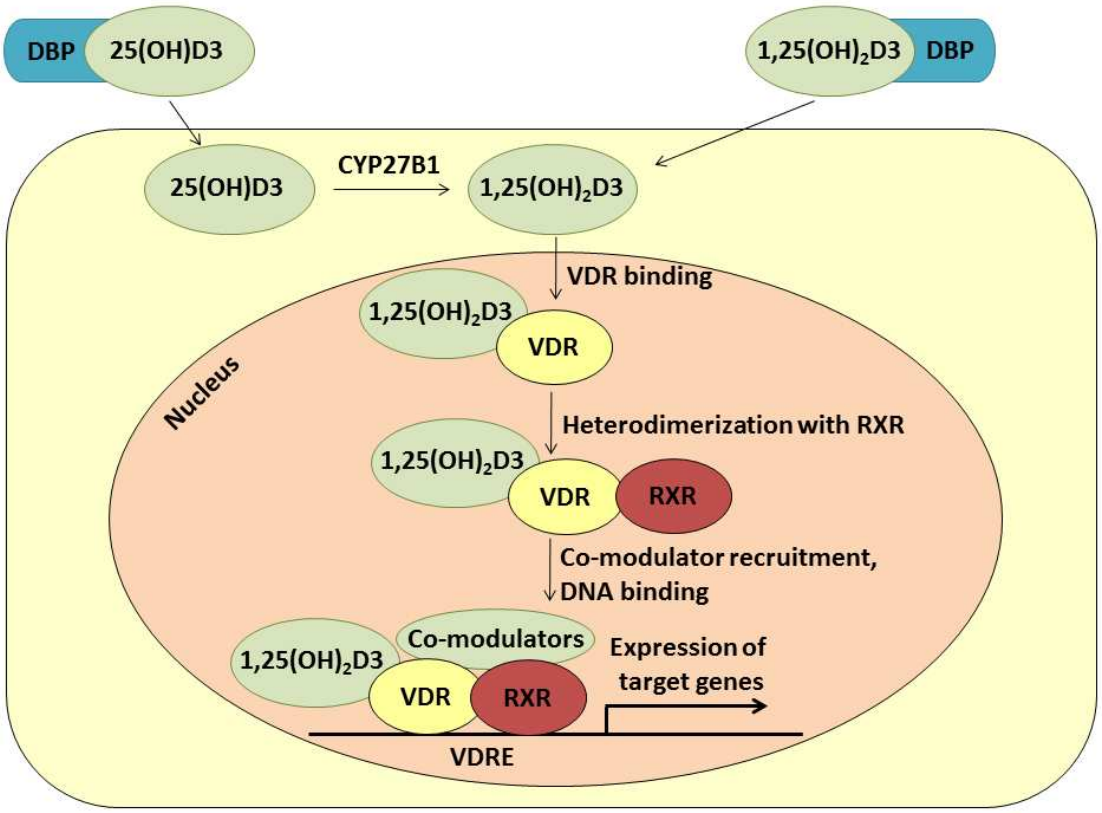

Figure 4: The mechanism of vitamin D receptor action. Adopted from Feldman *et al*. (2014). Calcifediol (25(OH)D3) and calcitriol ($1,25(OH)_2D3$) circulate in the blood stream bound to DBP. Free molecules enter the cell, where calcifediol is converted to calcitriol by CYP27B1. Calcitriol binds VDR, causing formation of VDR-RXR heterodimer. This heterocomplex recruits a battery of co-modulators and binds VDREs in promoters of target genes, thereby triggering their expression.

2.3.3 Vitamin D analogues and disease treatment

Besides the obligate functions of calcitriol in bone, calcium, and phosphate homeostasis, it is well known for its immunomodulatory, pro-differentiation and anti-proliferative effects on healthy and cancer cells. Unfortunately, these off-target effects of calcitriol are achieved at a supra-physiological concentration, which leads to hypercalcaemia, thus hindering the use of calcitriol as a drug. For this reason vitamin D analogues have been introduced in human pharmacotherapy.

Generally, vitamin D analogues are designed to attain desired effects as compared to calcitriol, but without the influence on calcium serum levels.

Several analogues are used to treat hyperproliferative diseases (psoriasis), some types of cancer, secondary hyperparathyroidism or bone disorders (osteoporosis). Officialy approved vitamin D analogues used to treat secondary hyperparathyroidism include paricalcitol (Martin *et al*., 1998) and doxercalciferol (Frazão *et al*., 2000), both vitamin D2 analogues. They mimic the ability of natural vitamin D to suppress the elevated PTH levels characteristic for this condition, but have little or no effect on calcium levels. Tacalcitol (Van de Kerkhof *et al*., 1996), 22-oxacalcitriol (maxacalcitol) (Barker *et al*.,1999) and calcipotriol (Kragballe *et al*., 1989) are used to manage psoriasis, an autoimmune hyperproliferative condition characterized by red itching areas of diseased skin. Alfacalcidol (1α-hydroxyvitamin D3) is used in the treatment of osteoporosis because this drug increases bone density (Shiraishi *et al*., 1999).

Due to their anti-proliferative effects, vitamin D analogues have also been tested in cancer therapy. Because they lack a cytotoxic effect, combined treatment with radiotherapy or chemotherapy was considered for many types of cancer. Seocalcitol (EB 1089) was intended for cancer treatment (Hansen *et al*., 2000). Although tests in mice and rats were promising, clinical trials in human patients revealed little or no improvement of cancer status after administration of any vitamin D analogue tested (Leyssens *et al*., 2014).

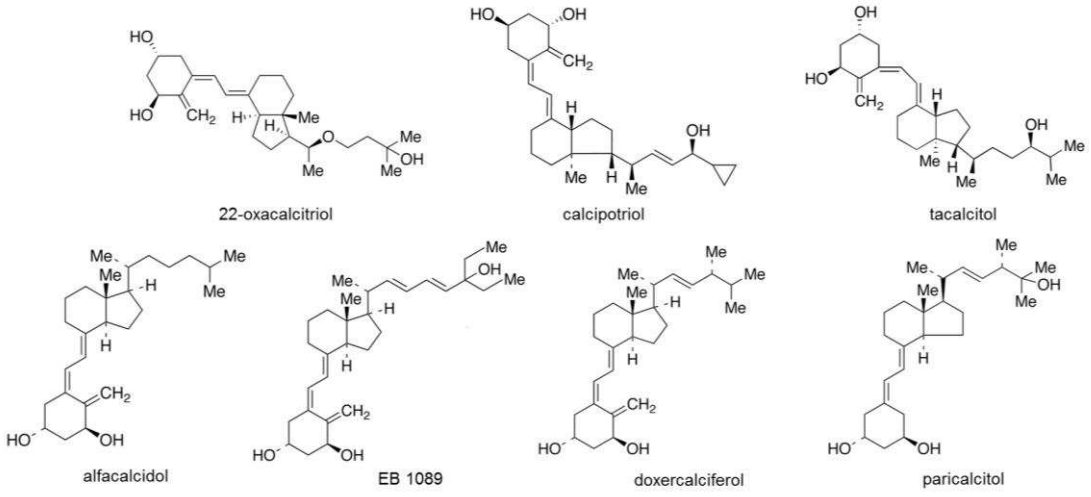

Figure 5: Structures of clinically approved analogues of vitamin D.

2.3.4 Role of VDR in xenobiotic metabolism

VDR has been found to influence the expression of xenobiotic-metabolising enzymes. Phase I enzymes CYP3A4, CYP2B6 and CYP2C9 were found to be regulated not only by pregnane X receptor (PXR) and constitutive androstane receptor (CAR) but also by VDR (Droucourt *et al*., 2002). VDR-binding response elements having ER6, DR3 and DR4 motifs were identified in the promoter regions of genes *CYP3A4*, *CYP2B6* and *CYP2C9*. Lithocholic acid (LCA), a secondary bile acid, was identified as an endogenous VDR ligand that induces the expression of CYP3A4 *in vivo* (Makishima *et al*., 2002). Pavek *et al*. (2010) found that the VDR-RXR heterodimer interacts with the CLEM4-ER6 module in the promoter region of the *CYP3A4* gene, thus inducing its expression in human intestinal cell lines.

On the other hand, the expression of *CYP24A1*, a target gene of human VDR, was found to be induced by PXR (Pascussi *et al*., 2005). *CYP24* mRNA was upregulated by murine PXR agonists dexamethasone and pregnenolone 16α-carbonitrile in mice and by human PXR agonists rifampicin and hyperforin in human hepatocytes, as well as in human embryonic kidney cells transfected with PXR. Vrzal *et al*. (2011) described augmentation of CYP24A1 expression by valproic acid (VPA), an activator of human PXR. Those authors demonstrated potentiated expression of *CYP24A1* mRNA after VPA treatment in human hepatocytes and in HEK293 cell line. VPA also increases basal and calcitriol-induced activity of the *CYP24* promoter in HepG2 cells in gene reporter assays; this result is suggestive of enhancement of VDR transcriptional activity by VPA treatment.

2.4 Endocrine disruption

Endocrine disrupting chemicals (EDCs) are exogenous compounds found in the environment and foods that possess the ability to mimic or disturb the normal hormone function, thus adversely affecting human physiological functions. The endocrine imbalance caused by EDCs is associated with pathophysiological conditions such as obesity, diabetes, increased cholesterol levels, reproductive disorders, and cancers.

Endocrine disruption can be caused by numerous structurally and functionally diverse compounds (Casals-Casas *et* Desvergne, 2011). For instance, pesticides include hundreds of chemicals (e.g. organophosphates, organochlorines, carbamates, and triazines) used worldwide in agriculture, and therefore easily accessible by humans. EDCs are often persistent in the environment and they can cause severe damage to living organisms. The examples are dioxins (e.g. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD), polychlorinated dibenzofurans, and polychlorinated biphenyls (PCBs). Other groups of EDCs include organotins, and plasticizers (e.g. bisphenol A, phthalates) etc.

EDCs can interfere with multiple steps in hormonal signalling, i.e. production of a hormone, its release, metabolism and elimination, or directly by binding to various NRs (Tabb *et* Blumberg, 2006). The most common mechanism of endocrine disruption is a direct interaction of endocrine disruptors with members of the steroid hormone receptor family. EDCs were found to bind ER, AR, TR and GR (Casals-Casas *et* Desvergne, 2011). Some commonly known EDCs were found to modulate the metabolism rate of steroid hormones by changes in the expression of CYP3A4, CYP2B6 and CYP2C9 via PXR and CAR. Bisphenol A and diethylstilbestrol were found to induce human PXR transcriptional activity and the expression of CYP3A4 (Blumberg, *et al*., 1998; Takeshita *et al*., 2001). Rat PXR and CAR were shown to be activated by DDT in the rat liver (Kiyosawa *et al*., 2008). Aryl hydrocarbon receptor (AHR) was identified as a target for polyaromatic hydrocarbons (PAHs), dioxins and PCBs (Harper *et al*., 1991; Murk *et al*., 1996). Induction of AHR transcriptional activity leads to increased expression of the *CYP1A1* gene, which is associated with the onset of various types of cancer (Androutsopoulos *et al*., 2009).

2.5 A gene reporter assay and stable transfection

The technique of the gene reporter assay has been introduced as a reliable *in vitro* tool for screening of transcriptional activation in general. Gene reporter assays are based on the introduction (transfection or transduction) of an expression vector and reporter plasmid into appropriate cells in culture *in vitro*,

although *in vivo* applications are employed as well. The reporter plasmid is designed by combining an appropriate reporter gene under transcriptional control of a promoter sequence derived from the gene of interest. There are several requirements for an optimal reporter gene: it has to be easily and rapidly detectable, sensitive, non-toxic to host cells and not endogenously expressed in target cells. The first reporter gene to be used was chloramphenicol acetyltransferase (*CAT*), an *Escherichia coli* enzyme (Gorman *et al*., 1982). Since 1980 other reporter genes have been introduced, including various luciferases, β-galactosidase, β-glucuronidase, alkaline phosphatase (*AP*), secreted alkaline phosphatase (*SEAP*), and green fluorescent protein (*GFP*) (Schenborn *et* Groskreutz, 1999).

There are several different ways to deliver the reporter vector into target mammalian cells (Kim *et* Eberwine, 2010). Probably the most common chemical method of transfection is the lipofection/lipid-mediated gene delivery. The transfer of the reporter vector is mediated by liposomes made from phospholipid bilayer that can merge with the cell membrane. The process of lipofection is highly efficient and easy to use, but it is not applicable to all cell types. Another technique is based on calcium phosphate which is mixed with cultured cells, and the DNA is taken up by the cells by endocytosis. Non-chemical (physical) techniques include electroporation, magnetic nanoparticles, and direct injection of biolistic particles. In general, these techniques require a more specific material, proper equipment, and experimental skills. The third group of transfection methods is represented by virus-mediated transfection (or transduction), which is highly efficient and easy to use, but has many serious disadvantages that include potential insertional mutagenesis, limited DNA size or immunogenicity and cytotoxicity.

The significant drawback of the reporter assays, in particular when intended for large-scale experimentation, is the need for transfection of a plasmid. This problem can be overcome by means of the stable transfection approach, a process of permanent integration of a reporter vector into the cell. Because all the cells in the culture are stably transfected with a reporter (100% efficacy), this approach has several advantages (Novotna *et al*., 2011):

1. There is no need to transiently transfect the cells for each experiment: therefore, stable transfection is much less time- and material-consuming than transient transfection.
2. There is no need for internal control of transfection efficiency.
3. The assay is much more sensitive in comparison with transient transfection.

The process of stable transfection involves a systematic process of selection under the pressure of a selection antibiotic. The selection marker (gene of drug resistance) can be encoded either in the reporter plasmid or in a separate plasmid that needs to be co-transfected along with the reporter plasmid. The first population of antibiotic-resistant cells obtained by the selection process is called polyclonal population, because it is derived from the original transfected culture containing all the successfully transfected clones. Given that the individual clones in the polyclonal population may differ in cell morphology, functionality and responsiveness, the generation of monoclonal populations (derived from a defined single clone) by limiting dilution should be the next step for the development of a stable cell line.

Recently, CRISPR/Cas9 genome editing was used successfully to generate stably transfected cell lines. The microbial CRISPR/Cas immune system is composed of clustered regularly interspaced short palindromic repeats (CRISPR) loci together with the CRISPR-associated (cas) genes and is responsible for cleavage of foreign genetic elements (Garneau *et al*., 2010). The Cas nucleases are targeted to specific DNA sequences by noncoding RNAs that aim the Cas nuclease to its target through Watson-Crick base pairing. After a simple modification in the sequence of guide RNA, this system can be easily used for gene editing in a huge variety of cell cultures and experimental systems. Lee *et al*. (2015) used this novel approach to stably transfect Chinese hamster ovary (CHO) cells, demonstrating precise insertion of a 3.7-kb gene expression vector at defined loci in CHO cells, followed by a simple selection process that resulted in homogeneous transgene expression throughout the cell culture.

In the past, diverse methods were used to evaluate the effect of exogenous compounds on the transcriptional activity of human AR. *In vivo* experiments were conducted in rats (Ostby *et al.*, 1999) or transient transfections procedures were used (Vinggaard *et al*., 1999, Vinggaard *et al.*, 2008). Aside from these costly and time-consuming approaches, several stably transfected cell lines were employed. The PALM cell line was developed by stable co-transfection of the PC-3 cell line with human AR and a reporter plasmid containing the firefly luciferase gene under the control of the androgen-dependent mouse mammary tumour virus (*MMTV*) promoter (Térouanne *et al*., 2000). Blankvoort *et al*. (2001) published stable reporter cell line AR-LUX derived from human breast cancer cell line T47D that was constructed using a reporter plasmid pBRARE2tataluc^{+} containing two AREs from rat probasin gene (Blankvoort *et al*., 2001). The response of the AR-LUX cell line to model androgen R1881 was maximal five-fold induction. The MDA-kb2 cell line was constructed by a transfection of human breast cancer cell line MDA-MB-453 with reporter vector MMTV.luciferase.neo (Wilson *et al*., 2002). The AR CALUX bioassay was developed by Sonneveld *et al*. (2005) from human osteosarcoma cell line U2-OS by co-transfection of reporter plasmid 3xHRE-TATA-Luc and expression plasmid pSG5-neo-AR. This stably transfected cell line was tested for the responsiveness after DHT treatment, maintenance of inducibility and stability (Sonneveld *et al*., 2005).

Different strategies have been used for evaluation of VDR transcriptional activity, including commercially available systems. To date, they have been mostly based on transient transfection of human cancer cell lines with reporter constructs (Vrzal *et al*., 2011; Salimgareeva *et al*., 2014, Mano *et al*., 2015).

3 OBJECTIVES

The aim of this thesis was to develop stably transfected human reporter cell lines that could be used in toxicological, pharmacological and environmental studies. In particular

1. Construction and characterization of human reporter cell line for the assessment of human androgen receptor (AR) transcriptional activity.

2. Construction and characterization of human reporter cell line for the assessment of vitamin D receptor (VDR) transcriptional activity.

4 EXPERIMENTAL PART

4.1 Biological material

4.1.1 22Rv1 cell line

Human prostate carcinoma epithelial cell line 22Rv1 (ECACC No. 105092802) was purchased from the European Collection of Cell Cultures (ECACC) and cultured in RPMI-1640 medium supplemented with 10% of foetal bovine serum, 100 U/mL streptomycin, 100 µg/mL penicillin, 4 mM L-glutamine and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.1.2 LS180 cell line

Human Caucasian colon adenocarcinoma cell line LS180 (ECACC No. 87021202) was purchased from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% of foetal bovine serum, 100U/mL streptomycin, 100 µg/mL penicillin, 4 mM L-glutamine, 1% non-essential amino acids and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.2 Materials and reagents

Dulbecco′s Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI-1640), dimethyl sulfoxide (DMSO), hygromycin B, thiazolyl blue tetrazolium bromide (MTT), foetal bovine serum (FBS), charcoal stripped foetal bovine serum (CS-FBS), non-essential amino-acids, penicillin, streptomycin, L-glutamine, D-luciferin, coenzyme A, adenosine triphosphate (ATP), testosterone, spironolactone, cortisol, dexamethasone, beclomethasone, betamethasone, corticosterone, aldosterone, prednisolone, methylprednisolone, 17α progesterone, 3,3′,5-triiodo-L-thyronine, progesterone, 17β-estradiol,

diethylstilbestrol and 4-hydroxytamoxifen were purchased from Sigma-Aldrich (Prague, Czech Republic). Danazol, cyproterone acetate, mifepristone, triamcinolone, genistein, raloxifene hydrochloride, tamoxifen citrate salt, all-trans-retinoic acid, 9-cis-retinoic acid, 7-dehydrocholesterol, vitamin D3 (cholecalciferol), EB 1089 (seocalcitol), 1α,25-dihydroxyvitamin D3 (calcitriol), 1α,25-dihydroxyvitamin D2 (ergocalcitriol), ZK 159222 and 22-oxacalcitriol were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calcitroic acid, 25-hydroxyvitamin D3 (calcifediol), 1α-hydroxyvitamin D3 (alfacalcidol), 24R,25-dihydroxyvitamin D3 (secalciferol), tacalcitol monohydrate, ergosterol, vitamin D2 (ergocalciferol), 25-hydroxyvitamin D2 (ergocalcidiol) and 1α-hydroxyvitamin D2 (doxercalciferol) were purchased from Toronto Research Centre Inc. (Toronto, Canada). Fugene HD transfection reagent was purchased from Roche (Basel, Switzerland). Reporter lysis buffer and Nano-Glo Luciferase Assay System were from Promega (Hercules, CA, USA). All other reagents were of the highest quality commercially available.

4.3 Reporter plasmids

4.3.1 p3ARR/ARE-luc2P/minP/hygro

Reporter plasmid p3ARR/ARE-luc2P/minP/hygro was designed as follows: three copies of the ARR sequence followed by a single copy of ARE sequence from the promoter region of the human prostate-specific antigen (*PSA*) gene were inserted into the pGL4.27 [luc2P/minP/hygro] vector (Cat. No. E8451; Promega, Hercules, CA), using Kpn-1 and XhoI restriction enzymes.

3ARR/ARE insert (84 bp)

5′-CAGGGATCAGGGAGTCTCACACAGGGATCAGGGAGTCTCACACAGGGATCAGGGAGTCTCACAATGcAGAACAGCAAGTGCTAGC-3′

ARR: 5′-CAGGGATCAGGGAGTCTCACA-3′

ARE: 5′-TGCAGAACAGCAAGTGCTAGC-3′

4.3.2 CYP24_minP-pNL2.1[Nluc/Hygro]

Reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] was designed as follows: a partial sequence (-326/-46) from the promoter region of the human *CYP24A1* gene containing two VDREs, VDRE-I (-174/-151) and VDRE-II (-194/-174), followed by a minimal promoter sequence (41 bp) as a control of luciferase expression was inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and HindIII restriction enzymes.

CYP24_minP insert (333 bp)

5′-CTCGAGCCTGCGCCGGGGGAGGGCGGGGAGGCGCGTTCGAAGCACAC
CCGGTGAACTCCGGCGTTCGCATGCCTTCCTGGGGGTTATCTCCGGGGT
GGAGTGCTGCCGCCCCCACCCCACCTCCCGCGCCCAGCGAACATAGCCC
CGGTCACCCCAGGCCCGGACGCCCTCGCTCACCTCGCTGACTCCATCCT
CCTTCCACCCCCCCTCCCCTGGGTCCGCGTCCCTCGGAGTCTGGCCAGC
CGGGGGCCACTCCGCCCTCCTCTGCGTGCTCATTGGCCACCCAAGACACT
AGAGGGTATATAATGGAAGCTCGACTTCCAGCTTAAGCTT-3′

VDRE-I: 5′-GACGCCCTCGCTCACCTCG-3′

VDRE-II: 5′-CACACCCGGTGAACT-3′

4.3.3 VDREI3_SV40-pNL2.1[Nluc/Hygro]

Reporter plasmid VDREI3_SV40-pNL2.1[Nluc/Hygro] was designed as follows: three copies of VDRE-I (-174/-151) from the promoter region of the human *CYP24A1* gene followed by the basic SV40 promoter with a deleted 5′-enhancer region as a control of luciferase expression was inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and HindIII restriction enzymes.

VDREI3_SV40 insert (197 bp)

5′-CTCGAGGACGCCCTCGCTCACCTCGGACGCCCTCGCTCACCTCGGACG
CCCTCGCTCACCTCGTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCC
CTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCC
GCCCCATCGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTC
GGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAG
GCTTTTGCAAAAAGCTT-3′

VDRE-I: 5′-GACGCCCTCGCTCACCTCG-3′

4.4 Generation of stably transfected cell lines

Human prostate carcinoma epithelial cell line 22Rv1 was transfected with reporter plasmid p3ARR/ARE-luc2P/minP/hygro (for AIZ-AR cell line). Human colon adenocarcinoma cell line LS180 was transfected with reporter plasmids CYP24_minP-pNL2.1[Nluc/Hygro] (for IZ-CYP24 cell line) or VDREI3_SV40-pNL2.1[Nluc/Hygro] (for IZ-VDRE cell line). All the transfection procedures involved the Fugene HD transfection reagent at a ratio 3:1 (reagent/DNA) and cells were seeded in 60-mm culture dishes at a density of 8×10^5 (22Rv1 cell line) and 10^6 (LS180 cell line), respectively. Following stabilization, the culture medium was replaced by the selection medium containing selection antibiotic hygromycin B at concentration 0.5 mg/mL (22Rv1 cell line) or 0.25 mg/mL (LS180 cell line). The selection medium was changed every 3 or 4 days for the period of 3 weeks until polyclonal populations of stably transfected cells were obtained.

The polyclonal populations with the highest induction of luciferase signal after treatment with model agonists of AR (100 nM 5α-dihydrotestosterone, DHT) and VDR (50 nM 1α,25-dihydroxyvitamin D3, calcitriol) were selected for the production of monoclonal populations. For this purpose, cells were seeded in 100-mm culture dishes at a density of 300–700 (22Rv1 cell line) and 100–1000 (LS180 cell line) cells per culture dish and were cultured for additional 2 or 3 weeks, respectively, until small individual colonies of cells became visible. Thereafter, individual colonies were sub-cloned in 24-well plates and cultured

for additional 3 weeks in the selection medium to obtain monoclonal populations of stably transfected cells. Two clones of the AIZ-AR cell line and one clone of each of cell lines IZ-CYP24 and IZ-VDRE were selected for detailed characterization. The use of GMO in the Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref. 91997/ENV/10).

4.5 The cytotoxicity assay (MTT assay)

Cells were seeded in 96-well plates at a density of 5×10^4 (AIZ-AR cell line) and 2.5×10^4 (IZ-CYP24 and IZ-VDRE cell lines) cells per well in charcoal-stripped culture medium. After 24 h of stabilization, the cells were treated with increasing concentrations of a tested compound (in the range 1 pM to 10 μM), vehicle (DMSO; 0.1% v/v) and Triton X-100 (2% v/v) as a positive control. After 24 h of incubation, the medium was replaced with a medium supplemented with 10% of MTT at a final concentration of 1 mg/mL (AIZ-AR cell line) or 0.3 mg/mL (IZ-CYP24 and IZ-VDRE cell lines) and incubated in 37 °C for additional 2 h or 30 min, respectively. At the end of incubation, purple formazan was dissolved in DMSO. The MTT assay results were measured spectrophotometrically at 540 nm using a Tecan Infinite M2000 plate luminometer.

4.6 The luciferase inhibition assay

Cells were incubated with model agonists for 24 h. Cell lysates containing high catalytic activity of firefly luciferase (AIZ-AR cell line) or Nano luciferase (IZ-CYP24 and IZ-VDRE cell lines) were obtained. The tested compounds at the highest concentration used during the experiments were added to the cell lysates and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.7 The gene reporter assay

Cells were seeded in 96-well plates at a density of 5×10^4 (AIZ-AR cell line) and 2.5×10^4 (IZ-CYP24 and IZ-VDRE cell lines) cells per well in a charcoal-stripped culture medium. Following 24 h of stabilization, the cells were incubated with the tested compounds. After the incubation, the cells were lysed and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.8 Statistical analyses

Student's paired t-test as well as calculations of EC_{50} and IC_{50} values were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA (www.graphpad.com).

5 RESULTS

5.1 Construction and characterization of stably transfected reporter cell line AIZ-AR

5.1.1 Generation of stably transfected AIZ-AR monoclonal populations

Human prostate carcinoma epithelial cell line 22Rv1 was transfected with reporter plasmid p3ARR/ARE-luc2P/minP/hygro by lipofection. Stably transfected monoclonal populations were selected using hygromycin B as described in section 4.4. Hygromycin B-resistant clones (23 clones), displaying the same morphology as the parental 22Rv1 cell line were obtained after the selection process (Figure 6).

Figure 6: Comparison of morphological features of the parental 22Rv1 cell line and AIZ-AR cell line. A: The parental 22Rv1 cell line (passage 22), B: AIZ-AR cell line clone 8 (passage 12), C: AIZ-AR cell line clone 14 (passage 12).

All the selected clones were tested for responsiveness to a model agonist of AR, 5α-dihydrotestosterone (DHT). For this purpose, the cells were seeded in 96-well plates and after stabilization, the cells were incubated with 100 nM DHT or vehicle (DMSO; 0.1% v/v) for 24 h. Four of the 23 clones showed induction of luciferase between 1500 and 4000 relative luciferase units (RLU) and fold induction between 7.6 and 12.1 (Figure 7). Based on the strength of the signal, clones 8 and 14 were selected for further characterization and termed as the AIZ-AR cell line.

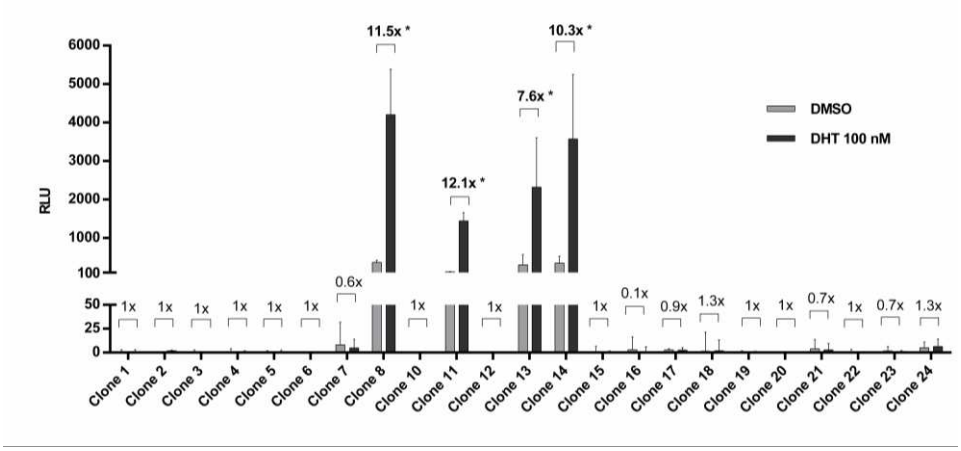

Figure 7: Responsivity of hygromycin B-resistant AIZ-AR clones to 100 nM DHT after 24 h. The cells were seeded in 96-well plates at a density of 5×10^4 cells per well. After stabilization, cells were treated with 100 nM DHT and vehicle (DMSO; 0.1% v/v). After 24 h of incubation, cells were lysed and luciferase activity was measured. Experiments were performed in triplicate measurements and data are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and it is indicated above each clone in the bar graph. Similar data were obtained in three independent experiments (three consecutive passages).

5.1.2 Maintenance of luciferase inducibility after cryopreservation

As the initial step of the characterization process, the AIZ-AR cell line was tested for the maintenance of functionality after cryopreservation (a freeze–thaw cycle). To this end, the cells were frozen in the mixture of FBS and DMSO as a cryo-protectant in the ratio 9:1 and were stored at -80 °C for 1 week. After thawing, both fresh and cryopreserved cells were treated for 24 h with AR agonists: DHT, testosterone and danazol. No significant difference was observed between fresh and cryopreserved cells in terms of fold induction relative to DMSO-treated cells and EC_{50} values (Figure 8). Therefore, AIZ-AR cells can be considered fully functional after cryopreservation.

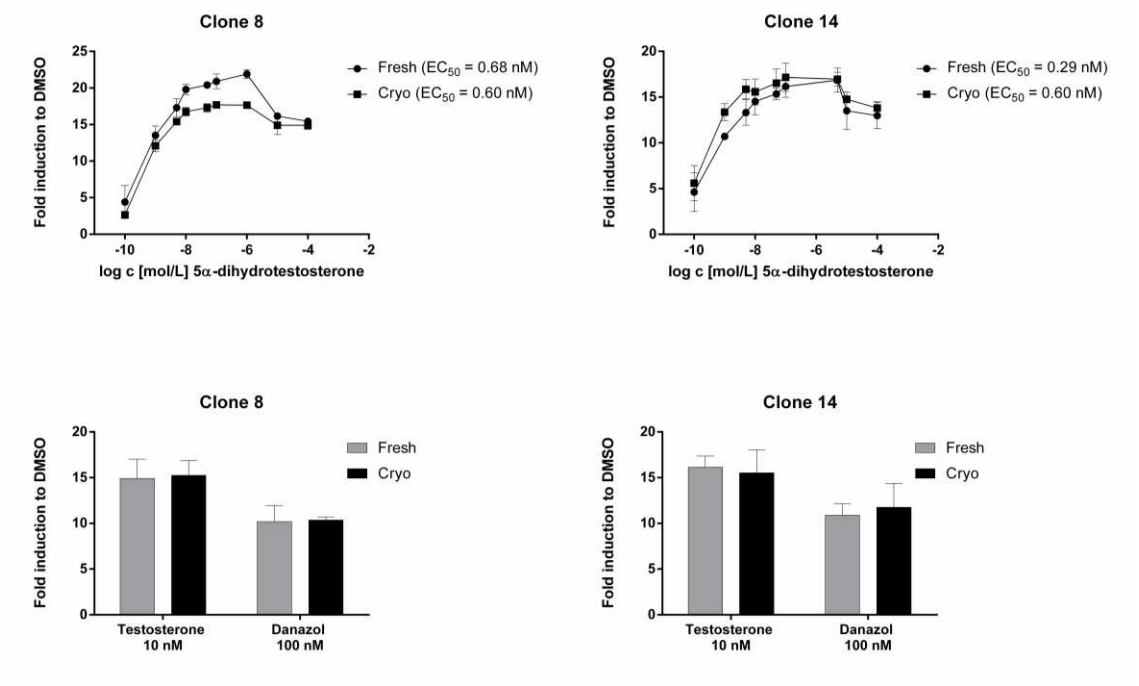

Figure 8: Luciferase inducibility in AIZ-AR cells after cryopreservation. The cells were treated for 24 h with DHT (0.1 nM to 10 µM), testosterone (10 nM) or danazol (100 nM). Data are the mean of triplicate measurements and are expressed as a fold induction over DMSO-treated cells. Similar data were obtained in three independent experiments (three consecutive passages).

5.1.3 Long-term maintenance of luciferase activity in AIZ-AR cells

At the next step, long-term responsiveness of AIZ-AR cells to DHT was examined. For this purpose, the response of AIZ-AR cells to 100 nM DHT (24-h treatment) was assessed after each passage. The results indicate that both clones induction of luciferase activity in the AIZ-AR cell line remained stable for more than 2 months in the cell culture that corresponds to 27-28 passages. Although some variability was observed in between individual passages, no systematic decline of luciferase activity was noticed in terms of both RLU values and fold induction magnitude (Table 1).

Table 1: Long-term maintenance of responsiveness of AIZ-AR cells to 100 nM 5α-dihydrotestosterone.

Days in culture	Clone 8			Clone 14		
	Passage	RLU	FOLD	Passage	RLU	FOLD
8	4	3368.8	10.4	3	2383.6	10.9
10	5	5039.6	12.4	4	4757.6	10.1
15	7	2489.2	11.2	6	2133.0	11.8
22	9	1759.2	11.1	8	n.d.	n.d.
25	10	2787.5	20.5	9	2297.8	20.5
30	12	2517.2	22.4	11	2185.0	20.7
32	13	3396.4	29.2	12	2127.2	23.2
37	15	3976.5	21.1	14	2121.4	23.9
39	16	4506.3	16.9	15	4517.0	16.8
44	18	4269.3	6.5	17	3470.3	5.7
46	19	4799.0	13.8	18	4023.5	14.8
51	21	3918.0	20.6	20	4011.0	17.3
53	22	6534.5	21.9	21	5746.5	20.3
58	24	5244.3	18.6	23	4787.0	14.4
60	25	2241.8	20.5	24	2044.8	16.6
65	27	3225.8	18.7	26	3499.0	20.3
67	28	4043.5	18.5	27	2485.5	15.5

5.1.4 Time-course analyses of the AIZ-AR cell line

To identify optimal and minimal incubation time for reliable identification of AR agonists, the induction of luciferase activity in the AIZ-AR cell line after DHT treatment (in different periods of incubation) was measured. AIZ-AR cells were seeded in 96-well plates and after stabilization, the cells were incubated with DHT (100 nM) or vehicle (DMSO; 0.1% v/v) for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 48, and 72 h.

After long incubation periods (24, 48, and 72 h), a significant and steady increase in luciferase activity in terms of RLUs was observed (due to the accumulation of luciferase), but fold inductions remained constant (Figure 9A) regardless of the time of incubation. Therefore, maximum incubation time of 24 h is optimal because prolonged incubation did not add any additional value. On the other hand, identification of cytotoxic AR ligands can fail after 24 h of incubation due to the loss of viable cells. Therefore, the minimal incubation period allowing for reliable identification of AR ligands was studied as well.

A time-dependent increase of both RLU and fold induction after the treatment with DHT was observed. The plateau in fold induction was reached approximately after 16 h of incubation. Luciferase activity of ~1000 RLU and induction ~6-fold was attained after 8 h of incubation with DHT, suggesting that it is possible to test cytotoxic compounds within 8 h (Figure 9B).

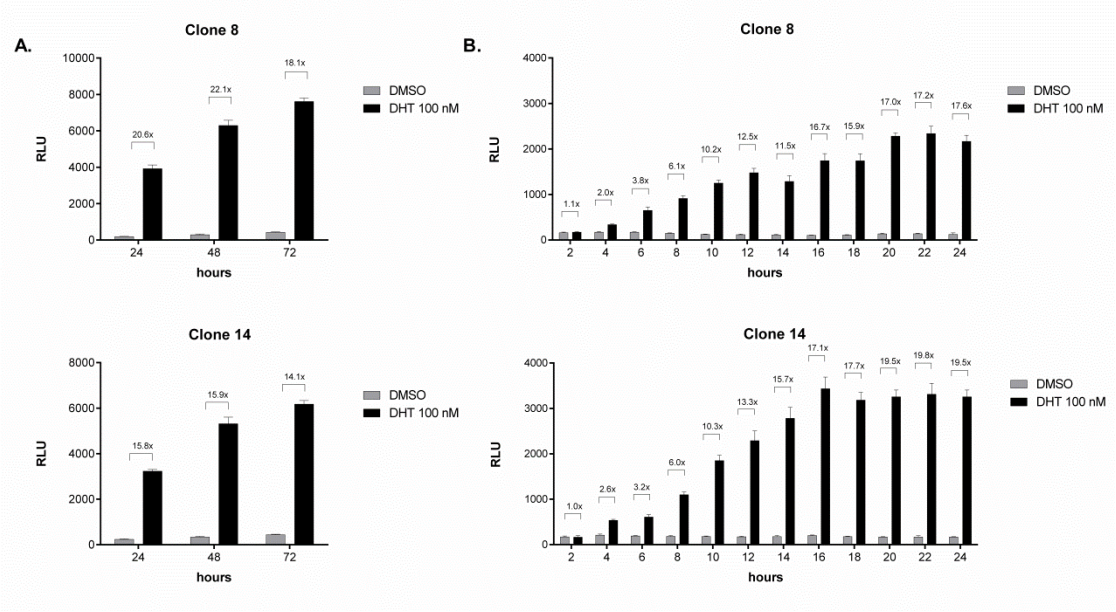

Figure 9: Time-course analyses of AIZ-AR cells after DHT treatment. The cells were treated with DHT (100 nM) or vehicle (DMSO; 0.1% v/v). Data are the mean of triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and is shown above the bars in the graph. Similar data were obtained from two consecutive passages. **Panel A:** Long-term time-course analyses; incubation periods: 24, 48, and 72 h. **Panel B:** Short-term time-course analyses; incubation periods: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h.

5.1.5 Dose-response analyses of AIZ-AR cells after the treatment with a panel of different steroid compounds

The selectivity of AIZ-AR cells towards androgens was tested by dose-response analyses using 23 endogenous and synthetic ligands for steroid receptors. Prior to the dose-response experiments, we examined

1. Cytotoxicity of the tested compounds up to 10 µM using standard MTT assay as described in Chapter 2. Cytotoxicity after 24 h was observed for spironolactone, mifepristone, raloxifene hydrochloride, 4-hydroxytamoxifen and tamoxifen citrate, with IC_{50} values ranging from 1.2 to 5.4 µM (Table 2).
2. Inhibition of a firefly luciferase assay catalytic activity as described in Chapter 2. Out of 23 steroid compounds selected for dose-response analyses, only genistein caused a significant decrease in luciferase activity (Figure 10).

Thus, cytotoxicity as well as inhibition of firefly luciferase catalytic activity should be taken in account while interpreting the data from dose-response analyses.

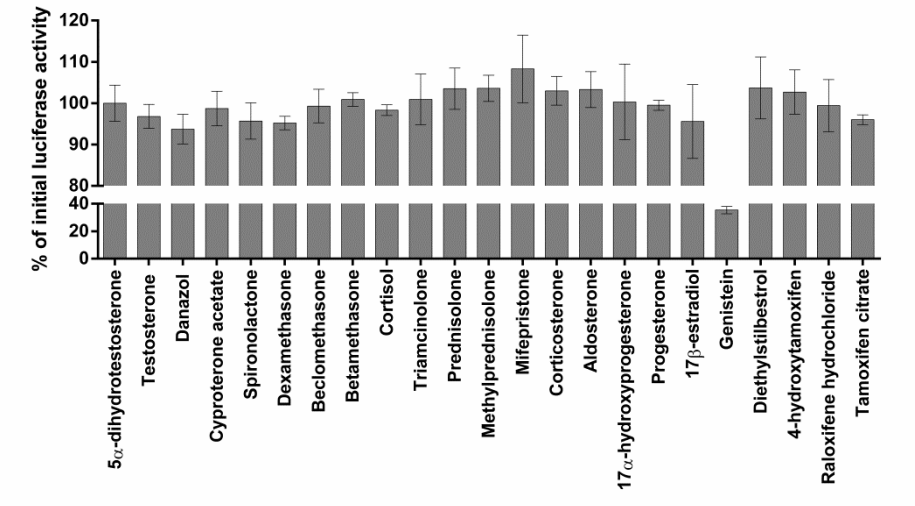

Figure 10: Inhibition of firefly luciferase by the tested steroids. For this purpose, cells were treated with DHT (100 nM). Following the incubation time, cell lysate was collected, incubated with the tested compounds and luciferase activity was measured. Data are the mean of triplicate measurements and they are expressed as a percentage of initial luciferase activity. Similar data were obtained in two independent experiments.

Table 2: Viability of AIZ-AR cells after treatment with endogenous or synthetic steroids. Data were calculated from triplicate measurements and are expressed as mean ± SD. Analyses were performed in two independent experiments.

	log c/ % of viability					
Compound	**-10**	**-9**	**-8**	**-7**	**-6**	**-5**
5α-dihydrotestosterone	100.0	107.5 ± 4.6	108.7 ± 2.1	114.4 ± 5.7	107.7 ± 2.6	104.1 ± 2.0
Testosterone	100.0	108.6 ± 1.0	108.0 ± 4.8	102.9 ± 5.6	105.4 ± 6.9	105.3 ± 1.0
Danazol	100.0	106.5 ± 1.5	108.1 ± 6.9	109.0 ± 10.6	108.2 ± 11.6	113.6 ± 12.1
Cyproterone acetate	100.0	105.3 ± 1.7	104.7 ± 2.0	105.8 ± 5.7	99.4 ± 7.8	89.5 ± 6.6
Spironolactone	100.0	107.7 ± 1.5	111.8 ± 5.7	112.6 ± 10.0	98.9 ± 5.5	58.9 ± 2.5
Dexamethasone	100.0	99.8 ± 3.9	114.0 ± 2.0	112.7 ± 1.1	111.4 ± 3.4	108.3 ± 2.6
Beclomethasone	100.0	99.9 ± 3.9	106.5 ± 6.1	109.2 ± 5.7	98.4 ± 0.9	92.3 ± 2.1
Betamethasone	100.0	104.9 ± 4.0	110.0 ± 7.3	109.1 ± 7.9	108.0 ± 14.6	101.3 ± 9.9
Cortisol	100.0	109.1 ± 4.1	106.4 ± 9.1	107.3 ± 13.2	107.4 ± 12.3	105.4 ± 10.4
Triamcinolone	100.0	101.0 ± 4.2	100.0 ± 8.2	105.2 ± 11.4	103.2 ± 11.0	101.2 ± 5.4
Prednisolone	100.0	100.7 ± 0.8	103.8 ± 3.7	106.9 ± 5.2	104.2 ± 5.2	101.0 ± 8.3
Methyl-prednisolone	100.0	106.8 ± 3.1	111.4 ± 9.4	104.8 ± 8.9	104.6 ± 14.3	104.9 ± 16.0
Mifepristone	100.0	104.0 ± 2.8	102.2 ± 9.7	103.9 ± 18.2	94.7 ± 9.5	41.7 ± 1.1
Corticosterone	100.0	104.0 ± 0.0	107.3 ± 2.5	110.8 ± 5.7	109.2 ± 6.5	105.2 ± 3.4
Aldosterone	100.0	110.4 ± 1.4	99.4 ± 1.0	99.0 ± 1.4	98.6 ± 0.8	97.0 ± 5.4
17α-progesterone	100.0	98.2 ± 2.6	95.0 ± 4.0	95.0 ± 4.0	92.0 ± 3.7	92.0 ± 3.7
Progesterone	100.0	98.7 ± 7.3	98.7 ± 12.2	99.8 ± 14.1	98.1 ± 13.9	91.0 ± 9.5
17β-estradiol	100.0	106.6 ± 4.3	110.5 ± 3.2	110.2 ± 6.2	110.2 ± 6.2	116.4 ± 16.4
Genistein	100.0	96.5 ± 2.4	96.7 ± 3.3	102.9 ± 7.5	102.9 ± 7.5	119.9 ± 6.4
Diethylstilbestrol	100.0	100.7 ± 5.1	102.1 ± 7.6	101.7 ± 5.1	98.0 ± 4.3	99.9 ± 7.0
4-hydroxytamoxifen	100.0	101.8 ± 1.0	100.0 ± 5.4	103.6 ± 4.4	105.9 ± 3.8	9.2 ± 7.7
Raloxifene hydrochloride	100.0	102.9 ± 8.5	99.8 ± 4.3	105.7 ± 6.8	95.9 ± 10.3	7.1 ± 0.2
Tamoxifen citrate	100.0	102.7 ± 4.3	107.0 ± 4.6	105.5 ± 3.8	88.5 ± 2.4	1.7 ± 0.8

Dose-response analyses were performed in two experimental layouts. In the agonist mode, cells were incubated with the tested compounds in the concentration range of 100 pM to 10 μM, and EC_{50} (half maximal effective concentration) values were calculated where appropriate. In the antagonist mode, cells were co-incubated with the tested compounds and a model AR agonist DHT (100 nM) or testosterone (10 nM). IC_{50} (half maximal inhibitory concentration) values were calculated where appropriate.

In the agonist mode, androgens DHT, testosterone and danazol yielded typical dose-dependent sigmoid curves. Antiandrogens spironolactone and cyproterone acetate exerted partial agonist and antagonist patterns, respectively. Progesterone, 17β-estradiol and some glucocorticoids, in particular triamcinolone, corticosterone and cortisol, also induced luciferase activity but with potency 2–3 orders of magnitude weaker in comparison with androgens (Figure 11, Table 3).

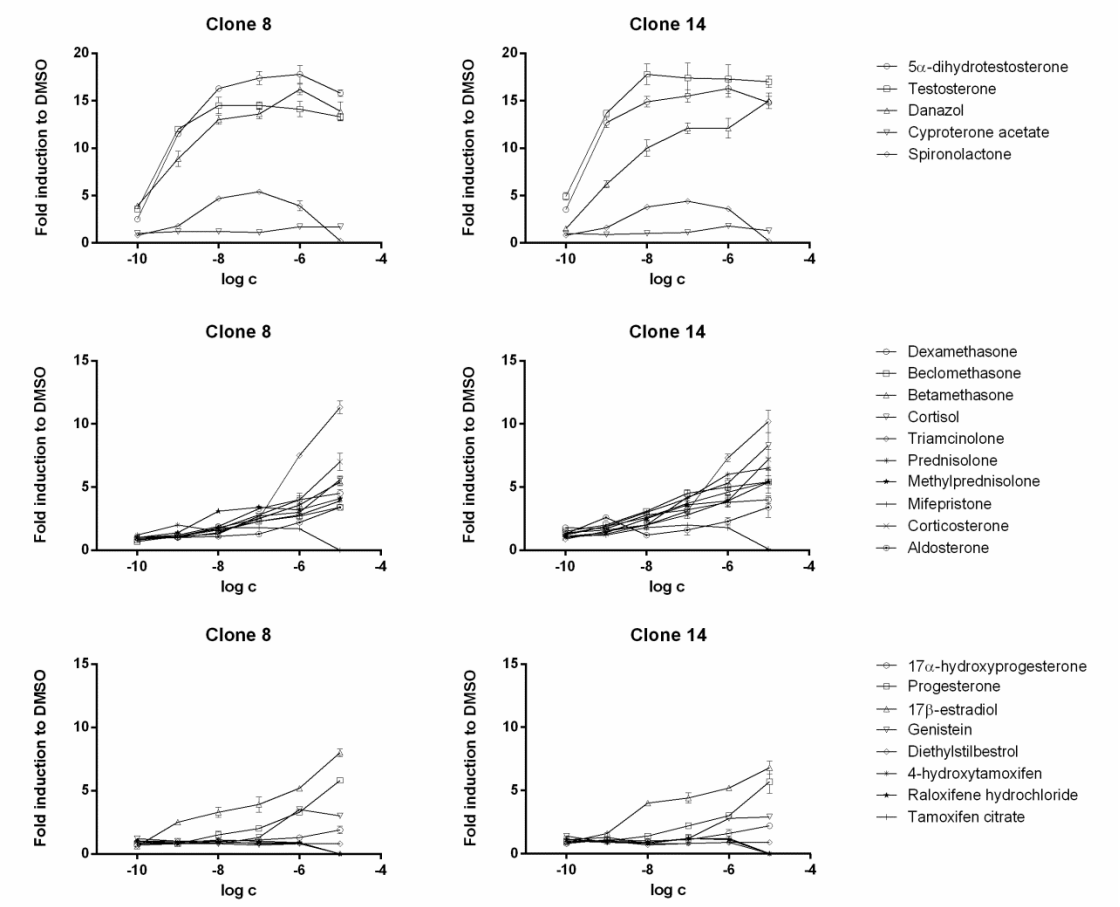

Figure 11: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: agonist mode. The cells were treated for 24 h with endogenous or synthetic steroids. The cells were lysed and luciferase activity was measured. Data are the mean of triplicate measurements and are expressed as a fold induction over DMSO-treated cells. Similar data were obtained from three consecutive cell passages. **Top plots**: androgens and antiandrogens, **middle plots**: corticoids, **bottom plots**: gestagens and oestrogens.

Table 3: Characteristics of AIZ-AR cells in comparison with published data. EC_{50}: half maximal effective concentration; IC_{50}: half maximal inhibitory concentration; n.c.: not calculated; n.d.: not determined. Data were calculated from triplicate measuments and are expressed as mean ± SD. Analyses were performed in three independent experiments for each clone.

	Log EC_{50}		Log IC_{50} (DHT 100nM)		Log IC_{50} (Testosterone 10 nM)		Literature Log EC_{50}	
Compound	**Clone 8**	**Clone 14**	**Clone 8**	**Clone 14**	**Clone 8**	**Clone 14**	**Sedlak *et al*.**	**Wilkinson *et al.***
5α-dihydrotestosterone	-9.16 ± 0.09	-9.19 ± 0.10	-	-	> -5.00	> -5.00	-11.38 ± 0.21	-9.52
Testosterone	-8.92 ± 0.33	-9.10 ± 0.09	> -5.00	> -5.00	-	-	-10.00 ± 0.26	-11.37
Danazol	-8.29 ± 0.34	-8.13 ± 0.12	> -5.00	> -5.00	> -5.00	> -5.00	-9.38 ± 0.17	-9.47
Cyproterone acetate	-6.69 ± 0.17	-6.65 ± 0.51	-6.75 ± 0.05	-6.62 ± 0.02	-7.50 ± 0.04	-7.52 ± 0.06	-8.02 ± 0.19	> -5.00
Spironolactone	-8.80 ± 0.41	-8.36 ± 0.26	-6.37 ± 0.10	-6.17 ± 0.19	-6.72 ± 0.12	-6.65 ± 0.04	-7.52 ± 0.12	> -5.00
Dexamethasone	-7.58 ± 0.31	-7.59 ± 0.27	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
Beclomethasone	-7.59 ± 0.41	-7.70 ± 0.10	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Betamethasone	-6.62 ± 0.04	-7.15 ± 0.26	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Cortisol	-6.35 ± 0.11	-6.49 ± 0.04	n.c.	n.c.	n.c.	n.c.	> -5.00	-8.22
Triamcinolone	-6.37 ± 0.13	-6.56 ± 0.19	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Prednisolone	-6.61 ± 0.09	-7.10 ± 0.38	n.c.	n.c.	n.c.	n.c.	n.d.	n.d.
Methyl-prednisolone	-7.60 ± 0.43	-7.48 ± 0.30	n.c.	n.c.	n.c.	n.c.	n.d.	-8.75
Mifepristone	-5.81 ± 0.05	-5.86 ± 0.06	-6.15 ± 0.10	-6.08 ± 0.03	-6.88 ± 0.20	-6.95 ± 0.21	-7.89 ± 0.13	> -5.00
Corticosterone	-6.22 ± 0.08	-6.25 ± 0.11	n.c.	n.c.	n.c.	n.c.	> -5.00	-8.22
Aldosterone	-6.03 ± 0.04	-6.11 ± 0.02	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
17α-progesterone	-6.45 ± 0.16	-6.29 ± 0.03	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	n.d.
Progesterone	-6.13 ± 0.08	-6.22 ± 0.12	> -5.00	> -5.00	> -5.00	> -5.00	-7.85 ± 0.11	-7.55
17β-estradiol	-7.19 ± 0.20	-7.54 ± 0.01	> -5.00	> -5.00	> -5.00	> -5.00	-7.30 ± 0.25	> -5.00
Genistein	-6.62 ± 0.42	-6.79 ± 0.23	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
Diethylstilbestrol	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
4-hydroxytamoxifen	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
Raloxifene hydrochloride	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
Tamoxifen citrate	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	n.d.

In the antagonist mode, antiandrogens cyproterone acetate and spironolactone caused dose-dependent inhibition of testosterone or DHT-induced luciferase activity. Moreover, the IC_{50} values for antiandrogens were 10-fold lower in DHT-treated cells as compared to testosterone-treated cells (Figures 12 and 13, Table 3). Mifepristone was the only corticoid that caused a significant decrease in the luciferase signal as compared to agonist-treated cells. All the other tested corticoids had an additive effect to at concentrations up to 10^{-6} M, resulting in an increase of luciferase activity in comparison with agonist-treated cells (DHT or testosterone). Furthermore, luciferase activity was significantly augmented to 120-180% at the concentration 10^{-5} M when DHT served as the agonist, but it dropped in the presence of testosterone to 60-120% of initial agonist-activity. Some gestagens and oestrogens yielded an inhibitory effect, but this phenomenon could be attributed to their cytotoxicity rather than any intrinsic antiandrogen effect. Genistein strongly augmented agonist-induced activity (DHT or testosterone), but the massive drop at 10^{-5} M was observed probably because of inhibition of the firefly luciferase (Figures 12 and 13, Table 3).

Therefore, AIZ-AR cells allow for effective detection of compounds with androgenic activity. Some cross-reactivity with glucocorticoids was observed at concentrations of 2-3 orders of magnitude higher as compared to androgens. In the antagonist mode, cytotoxicity, different profiles depending on the agonist used, and luciferase inhibition must be taken in account during data interpretation.

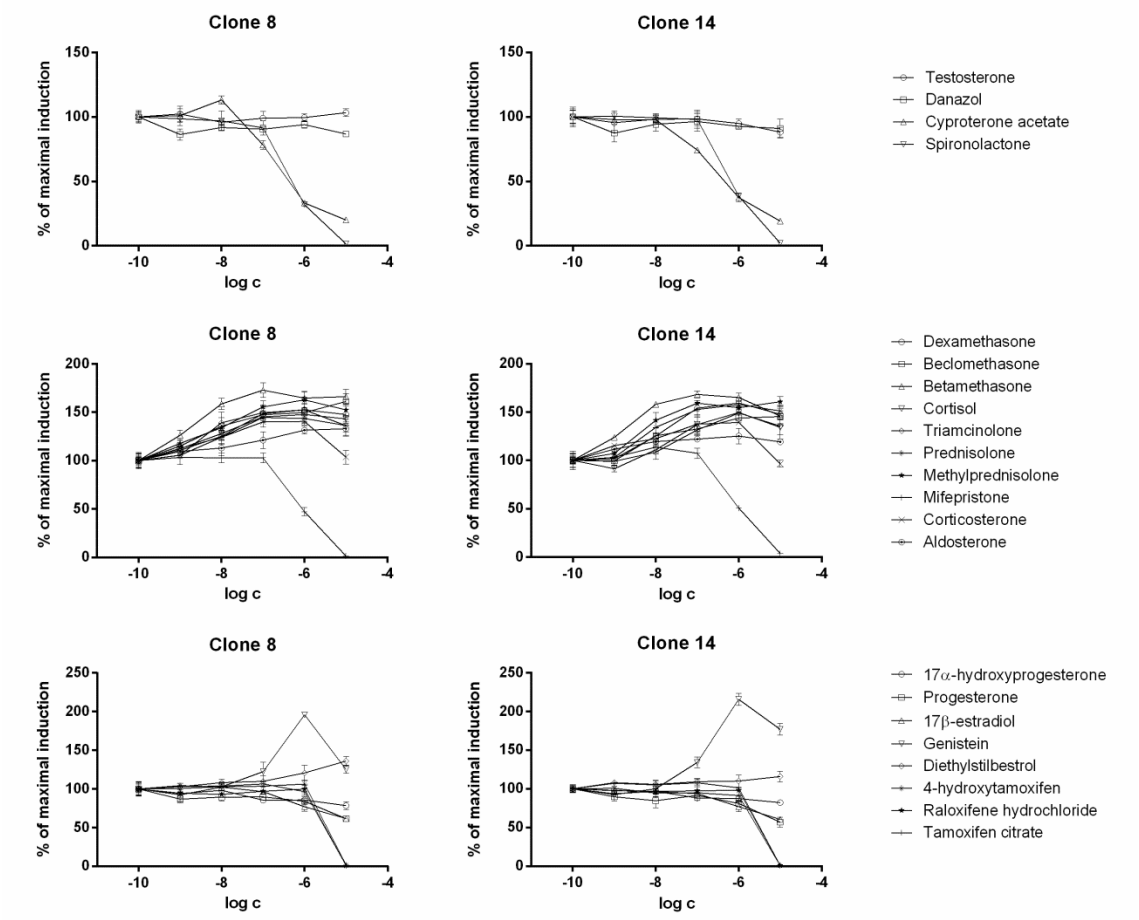

Figure 12: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: antagonist mode with 5α-dihydrotestosterone. The cells were treated for 24 h with endogenous and synthetic steroids in the presence of 5α-dihydrotestosterone (DHT; 100 nM). The cells were lysed and luciferase activity was measured. Data are the mean of triplicate measurements and are expressed as a percentage of maximal induction by DHT. Similar data were obtained from three consecutive cell passages. **Top plots**: androgens, **middle plots**: corticoids, **bottom plots**: gestagens and oestrogens.

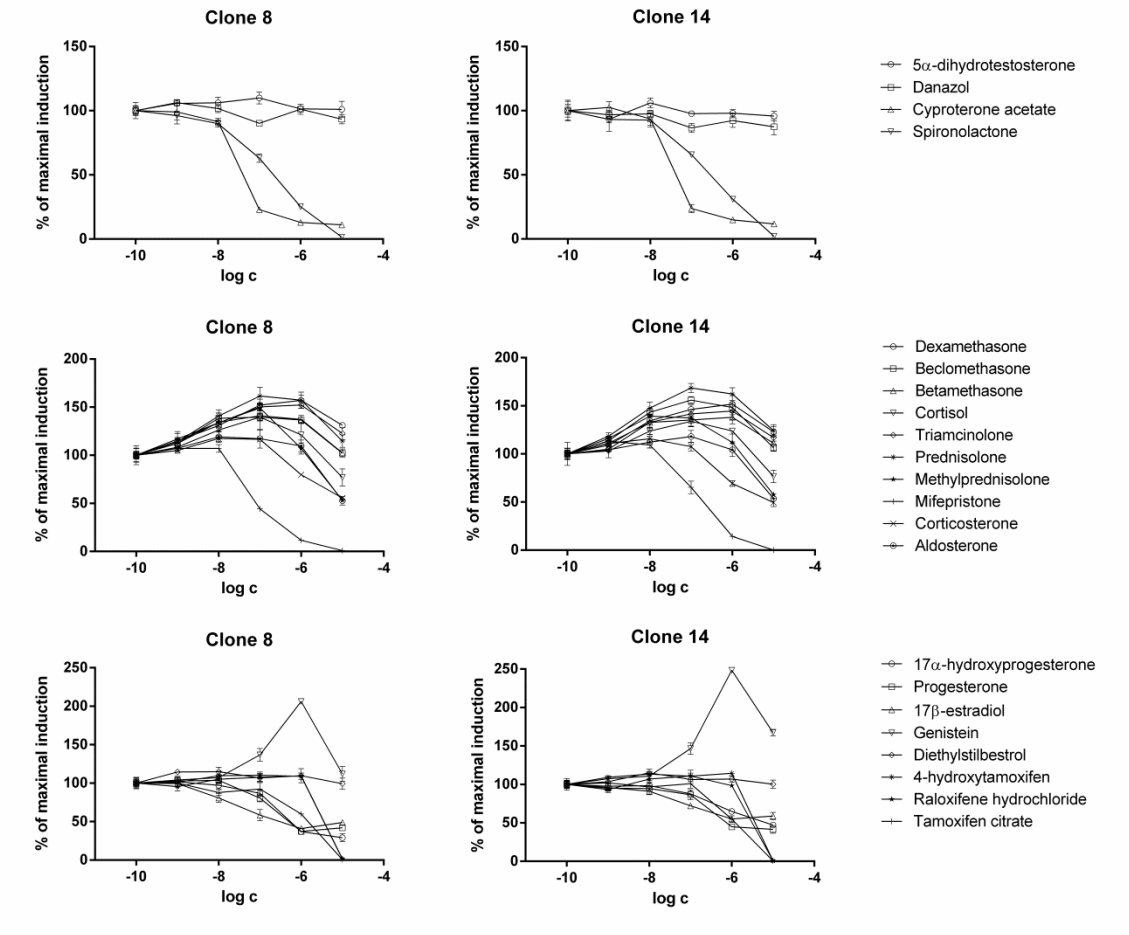

Figure 13: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: antagonist mode with testosterone. The cells were treated for 24 h with endogenous and synthetic steroids in the presence of testosterone (10 nM). The cells were lysed and luciferase activity was measured. Data are the mean of triplicate measurements and are expressed as a percentage of maximal induction by DHT. Similar data were obtained from three consecutive cell passages. **Top plots**: androgens, **middle plots**: corticoids, **bottom plots**: gestagens and oestrogens.

5.2 Construction and characterization of stably transfected reporter cell lines IZ-CYP24 and IZ-VDRE

5.2.1 Generation of stably transfected IZ-CYP24 and IZ-VDRE monoclonal populations

Human Caucasian colon adenocarcinoma cell line LS180 was transfected using lipofection with reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] or VDREI3_SV40-pNL2.1[Nluc/Hygro] to develop stably transfected IZ-CYP24 or IZ-VDRE cell line, respectively. Stably transfected monoclonal populations were selected as described in the Materials and Methods section. 11 and 30 hygromycin B-resistant clones of IZ-CYP24 and IZ-VDRE cells were obtained in the selection process, respectively, that had the same morphology as the parental LS180 cell line (Figure 14).

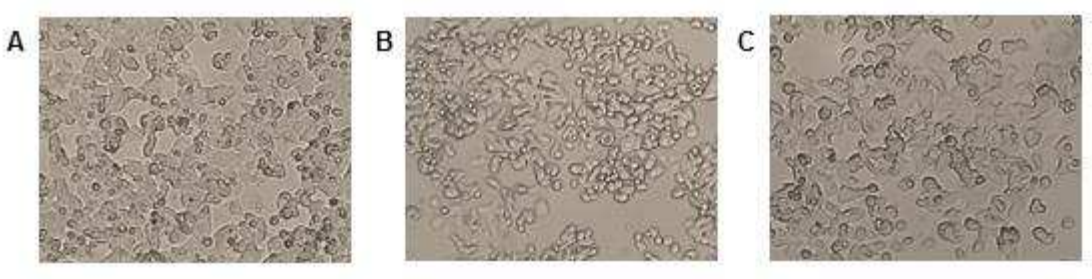

Figure 14: Comparison of morphology of parental LS180, IZ-CYP24 and IZ-VDRE cell lines. A: The parental LS180 cell line (passage 15), B: the IZ-CYP24 cell line (passage 20), C: the IZ-VDRE cell line (passage 12).

All hygromycin B-resistant monoclonal populations were tested for responsiveness to model VDR agonist 1α,25-dihydroxyvitamin D3 (calcitriol). To this end, the cells were seeded in 96-well plates at density 2.5×10^4 cells per well and after 16 h of stabilization, the cells were incubated with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v) for 24 h. Three clones of each cell line inducible by calcitriol were acquired, displaying fold induction over DMSO-treated cells in the range 29- to 73-fold (10^3-10^4 RLU) and 5- to 11-fold (10^6-10^7 RLU) for the IZ-CYP24 and IZ-VDRE cell lines, respectively (Figure 15A).

Based on RLU values and fold induction levels, clones 2 and 4 were selected for detailed characterization of IZ-CYP24 and IZ-VDRE cells, respectively.

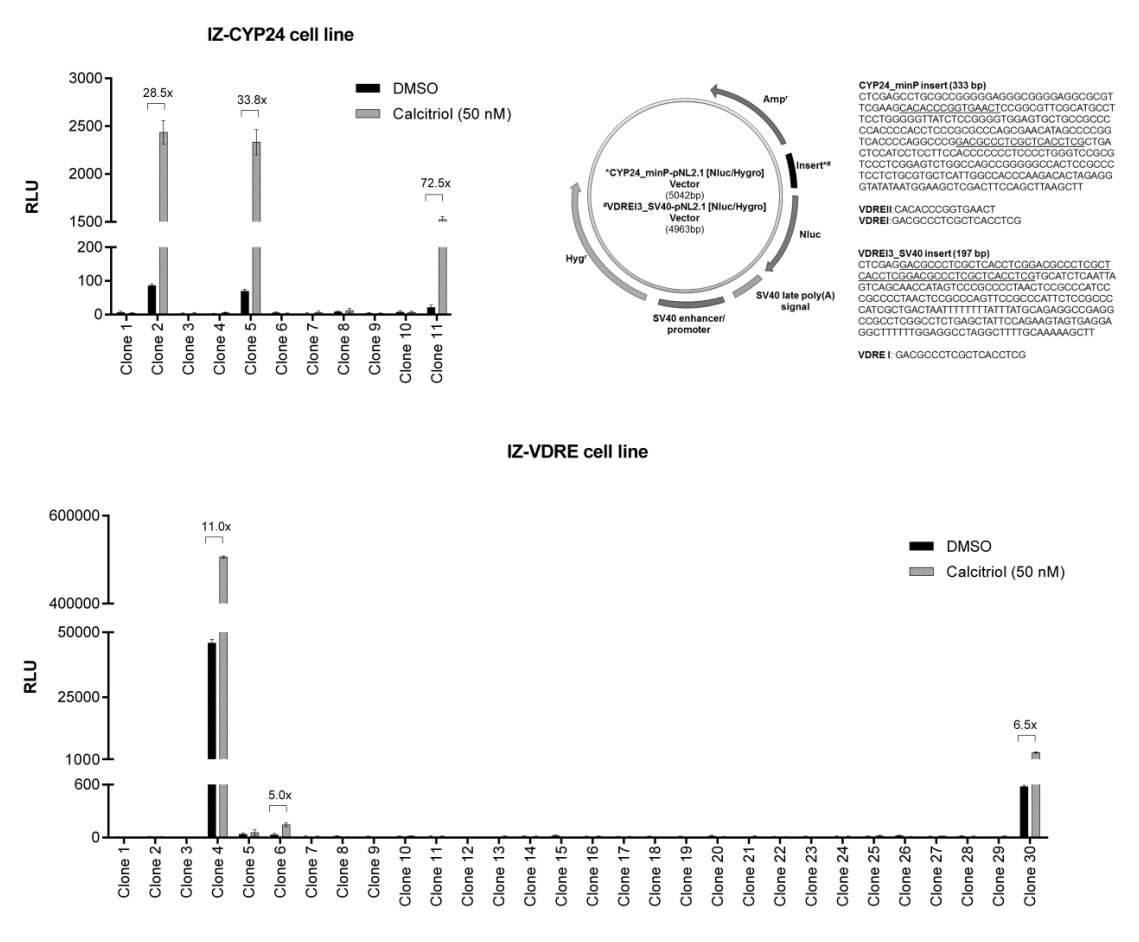

Figure 15A: Monoclonal populations of IZ-CYP24 and IZ-VDRE cell lines. Cells were treated for 24 h with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v). Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and is indicated above calcitriol-inducible clones in the bar graphs. Similar data were obtained from three consecutive cell passages. **Top left panel**: the IZ-CYP24 cell line, **Top right panel**: the scheme of reporter plasmids used for stable transfection, **bottom panel**: the IZ-VDRE cell line.

We compared the responsivness to calcitriol between transiently and stably transfected LS180 cells. We found that the stable transfection has either no effect (IZ-VDRE cell line) or improves (IZ-CYP24 cell line) the responsivness of cells to calcitriol. Sensitivity (RLU units) of IZ-VDRE cells was similar to that of LS180 cells transiently transfected with the VDREI3_SV40-pNL2.1[Nluc/Hygro] plasmid. On the contrary, sensitivity of IZ-CYP24 cells decreased as compared

to transiently transfected cells transiently transfected with the CYP24_minP-pNL2.1[Nluc/Hygro] plasmid (Figure 15B).

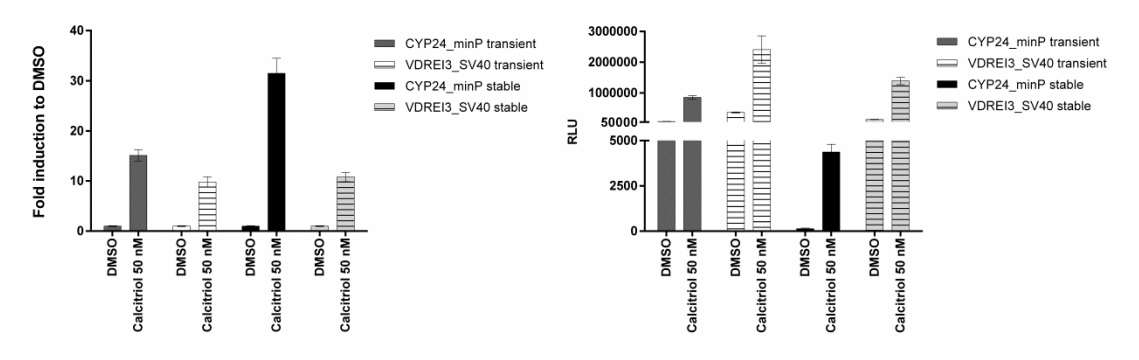

Figure 15B: Comparison of transient and stable transfection of LS180 cells. Cells were treated for 24 h with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v). Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as relative luciferase units (RLU) and fold induction over DMSO-treated cells. Similar data were obtained from three independent experiments.

5.2.2 Functionality of IZ-CYP24 and IZ-VDRE cells after cryopreservation

The maintenance of luciferase induction by VDR agonists in IZ-CYP24 and IZ-VDRE cell lines after cryopreservation (freeze–thaw cycle) was tested. The cells were frozen in the mixture of FBS and DMSO as a cryo-protectant in the ratio 9:1 and stored at -80 °C for 2 weeks. Both thawed and freshly passaged IZ-CYP24 and IZ-VDRE cells were seeded in 96-well plates at a density of 2.5×10^4 cells per well, stabilized for 16 h, and then treated with calcitriol or cholecalciferol in the concentration range of 0.1 nM to 10 µM. Although a slight drop of fold induction was observed after cryopreservation as compared to fresh cells, potency (EC_{50} values) was not altered (Figure 16). Therefore, both IZ-CYP24 and IZ-VDRE cell lines seem to remain fully functional after cryopreservation.

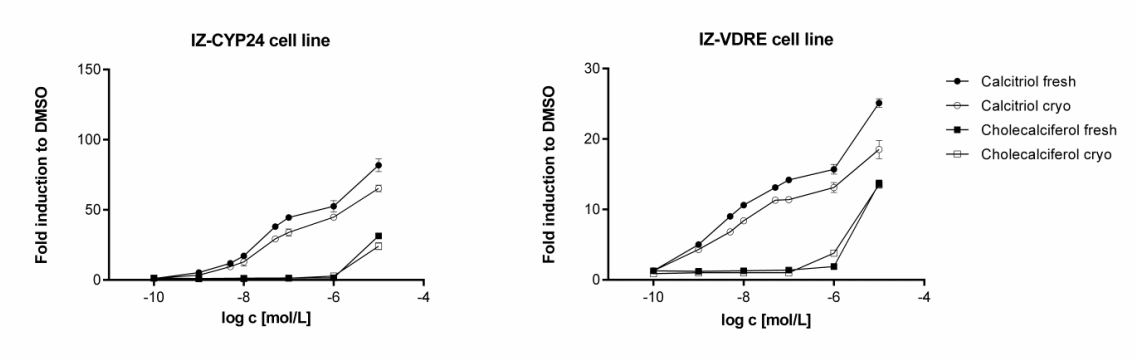

Figure 16: Inducibility of IZ-CYP24 and IZ-VDRE cells after cryopreservation. Both fresh cells and cells after a freeze–thaw cycle were treated with calcitriol or cholecalciferol (from 0.1 nM to 10 µM) or vehicle (DMSO; 0.1% v/v) for 24 h. The cells were lysed and luciferase activity was measured. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

5.2.3 Long-term maintenance of luciferase inducibility in IZ-CYP24 and IZ-VDRE cell lines

During the characterization process, IZ-CYP24 and IZ-VDRE cells were tested for long-term maintenance of responsivness to calcitriol. For this purpose, the cells were seeded in 96-well plates after each passage and incubated with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v) for 24 h. Both cell lines remained functional for more than 78 days, which corresponds to 32 passages (Table 4).

Table 4: Long-term maintenance of the responsiveness of IZ-CYP24 and IZ-VDRE cell lines to calcitriol.

Days in culture	IZ-CYP24			IZ-VDRE		
	Passage	RLU	FOLD	Passage	RLU	FOLD
7	4	6984	31.0	3	505902	11.0
20	8	1930	21.7	8	217855	8.9
22	9	2438	28.5	9	458518	10.1
27	11	2977	33.4	11	440674	13.1
29	12	5350	33.0	12	925705	13.4
34	14	1687	32.6	14	513340	9.5
36	15	2820	38.0	15	875467	11.4
41	17	2300	42.8	17	554728	7.4
48	20	1563	34.1	19	747650	7.2
50	21	3204	36.7	20	787154	10.6
55	23	1897	29.4	22	499042	8.9
57	24	1620	33.4	23	1307103	8.0
63	26	1452	22.2	25	652532	10.8
69	28	966	30.8	26	544402	10.9
71	29	1078	30.2	28	386239	9.0
76	31	552	27.6	29	771601	10.2
78	32	830	26.5	31	349611	11.3

5.2.4 Time-course analyses of luciferase induction in IZ-CYP24 and IZ-VDRE cell lines

The luciferase induction by calcitriol after long and short periods was analysed, to determine minimal and optimal incubation period for reliable identification of VDR ligands. To this end, IZ-CYP24 and IZ-VDRE cells were seeded in 96-well plates at a density 2.5×10^4 cells per well, stabilized for 16 h, and treated with calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v) for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 48, and 72 h.

After long incubation periods (24, 48, and 72 h), a progressive increase of the luciferase signal was observed, with RLU values ranging approximately from 4×10^3 to 1.4×10^4 RLU and from 1.3×10^6 to 7.1×10^6 RLU in IZ-CYP24 and IZ-VDRE cells, respectively. Similarly, fold induction levels over DMSO-treated cells also increased with time of incubation, ranging from 32- to 58-fold and from 11- to 27-fold in IZ-CYP24 and IZ-VDRE cells, respectively (Figure 17A). In both cell lines, RLUs as well as fold induction were high enough after 24 h to allow for reliable luciferase assays. Therefore, the incubation period of 24 h was

assumed to be the optimal time of incubation and was used in all subsequent experiments.

For testing of cytotoxic compounds, it is important to determine the minimal incubation time needed for reliable detection of VDR activation. Therefore, short-term analyses were also performed, and luciferase activity was measured every 2 h in a time range of 2 h to 24 h. In both cell lines incubated with calcitriol, there was a time-dependent rise of the luciferase signal in terms of RLU as well as fold induction. Differential induction of luciferase was observed in the either cell line incubated for 24 h with calcitriol. In IZ-VDRE cells, there was very strong signal (~10^6 RLU) but moderate magnitude of induction (11-fold). In contrast, moderate signal (~10^3 RLU) and high magnitude of induction (32-fold) was achieved in IZ-CYP24 cells. The minimal incubation times were estimated to be 6–8 and 8–10 h for IZ-CYP24 and IZ-VDRE cells, respectively. Overall, incubation for 8 h can be considered as minimal (for both cell lines), to reliably identify VDR activators (Figure 17B).

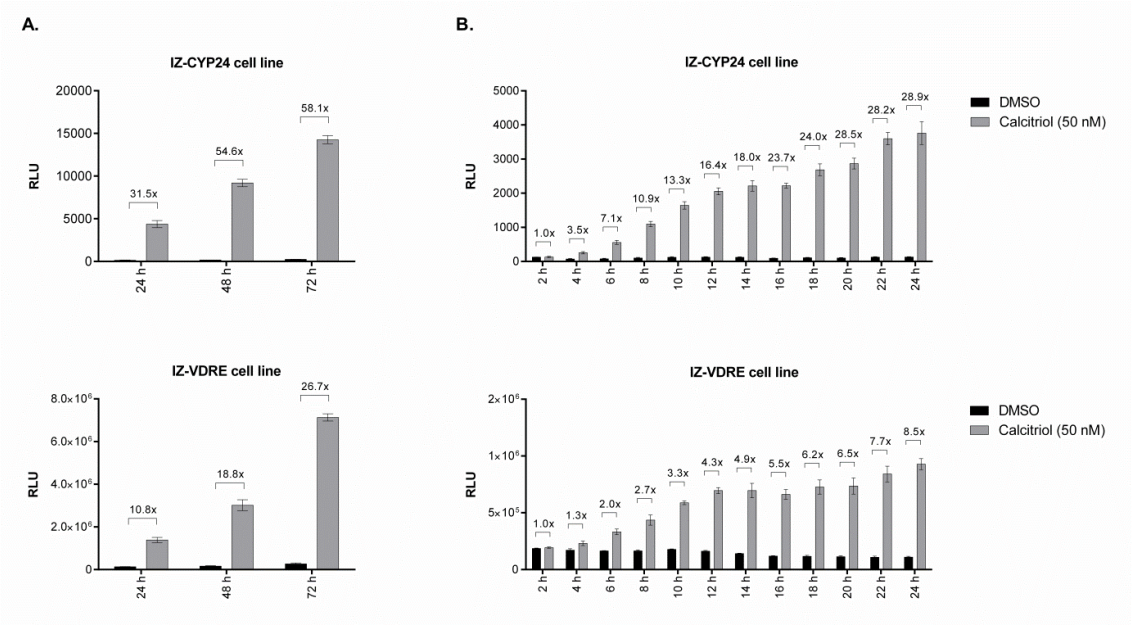

Figure 17: Time-course analyses of luciferase induction in the IZ-CYP24 and IZ-VDRE cell lines. The cells were incubated with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v). After the incubation, luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and is shown above the bars in the graph. Similar data were obtained from two consecutive cell passages. **Panel A:** Long-term analyses; incubation periods 24, 48, and 72 h. **Panel B:** Short-term analyses; incubation times 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h.

5.2.5 Assay performance indicators

These indicators were calculated according to Iversen et al. (2006). Minimal and maximum signal (in RLU) were obtained from cells incubated with calcitriol (50 nM) or vehicle (DMSO; 0.1% v/v) for 24 h. For each cell line, 10 individual experiments, comprising 8 or 12 samples, assay variability ratio (AVR), signal window (SW) and Z′ factor were calculated (Table 5). The values of SW in IZ-CYP24 and IZ-VDRE cell lines ranged from 4.9 to 14.6 and from 4.8 to 29.4, with averages 8.2 and 14.3, respectively. In both cases, SW was considerably higher than the recommended value (SW > 2). The values of Z′ factor in IZ-CYP24 and IZ-VDRE cells ranged from 0.60 to 0.81 and from 0.60 to 0.90, with an average of 0.70 and 0.75, respectively. Because assays with Z′ factor values exceeding 0.5 are considered “excellent”, both cell lines easily satisfied this

criterion. Given that AVR is reliant on the Z′ factor, both cell lines manifested the AVR values lower than the recommended value (AVR < 0.6).

Table 5: Control means and standard deviations for IZ-CYP24 and IZ-VDRE cell lines. Values of assay variability ratio (AVR), signal window (SW) and Z′ factor were calculated according to Iversen *et al*. (2006). In the table, *n* represents number of samples; calculations were performed on data from 10 independent experiments.

IZ-CYP24 cell line

	Maximum signal		Minimum signal				
n	**Mean**	**SD**	**Mean**	**SD**	**AVR**	**SW**	**Z′ factor**
8	4195.5	388.4	144.7	29.3	**0.31**	**7.20**	**0.69**
8	4209.5	491.1	210.3	40.4	**0.40**	**4.90**	**0.60**
8	2394.4	202.3	177.3	32.2	**0.32**	**7.48**	**0.68**
8	3835.1	336.4	146.9	24.2	**0.29**	**7.75**	**0.71**
8	3210.9	257.8	81.9	9.3	**0.26**	**9.03**	**0.74**
8	1894.5	101.6	68.3	11.8	**0.19**	**14.63**	**0.81**
8	1352.7	155.9	68.8	16.7	**0.40**	**4.91**	**0.60**
12	16784.4	1383.0	493.9	32.1	**0.26**	**8.71**	**0.74**
12	10397.2	637.9	297.5	57.3	**0.21**	**12.56**	**0.79**
12	14583.6	1704.6	367.7	83.2	**0.38**	**5.19**	**0.62**

IZ-VDRE cell line

	Maximum signal		Minimum signal				
n	**Mean**	**SD**	**Mean**	**SD**	**AVR**	**SW**	**Z′ factor**
8	2188403.4	161344.4	306926.1	13926.0	**0.28**	**8.40**	**0.72**
8	1511659.3	65439.6	212762.6	8612.4	**0.17**	**16.45**	**0.83**
8	901158.6	73182.4	118792.0	11637.2	**0.33**	**7.21**	**0.67**
8	946285.6	28440.9	69581.2	2103.1	**0.10**	**27.60**	**0.90**
8	504835.8	13683.8	54551.7	2205.5	**0.11**	**29.42**	**0.89**
8	521925.5	38139.3	76799.6	3366.2	**0.28**	**8.41**	**0.72**
12	2407244.0	231765.3	229513.8	21037.0	**0.35**	**6.12**	**0.65**
12	2263459.0	62808.0	220615.8	14784.5	**0.11**	**28.82**	**0.89**
12	1454875.0	134865.5	176279.1	26582.6	**0.38**	**5.89**	**0.62**
12	1369790.0	153760.6	125924.0	12592.8	**0.40**	**4.84**	**0.60**

Acceptance criteria (Iversen *et al*., 2006)

SW

Recommended: SW > 2

Acceptable: SW > 1

Unacceptable: SW < 1

Z′ factor

Excellent: Z′ > 0.5

Do-able: 0 < Z′ < 0.5

Yes/No Assay: Z′ = 0

Unacceptable: Z′ < 0

AVR

Recommended: AVR < 0.6

Unacceptable: AVR > 0.6

5.2.6 Selectivity of IZ-CYP24 and IZ-VDRE cell lines for VDR activators

The selectivity of IZ-CYP24 and IZ-VDRE cells was tested by incubation with model ligands of glucocorticoid receptor (GR; dexamethasone), androgen receptor (AR; 5α-dihydrotestosterone), mineralocorticoid receptor (MR; aldosterone), progesterone receptor (PR; progesterone), oestrogen receptor (ER; 17β-estradiol), retinoid X receptor (RXR; 9-*cis* retinoic acid), retinoic acid receptor (RAR; all-*trans* retinoic acid) and thyroid hormone receptor (TR; 3,3′,5-triiodo-L-thyronine). Prior to dose-response analyses, an MTT assay and luciferase inhibition assay were performed as described in the Materials and methods section. In the tested concentration range, neither cytotoxicity (Figure 19; upper plots) nor inhibition of Nano luciferase (Figure 18) was observed for any compound.

Dose-response analyses were carried out in two experimental layouts. In the agonist mode, cells were treated with increasing concentrations of tested compounds in ranging from 0.1 nM to 10 μM. In the antagonist mode, a combination of calcitriol (50 nM) and increasing concentrations of tested compounds were applied.

In the agonist mode, no induction of luciferase activity in either cell line was observed after the incubation with dexamethasone, aldosterone, progesterone, 17β-estradiol, 5α-dihydrotestosterone, 9-*cis* retinoic acid or all-*trans* retinoic acid. 3,3′,5-triiodo-L-thyronine showed dose-response effects on luciferase activity, with induction levels of approximately 5-fold in both cell lines, which corresponds to approximately 15% and 50% of those caused by 50 nM calcitriol in IZ-CYP24 and IZ-VDRE cells, respectively. Such behaviour indicates binding of TR to a sequence different from VDREs in reporter plasmids used for stable transfection (Figure 19; middle plots).

In antagonist mode, aldosterone, 17β-estradiol, 9-*cis* retinoic acid and all-*trans* retinoic acid did not significantly influence calcitriol-mediated luciferase induction in either cell line. Dexamethasone, 5α-dihydrotestosterone and progesterone slightly augmented calcitriol-mediated luciferase induction in IZ-CYP24 cells, but the potentiation occurred only at supra-physiological concentrations of hormones (10^{-5}–10^{-6} M). On the contrary, these effects were

not observed in IZ-VDRE cells, with the exception of a slight increase by 10 μM dexamethasone. 3,3′,5-triiodo-L-thyronine yielded a dose-dependent additive effect at concentrations from 10^{-9} to 10^{-5} M (Figure 19; lower panels) in both cell lines. Thus, both cell lines allow for detection of VDR activators, with relatively high selectivity. Some cross-reactivity with thyroid hormone was observed in both lines; therefore, it must be taken in account while interpreting the experimental data.

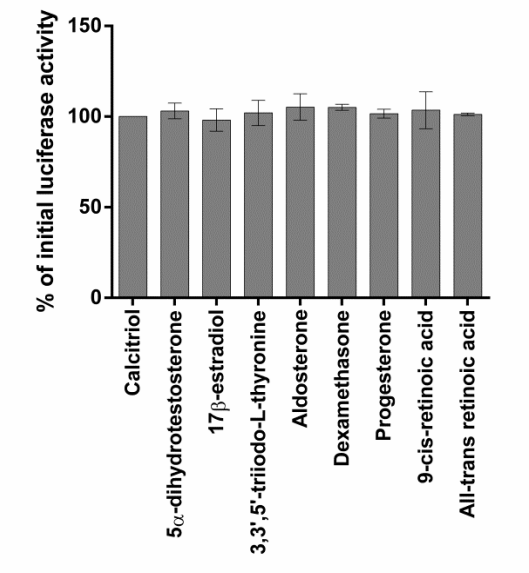

Figure 18: Inhibition of Nano luciferase by model agonists of other nuclear receptors. For this purpose, cells were treated with calcitriol (100 nM). Following the incubation, cell lysate was collected, treated with tested compounds and luciferase activity was measured. Data are the mean of triplicate measurements, and are expressed as a percentage of initial luciferase activity. Similar data were obtained in two independent experiments.

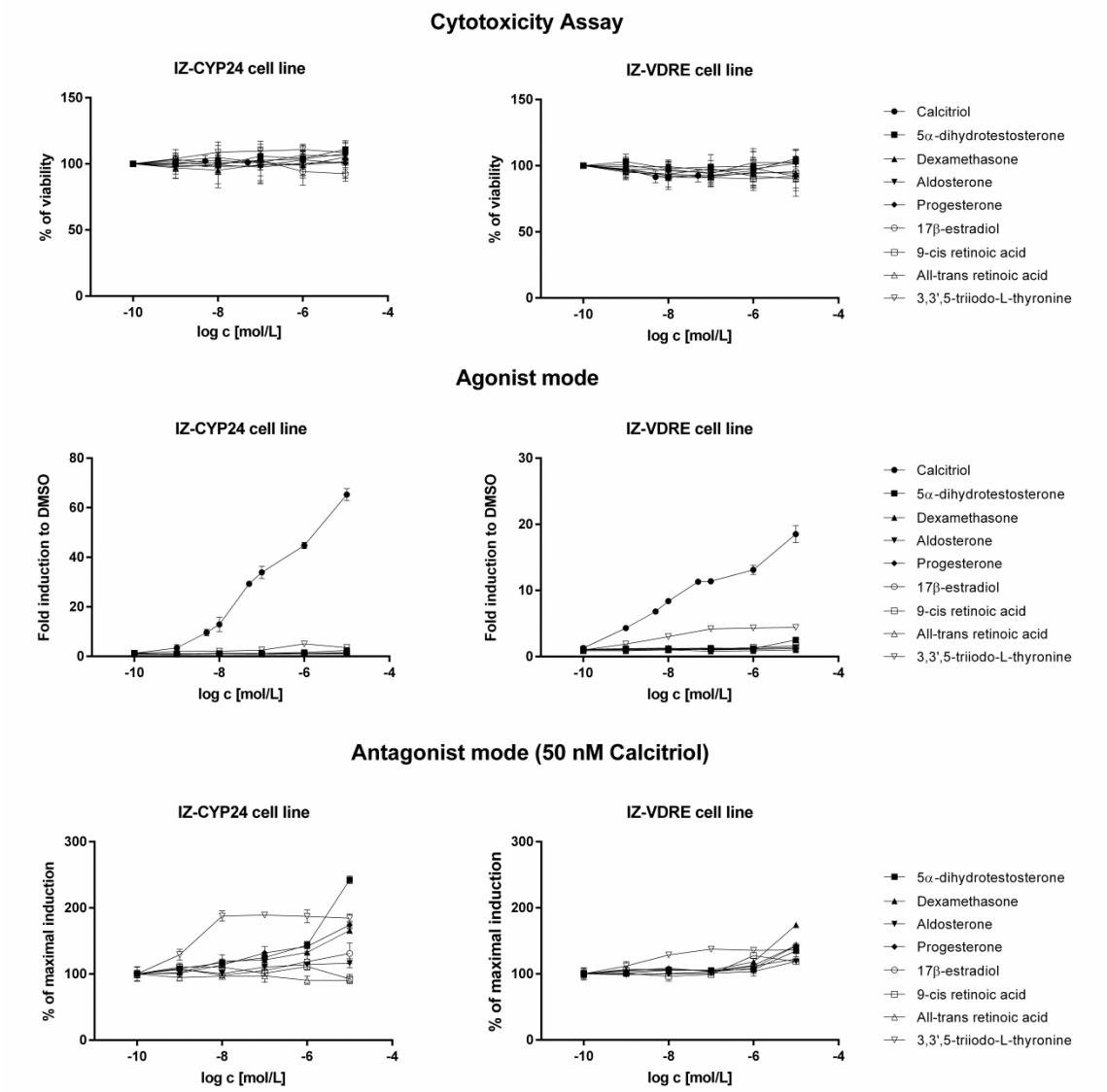

Figure 19: Selectivity of IZ-CYP24 and IZ-VDRE cells. Cells were treated for 24 h with model ligands of selected nuclear and steroid receptors in the concentration range of 0.1 nM to 10 µM in the presence (antagonist mode) or absence (cytotoxicity assay, agonist mode) of calcitriol (50 nM). The vehicle was DMSO (0.1% v/v). **Top panel:** Cytotoxicity assay. The MTT assay was performed and absorbance was measured at 540 nm. The data are the mean of experiments from three consecutive passages of cells and are expressed as a percentage of viability of control cells. **Middle panel:** Agonist mode. Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained in three independent experiments cell passages. **Bottom panel:** Antagonist mode. Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as a percentage of maximal induction attained by calcitriol. Similar data were obtained in three independent cell passages.

5.2.7 Profiling of vitamin D3 and vitamin D2 intermediates and analogues in IZ-CYP24 and IZ-VDRE cell lines

As a proof of concept, the novel IZ-CYP24 and IZ-VDRE cell lines were used for profiling of 17 vitamin D3 and vitamin D2 metabolic intermediates and analogues against VDR transcriptional activity (Table 6).

The MTT assay and luciferase inhibition assay were conducted prior to dose-response analyses. Out of the 17 tested compounds, significant cytotoxicity was observed for 10 µM calcifediol in both cell lines and was accompanied with a drop of luciferase activity in gene reporter assays. Moderate cytotoxicity was observed for 10 µM alfacalcidol and doxercalciferol in both cell lines (Figures 21–23). No inhibition of luciferase catalytic activity was observed for any of the tested compounds (Figure 20).

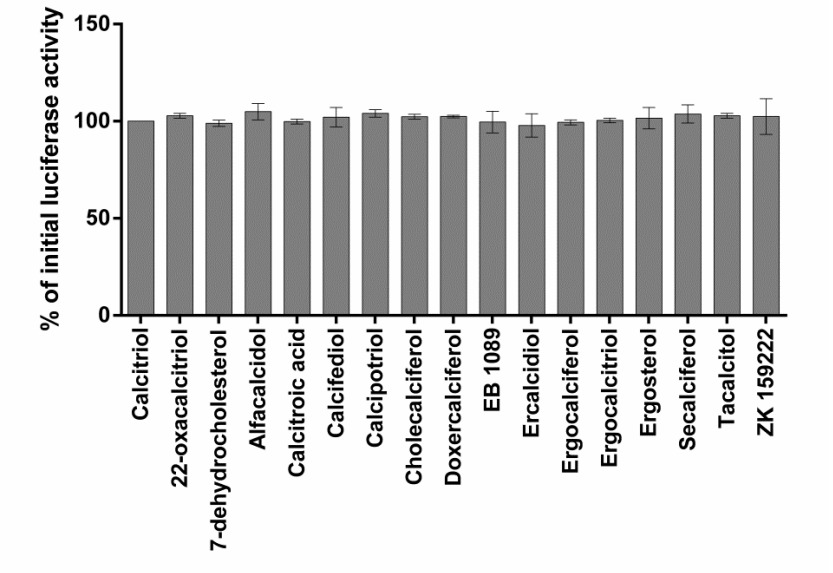

Figure 20: Inhibition of Nano luciferase by vitamin D intermediates and analogues. For this purpose, cells were treated with DHT (100 nM). Following the incubation, the cell lysate was collected, treated with tested compounds and luciferase activity was measured. Data are the mean of triplicate measurements and are expressed as a percentage of initial luciferase activity. Similar data were obtained from two independent experiments.

Within the vitamin D3 metabolic pathway, dose-response analyses of precursors (7-dehydrocholesterol, cholecalciferol, calcifediol), the obligatory VDR ligand (calcitriol), its by-product (secalciferol), and the ultimate metabolite (calcitroic acid) were performed. The effects of individual compounds were similar in the two cell lines. All the tested compounds induced the luciferase activity, but with different dose-response profiles, potency and efficacy. Early metabolic precursors of calcitriol, i.e. 7-dehydrocholesterol and cholecalciferol, induced VDR transcriptional activity only at 10 μM concentrations, with efficacy from 20% to 70% as compared to calcitriol. Late precursor calcifediol activated VDR with EC_{50} between 10^{-7} and 10^{-6} and with efficacy similar to that of calcitriol. The most potent and effective activator of VDR was calcitriol, yielding EC_{50} of 7 and 36 nM in IZ-VDRE and IZ-CYP24 cells, respectively. A by-product arising from calcifediol after 24-hydroxylation – secalciferol – dose-dependently activated VDR, with EC_{50} of approximately 150 nM and with efficacy similar to that of calcitriol. A common degradation product calcitroic acid induced luciferase activity only at 10 μM and with negligible efficacy (Figure 21, Table 7). Dose-response effects of the tested compounds were somewhat two-staged, when after reaching a plateau between 10^{-7} and 10^{-6} M, and additional increase was observed at concentration 10^{-5} M (probably off-target effects).

Within the vitamin D2 pathway, dose-response analyses were performed for precursors (ergosterol, ergocalciferol, ergocalcidiol), obligatory VDR ligand (ergocalcitriol), and a common ultimate degradation product (calcitroic acid). Early precursor ergosterol did not activate VDR at the entire range of tested concentrations, while later precursor ergocalciferol did increase luciferase activity only at the 10 μM in both cell lines. The canonical ligand and activator of VDR, ergocalcitriol, dose-dependently activated VDR with EC_{50} of approx. 10^{-7} M. Its progenitor ergocalciferol, activated VDR with similar potency and efficacy (Figure 22, Table 7).

Dose-response effects of seven commercially available VD3/VD2 analogues (six agonist and 1 antagonist) were measured. All the tested VDR agonists elicited a dose-responsive increase of luciferase activity in both cell lines, and the maximal fold induction values were similar to those caused by calcitriol. The values of EC_{50} decreased in the order: Alfacalcidiol = Doxecalciferol (~10^{-7}–10^{-6}

M) > Calcitriol = Calcipotriol = 22-oxacalcitriol = tacalcitol (~10^{-8}–10^{-7} M) > EB1089 (~10^{-10}–10^{-9} M). Moreover, when assaying VDR antagonist ZK159222 in combination with 50 nM calcitriol, the IC_{50} of 0.41±0.12 μM was observed in IZ-CYP24 cells, while it was not attained in IZ-VDRE cells even at the 10 μM concentration (Figure 23, Table 7).

Table 6: Vitamin D intermediates and analogues used for dose-response analyses in IZ-CYP24 and IZ-VDRE cell lines.

Substance	Synonym	Chemical name	CAS No.
7-dehydrocholesterol	Provitamin D3	3β-hydroxyholesta-5,7-cdiene	434-16-2
Cholecalciferol	Vitamin D3	9,10-secocholesta-5,7,10(19)-trien-3-ol	67-97-0
Calcifediol	25-hydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-3,25-diol	19356-17-3
Calcitriol	1α,25-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol	32222-06-3
Secalciferol	24R,25-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol	55721-11-4
Calcitroic acid	1α-hydroxycalcioic Acid	1,3-dihydroxy-24-nor-9,10-secochola-5,7,10(19)-trien-23-oic Acid	71204-89-2
Alfacalcidol	1α-hydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3-diol	41294-56-8
Calcipotriol	Calcipotriene	24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1,3,24-triol	112965-21-6
Tacalcitol	1α,24R-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3,24-triol	57333-96-7
EB 1089	Seocalcitol	24a,26a,27a-trihomo-9,10-secocholesta-5,7,10(19),22,24-pentaene-1,3,25-triol	134404-52-7
22-oxacalcitriol	Maxacalcitol	9,10-seco-22-oxacholesta-5,7,10(19)-triene-1α,3β,25-triol	103909-75-7
Ergosterol	Provitamin D2	3β-hydroxyergosta-5,7,22-triene	57-87-4
Ergocalciferol	Vitamin D2	9,10-secoergosta-5,7,10(19),22-tetraen-3-ol	50-14-6
Ergocalcidiol	25-hydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-3,25-diol	21343-40-8
Ergocalcitriol	1α,25-dihydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-1,3,25-triol	60133-18-8
Doxercalciferol	1α-hydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-1,3-diol	54573-75-0

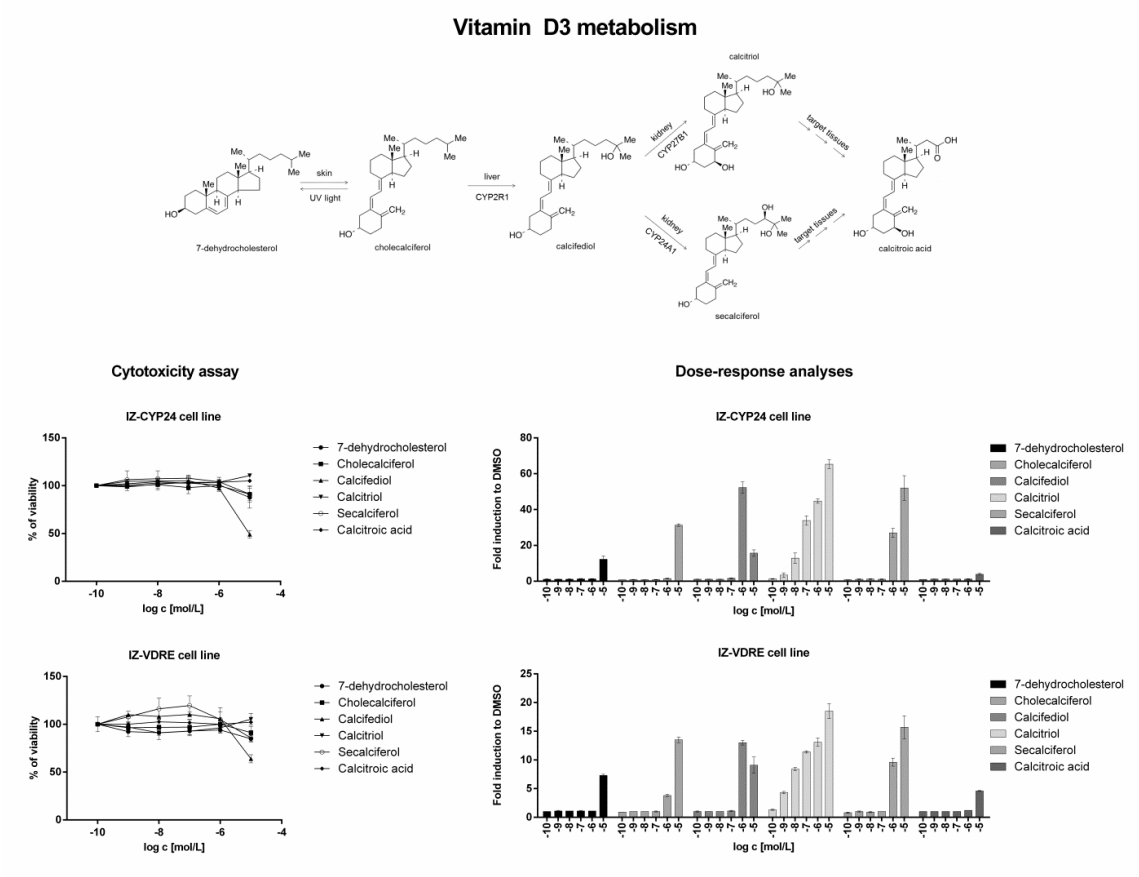

Figure 21: Profiling the vitamin D3 metabolic pathway. Cells were treated for 24 h with intermediates from the vitamin D3 pathway in a concentration range of 0.1 nM to 10 µM and vehicle (DMSO; 0.1% v/v). **Top panel:** A scheme of vitamin D3 metabolism. **Lower left panel:** A cytotoxicity assay. The MTT assay was performed and absorbance was measured at 540 nm. The data are the mean of experiments from three consecutive passages of cells and are expressed as a percentage of viability of control cells. **Lower right panel:** Dose-response analyses. Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

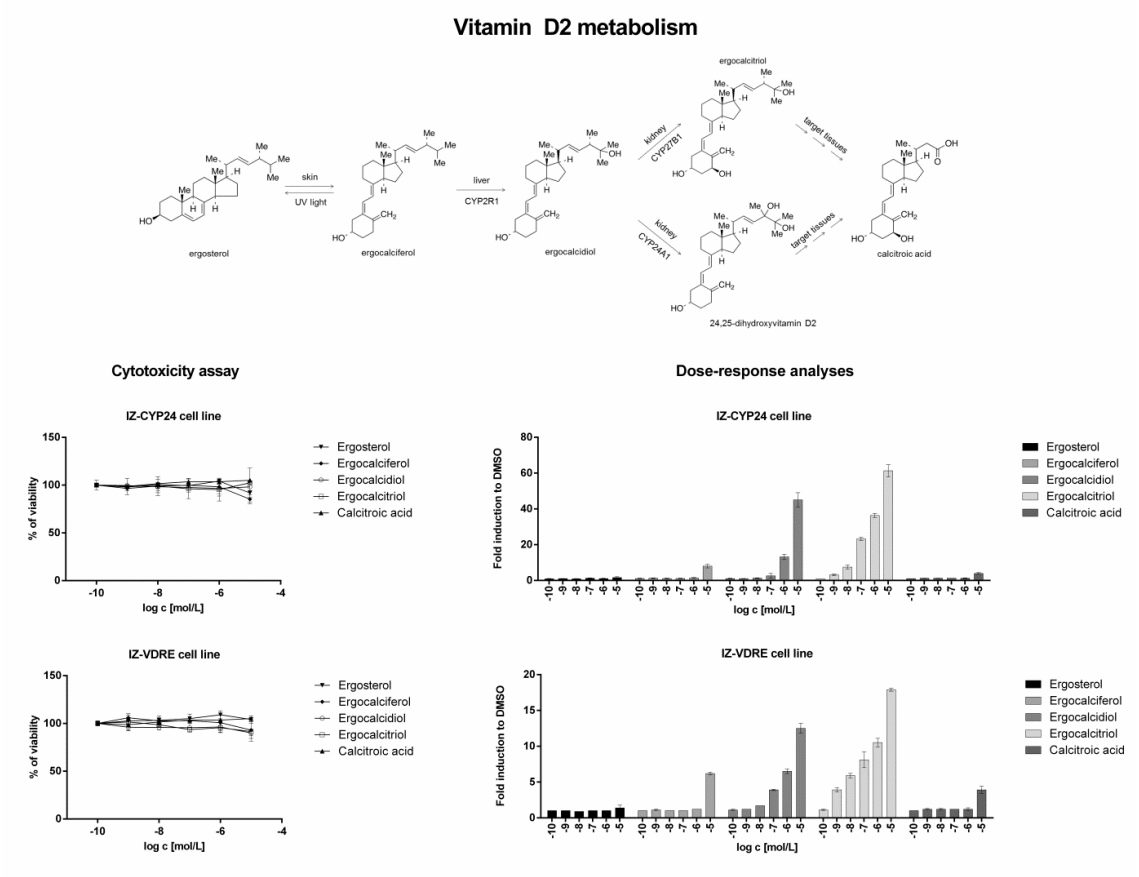

Figure 22: Profiling the vitamin D2 metabolic pathway. Cells were treated for 24 h with intermediates from the vitamin D2 pathway in a concentration range of 0.1 nM to 10 µM and vehicle (DMSO; 0.1% v/v). **Top panel:** A scheme of vitamin D2 metabolism. **Lower left panel:** A cytotoxicity assay. The MTT assay was performed and absorbance was measured at 540 nm. The data are the mean of experiments from three consecutive passages of cells and are expressed as a percentage of viability of control cells. **Lower right panel:** Dose-response analyses. Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

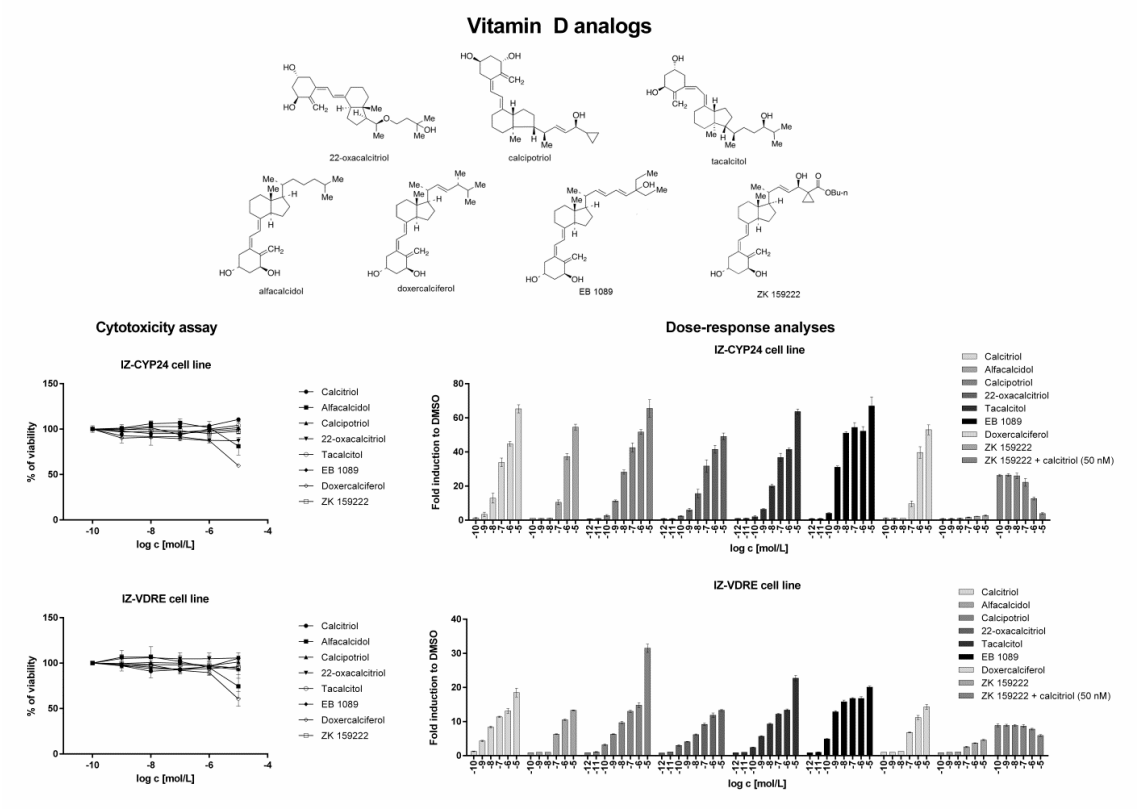

Figure 23: Profiling of vitamin D analogues. Cells were treated for 24 h with various vitamin D analogues in the concentration range of 1 pM to 10 µM in the presence or absence of calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v). **Top panel:** Chemical structures of vitamin D analogues. **Lower left panel:** A cytotoxicity assay. The MTT assay was performed and absorbance was measured at 540 nm. The data are the mean of experiments from three consecutive passages of cells and are expressed as a percentage of viability of control cells. **Lower right panel:** Dose-response analyses. Luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

Table 7: Half maximal effective concentrations (EC_{50}) for the tested vitamin D precursors, metabolites, and analogues. Data are expressed as mean ± SD, *n* represents the number of experiments. Values of EC_{50} were calculated using 1 µM as the highest concentration.

	EC_{50}			
Compound	**n**	**IZ-CYP24**	**n**	**IZ-VDRE**
7-dehydrocholesterol	3	> 1000 nM	3	> 1000 nM
22-oxacalcitriol	4	24.6 ± 3.3 nM	4	8.3 ± 2.1 nM
Alfacalcidol	3	126.6 ± 5.3 nM	3	88.5 ± 1.1 nM
Calcifediol	3	154.5 ± 16.0 nM	3	153.8 ± 13.8 nM
Calcitriol	3	36.2 ± 6.7 nM	3	7.2 ± 1.6 nM
Calcitroic acid	3	> 1000 nM	3	> 1000 nM
Calcipotriol	4	5.3 ± 1.9 nM	4	2.3 ± 0.7 nM
Cholecalciferol	3	136.8 ± 26.3 nM	3	143.5 ± 47.4 nM
Doxercalciferol	3	130.1 ± 15.3 nM	3	87.1 ± 2.5 nM
EB 1089	4	0.9 ± 0.1 nM	4	0.3 ± 0.1 nM
Ergocalcidiol	3	171.2 ± 12.1 nM	3	172.7 ± 41.9 nM
Ergocalciferol	3	316.3 ± 1.6 nM	3	368.7 ± 76.0 nM
Ergocalcitriol	3	53.4 ± 5.8 nM	3	11.4 ± 3.6 nM
Ergosterol	3	n.d.	3	n.d.
Secalciferol	3	166.1 ± 15.2 nM	3	140.8 ± 45.8 nM
Tacalcitol	4	14.0 ± 5.8 nM	4	2.7 ± 0.2 nM
ZK 159222	3	85.5 ± 12.6 nM	3	49.7 ± 25.4 nM

6 DISCUSSION

Endocrine disrupting chemicals (EDCs) along with other exogenous compounds, such as food additives and drugs, were described to influence the activity of nuclear receptor (NRs). Therefore, reliable, rapid, efficacious and sensitive tools for the detection of any disturbance of transcriptional activity of NRs are needed. In this thesis, I describe the construction and characterization of stably transfected human gene reporter cell lines for the detection of human AR and human VDR transcriptional activity.

Human stably transfected reporter cell line AIZ-AR was derived from human prostate carcinoma epithelial cell line 22Rv1 by transfection with a reporter plasmid containing three AREs followed by an ARR sequence from the promoter region of the human prostate specific androgen (*PSA*) gene. The AIZ-AR cell line remained fully functional for over 67 days in culture (this duration corresponds to 28 passages) as well as after a freeze–thaw cycle. Time-course analyses revealed the possibility of identification of AR ligands as soon as after 8 hours of the incubation. Dose-response analyses with 23 steroid compounds were performed to assess specificity of the AIZ-AR cell line. Luciferase activity of the AIZ-AR cell line was induced by androgens, but not by oestrogens or mineralocorticoids. Glucocorticoids and progesterone induced the luciferase signal, but with 2–3 orders of magnitude higher in comparison with androgens. Sensitivity of the AIZ-AR cell line allowed for experiments in 96-well plates. In comparison with previously published experimental models, the AIZ-AR cell line possesses several advantages:

1. The AIZ-AR cell line is an entirely human system: human prostate carcinoma epithelial cell line 22Rv1 expressing fully functional endogenous human AR was transfected with a reporter vector that is under control of AREs of the *PSA* gene.
2. The AIZ-AR cell line preserved the signalling stoichiometry. Because the expression of AR is endogenous in this cell line and no extra AR was cotransfected, the ratio between AR protein and regulatory proteins of transcriptional machinery remains unchanged.

The characteristic properties indicated above clearly describe the added value of AIZ-AR cell line as compared to previously published stably transfected cell lines. MDA-kb2 and AR-LUX cell lines both contain a reporter vector under the control of rodent promoter sequences, not human ones (Blankvoort *et al*., 2001; Wilson *et al*., 2002). In addition, human cell lines PALM and AR CALUX were co-transfected with expression vectors for AR (Térouanne *et al*., 2000; Sonneveld *et al*., 2005): therefore, the stoichiometry of cell signalling was significantly changed due to overexpression of AR.

In the second part of this thesis, I describe the construction and characterization of two novel stably transfected reporter cell lines for the evaluation of human VDR ligands. Human colon adenocarcinoma cell line LS180, which expresses fully functional VDR, was transfected with two different reporter vectors:

1. The IZ-CYP24 cell line was created by transfection with a reporter construct containing a partial sequence (-326/-46) from the promoter region of human *CYP24A1* gene. This promoter sequence contains two separate VDREs: VDRE-I (-174/-151) and VDRE-II (-194/-174). Because IZ-CYP24 cells contain a partial sequence of the CYP24A1 promoter, they may serve as a potential instrument for studies of CYP24A1 transcriptional regulation.
2. The IZ-VDRE cell line was developed by transfection with a reporter construct containing three copies of VDRE-I (-174/-151) from the promoter region of the human *CYP24A1* gene.

During the characterization, the IZ-CYP24 cell line yielded 30-fold induction: on the order of magnitude 10^3–10^4 RLU after treatment with 50 nM calcitriol. IZ-VDRE cells showed ~10-fold induction: on the order 10^6–10^7 RLU after the application of 50 nM calcitriol. This extremely high luciferase signal makes the IZ-VDRE cell line a good tool for sensitive and high-throughput screening for VDR activators and antagonists. Both cell lines were used in the 96-well plate format throughout the whole characterization process, with sufficiently strong signals. Both IZ-CYP24 and IZ-VDRE cell lines remained fully functional for more than 78 days in cell culture (corresponding to 32 passages). The responsiveness to calcitriol was fully retained after a freeze–thaw cycle. Time-

course analyses revealed 24 h of incubation with the tested compounds to be optimal time for the identification of VDR ligands, while the minimal time to obtain a sufficient luciferase signal seems to be 8 hours after the treatment, allowing for the testing of cytotoxic compounds. Selectivity of IZ-CYP24 and IZ-VDRE cells was tested by incubation with agonists of NRs (RAR, RXR, and TR) and steroid receptors (GR, MR, PR, ER, and AR). No induction of luciferase activity was observed after the treatment with aldosterone, dexamethasone, progesterone, 5α-dihydrotestosterone, 17β-estradiol, 9-cis retinoic acid, or all-trans retinoic acid, either in the presence (antagonist mode) or absence (agonist mode) of 50 nM calcitriol. This finding indicates no cross-talk with GR, MR, PR, AR, ER, RXR, or RAR in either cell line, implying no false positive results. Slight augmentation of the calcitriol-induced luciferase signal was observed after the treatment with dexamethasone, progesterone and 5α-dihydrotestosterone in the IZ-CYP24 cell line, but not in the IZ-VDRE cell line, suggesting possible roles of GR, PR, and AR in the regulation of *CYP24A1* gene expression. By contrast, non-genomic mechanisms are likely due to supra-physiological concentrations (10^{-5}–10^{-6} M) of hormones used during the treatments. In both cell lines, moderate cross-reactivity with thyroids was observed. This finding is consistent with induction of p-glycoprotein by levothyroxine in human intestinal cell lines LS180 and Caco-2 (Mitin *et al*., 2004). This observation must be taken into consideration while interpreting the experimental results.

Dose-response analyses of both cell lines treated with VDR agonists revealed a something like two-stage effect of VDR ligands. In both cell lines, calcitriol yielded a typical sigmoid curve with EC_{50} of 36 nM and 7 nM in the IZ-CYP24 and IZ-VDRE cell lines, respectively. The curve reached a plateau between concentrations 100 nM and 1 μM, and a further increase led to an additional rise of the luciferase signal. This effect can be an indicator of non-genomic effects of VDR ligands at supra-physiological concentrations (~1000-fold higher than EC_{50}). The values of calcitriol EC_{50} significantly differed among theexperimental models. The LanthaScreen TR-FRET VDR coactivator assay and the GeneBLAzer VDR assay (Invitrogen) produced 0.75 and 0.50 nM EC_{50} for calcitriol, respectively, 10-fold lower in comparison to cell lines IZ-CYP24 and IZ-VDRE. The Human VDR Reporter Assay System from Indigo

Biosciences using non-human cells yielded EC_{50} of 3.5 nM for calcitriol, which is close to the 7 nM obtained in IZ-VDRE cell line. In the transiently transfected CHO-K1 cell line, EC_{50} for calcitriol was found to be 1.7 nM (Wu-Wong *et al*., 2013), but the same group in the same system reported EC_{50} for calcitriol to be 15 nM (Chen *et al*., 2013). These data indicate enormous variability in EC_{50} values obtained by means of different experimental systems.

As proof of concept, the IZ-CYP24 and IZ-VDRE cell lines were used for profiling of vitamin D analogues and intermediates in vitamin D2 and vitamin D3 metabolic pathways in relation to VDR transcriptional activity. Our data revealed induction of luciferase activity not only by obligatory ligands, but also by their precursors and degradation products. Nevertheless, some intermediates and analogues significantly induced VDR at a supra-physiological concentration (above 10^{-5} M). A non-genomic mechanism may contribute to this phenomenon, apart from low efficacy of the tested compounds.

For both IZ-CYP24 and IZ-VDRE cell lines, assay performance indicators were calculated according to Iversen *et al*. (2006). The SW was on average 8.2 in the IZ-CYP24 cell line and 14.3 in the VDRE cell line. Given that the recommended value is SW > 2, both cell lines surpassed this cut-off excellently. The values of the Z′ factor in both cell lines substantially exceeded 0.5, the value considered to be “excellent”. In line with these results, the assay variability ratios (AVRs) were lower than 0.60, the recommended value, in all analysed experiments in both cell lines. Thus, both cell lines passed all the criteria for high-throughput models and they are comparable with already existing models. For example, the GeneBLAzer VDR assay yielded the Z′ factor of ~0.86 and the LanthaScreen TR-FRET VDR coactivator assay produced the Z′ factor of ~0.75 and SW ~5–6 (www.invitrogen.com). A comparison of different methods used for the screening of FXR ligands revealed similar Z′ factors for TRF (Z′ = 0.60), TR-FRET (Z′ = 0.85) and ALPHAscreen (Z′ = 0.75) (Glickman *et al*., 2002).

Overall, during my Ph.D. studies, I constructed and characterized human stably transfected reporter cell lines for the detection and evaluation of AR and VDR transcriptional activity. All the cell lines (AIZ-AR, IZ-CYP24, IZ-VDRE) represent a rapid, high-throughput, selective, and sensitive tool for the screening of AR

and VDR ligands. These cell lines have potential toxicological, environmental, food safety, pharmacological, and drug-discovery applications. Because of the involvement of both AR and VDR in cancer and tumour growth, these cell lines also provide a model for research on human oncology.

7 CONCLUSION

In the presented thesis, I developed and characterized unique novel human stably transfected reporter cell lines for evaluation of the transcriptional activity AR (AIZ-AR cell line) and VDR (IZ-CYP24 and IZ-VDRE cell lines). These cell lines represent a rapid, high-throughput, selective, sensitive, reproducible and reliable tool for the identification of AR and VDR ligands. The possible use of these cell lines in toxicological, environmental, food safety, pharmacological and drug-discovery applications is anticipated.

Lastly, AIZ-AR, IZ-CYP24 and IZ-VDRE cell lines are now commercially distributed by the Canadian company *Applied Biological Materials (ABM) Inc.*

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9 CURRICULUM VITAE

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Education

Present study

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Thesis topic: Construction of reporter cell lines for toxicological and environmental applications (supervisor: prof. RNDr. Zdeněk Dvořák, DrSc. et Ph.D.)

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2008-2011 Bachelor's program in Molecular and cell biology, Department of Cell Biology and Genetics, Faculty of Science, Palacky University Olomouc

Thesis topic: The analysis of polymorphic cross-species microsatellites for determination of paternity in selected Motacillidae birds.

2011-2013 Master′s program in Molecular and Cell Biology, Department of Cell Biology and Genetics, Faculty of Science, Palacky University Olomouc

Thesis topic: The analysis and characterization of polymorphic cross-species microsatellites in Great White Pelican (Pelecanus onocrotalus) and Spot-billed Pelican (Pelecanus philippensis)

Educational activity

Practical courses from cell biology II

Practical courses from molecular biology I

Practical courses from genetics

Research internship

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Short-term research stays at Medical University of Vienna, Department of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology & Immunology, Vienna (3 months)

Participation in projects (researcher)

- P303/12/G163 – Centre of Excellence: Centre of drug-dietary supplements interactions and nutrigenetics. Czech Scientific Foundation (2012 – 2018)
- 13-07711S – Role of a Hippo signalling pathway and organic toxicants in deregulation of hepatic cell functions and metabolism of xenobiotics, Czech Scientific Foundation (2013-2016)
- 17-02718S – Interactions of essential oils from culinary spices and herbs with endocrine and detoxication signalling pathways, Czech Scientific Foundation (2017-2019)

- OPVK CZ.1.07/2.3.00/20.0062 – Antabuse (disulfiram) as a pilot case of nonprofit drug (2011 – 2014)

Publications:

- Novotna, A, Kamenickova, A, Pecova, M, Korhonova, M, **Bartonkova, I**, Dvorak, Z (2014): Profiling of enantiopure drugs towards aryl hydrocarbon (AhR), glucocorticoid (GR) and pregnane X (PXR) receptors in human reporter cell lines. *Chem Biol Interact* 208, 64-76. [IF_{2013} 2.982]. Contributions to the published work: performed data analysis, conducted experiments.
- Novotna, A., Srovnalova, A, Svecarova, M, Korhonova, M, **Bartonkova, I**, Dvorak, Z (2014): Differential effects of omeprazole and lansoprazole enantiomers on aryl hydrocarbon receptor in human hepatocytes and cell lines. *PLoS One* 9, 98711- 98711. [IF_{2013} 3.534]. Contributions to the published work: performed data analysis, conducted experiments.
- Novotna, A, Korhonova, M, **Bartonkova, I**, Soshilov, A, Denison, M, Bogdanova, K, Kolar, M, Bednar, P, Dvorak, Z (2014): Enantiospecific effects of ketoconazole on aryl hydrocarbon receptor. *PLoS One* 9, 101832 – 101832. [IF_{2013} 3.534]. Contributions to the published work: performed data analysis, conducted experiments.
- Novotna, A, Krasulova, K, **Bartonkova, I**, Korhonova, M., Bachleda, P, Anzenbacher, P, Dvorak, Z (2014): Dual effects of ketoconazole cis-enantiomers on CYP3A4 in human hepatocytes and HepG2 cells. *PLoS One* 9, 111286-111286. [IF_{2013} 3.534]. Contributions to the published work: performed data analysis, conducted experiments.
- **Bartonkova, I**, Novotna, A, Dvorak, Z (2015): Novel stably transfected human reporter cell line AIZ-AR as a tool for an assessment of human androgen receptor transcriptional activity. *PLoS One* 10, 121316-121316. [IF_{2014} 3.234]. Contributions to the published work: participated in research design, performed data analysis, conducted experiments, wrote or contributed to the manuscript.

- **Bartonkova, I**, Grycova, A, Dvorak, Z (2016): Profiling of vitamin D metabolic intermediates toward VDR using novel stable gene reporter cell lines IZ-VDRE and IZ-CYP24. *Chem Res Toxicol* 29, 1211-1222. [IF_{2015} 3.025]. Contributions to the published work: participated in research design, performed data analysis, conducted experiments, wrote or contributed to the manuscript.

Conference reports:

- **Bartonkova, I**, Grycova, A, Dvorak, Z: Construction of a stably transfected human gene reporter cell line for assessment of Pregnane X receptor (PXR) transcriptional activity. 20th International Symposium on Microsomes and Drug Oxidations (MDO2014), 18th-22th May 2014, Stuttgart, Germany; p. 273.
- Korhonova, M, Novotna, A, **Bartonkova, I**, Dvorak Z: Enantiospecific effects of lansoprazole and omeprazole on the expression of human cytochrome P450 1A (CYP1A) via aryl hydrocarbon receptor (AhR). 20th International Symposium on Microsomes and Drug Oxidations (MDO 2014), 18th-22th May 2014, Stuttgart, Germany; p.140.
- **Bartonkova I**, Novotna A, Dvorak Z: Development of stably transfected human cell line for assessment of PXR transcriptional activity. Integration Seminar Nuclear Receptors and PAS proteins in Regulation of Xenobiotic-Metabolizing Enzymes and Cell Functions, 28th-29th May 2014, Brno, Czech Republic.
- **Bartonkova I**, Novotna A, Dvorak Z: Development of a novel human reporter cell line for assessing androgen receptor (AR) transcriptional activity. 19th Interdisciplinary Toxicological Conference (TOXCON 2014): Connecting for Safer Europe, 23th-26th September 2014, Stará Lesná, Slovakia; *Interdisciplinary Toxicology*, 7 (Suppl. 1), p. 26-27.
- **Bartonkova I**, Novotna A, Dvorak Z: Transgenic human gene reporter cell line for evaluating interactions between androgen receptor and xenobiotics. 17th European Congress of Endocrinology (ECE 2015), 16th-20th May 2015, Dublin, Ireland; *Endocrine abstracts* 37, p.338.

- **Bartonkova I**, Novotna A, Dvorak Z: Construction of human reporter cell line for assessment of androgen receptor transcriptional aktivity. Conference proceedings from scientific conference with international participation on ENDOCRINE DISRUPTORS, 4th-5th June 2015, Bratislava, Slovakia; p.31.
- **Bartonkova I**, Novotna A, Doricakova, A, Dvorak Z: Enantiospecific activation of AhR by ketoconazole. AhR 2016: The aryl hydrocarbon receptor as a central mediator of health and disease, August 3rd-6th, 2016, Rochester, NY, USA; p.59.
- **Bartonkova I**, Grycova A, Dvorak Z: Novel human stable reporter cell lines IZ-VDRE and IZ-CYP24: A tool for the assessment of vitamin D receptor transcriptional activity. 19th European Congress of Endocrinology (ECE 2017), 20th-23rd May 2017, Lisbon, Portugal; *Endocrine abstracts* 49, p.394.

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APPENDIX I:

Bartonkova I, Novotna A, Dvorak Z (2015): Novel stably transfected human reporter cell line AIZ-AR as a tool for an assessment of human androgen receptor transcriptional activity. *Plos One*, 10. 121316 - 121316 [IF_{2014} 3.234]

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RESEARCH ARTICLE

Novel Stably Transfected Human Reporter Cell Line AIZ-AR as a Tool for an Assessment of Human Androgen Receptor Transcriptional Activity

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Abstract

Androgen receptor plays multiple physiological and pathological roles in human organism. In the current paper, we describe construction and characterization of a novel stably transfected human reporter cell line AIZ-AR for assessment of transcriptional activity of human androgen receptor. Cell line AIZ-AR is derived from human prostate carcinoma epithelial cell line 22Rv1 that was transfected with reporter plasmid containing 3 copies of androgen response regions (ARRs) followed by a single copy of androgen response element (ARE) from the promoter region of human prostate specific antigen (PSA) gene. AIZ-AR cells remained fully functional for more than 60 days and over 25 passages in the culture and even after cryopreservation. Time-course analyses showed that AIZ-AR cells allow detection of AR ligands as soon as after 8 hours of the treatment. We performed dose-response analyses with 23 steroids in 96-well plate format. We observed activation of AR by androgens, but not by estrogens and mineralocorticoids. Some glucocorticoids and progesterone also induced luciferase, but their potencies were 2-3 orders of magnitude weaker as compared to androgens. Taken together, we have developed a rapid, sensitive, selective, high-throughput and reproducible tool for detection of human AR ligands, with potential use in pharmacological and environmental applications.

Introduction

Androgen receptor (AR, NR3C4) is a 110-kDa ligand-activated transcriptional factor that belongs to the steroid hormone receptor superfamily. It has broad physiological functions, including developmental and psychological. AR is also involved in several pathological situations, including genesis of prostatic hyperplasia and prostate cancer function or altered pubertal development due to its mutations [1]. In the absence of a ligand, AR primarily resides in the cytoplasm bound to chaperone proteins. Upon activation, AR translocates to the nucleus where it forms AR/AR homodimer, which binds specific DNA sequence known as androgen

response element (ARE) and stimulates expression of androgen-responsive genes [2, 3]. Endogenous ligands for AR are testosterone and 5α-dihydrotestosterone (DHT). There is an extensive need for identification of AR ligands, mainly for two reasons. Firstly, AR is a target for several drugs in human pharmacotherapy; therefore, identification and characterization of AR ligands as new lead compounds in drug discovery and development need effective experimental tool. Secondly, various environmental pollutants cause so called endocrine disruption in humans, which occurs often through interactions with steroid receptors signaling, including by AR [4, 5]. Hence, the development of *in vitro* experimental tool for analyses of androgenic and antiandrogenic effects of environmental matrices is of great importance.

Several approaches have been used to assess the effects of foreign compounds and mixtures on transcriptional activity of androgen receptor. In the past, *in vivo* experiments were carried out in rats [6] or transient transfections were performed [4, 7]. Both approaches are costly, time-consuming and they have low capacity for testing (low throughput). Therefore, several stably transfected gene reporter cell lines were introduced to provide reliable and high-throughput method of screening AR transcriptional activity. Térouanne *et al.* (2000) published a construction of prostatic cell line called PALM derived from human prostatic PC-3 cells stably co-transfected with human androgen receptor and reporter plasmid containing firefly luciferase gene under control of androgen-dependent promoter MMTV (mouse mammary tumor virus) [8]. Human breast cancer cell line T47D was used for development of AR-LUX reporter cell line, transfected with reporter plasmid pPBARE2tataluc^{+} containing two copies of the rat probasin androgen-response element. Response of AR-LUX cell line to androgens was maximally five-fold induction to R1881 [9]. MDA-kb2 cell line was constructed by transfection of human breast cancer cell line MDA-MB-453 with reporter gene construct MMTV.luciferase. neo and used for screening of compound for androgenic and glucocorticoid activity [10]. Sonneveld *et al.* (2005) established an androgen receptor gene reporter cell line named AR CALUX derived from human osteosarcoma cell line U2-OS using co-transfection of reporter plasmid 3xHRE-TATA-Luc and expression plasmid pSG5-neo-AR and they tested induction by DHT, maintenance of responsiveness and stability. After that, a panel of structurally and functionally diverse chemicals was tested for androgenic and anti-androgenic effects [11].

In the current paper, we present a novel stably transfected human gene reporter cell line for assessment of AR transcriptional activity. AIZ-AR cell line is derived from human prostate carcinoma epithelial cell line 22Rv1 expressing endogenous AR (no extra AR vector co-transfected) that was transfected with reporter plasmid containing sequence of androgen response element from promoter of human prostate-specific antigen (PSA). AIZ-AR cell line provides a tool for high-throughput (96-well plates) and sensitive identification and characterization of compounds with androgenic and anti-androgenic activity. Cell line AIZ-AR allows detection of androgens as soon as after 8 hours of incubation, and it remains fully functional for more than 28 passages and over 67 days in culture as well as after cryopreservation. Significant advancements and added value for AIZ-AR cell line are demonstrated, as compared to yet developed cell lines, e.g. human AR-LUX [9] and MDA-kb2 lines [10] transfected with reporters containing rodent promoters or human cell lines PALM [8] and AR CALUX [11] overexpressing AR vector.

Materials and Methods

Compounds and reagents

DMSO, hygromycin B, testosterone, spironolactone, dexamethasone, beclomethasone, betamethasone, cortisol, corticosterone, aldosterone, prednisolone, methylprednisolone, 17α-progesterone, progesterone, estradiol, diethylstilbestrol and 4-hydroxytamoxifen were

purchased from Sigma-Aldrich (Prague, Czech Republic). Danazol, cyproterone acetate, mifepristone, triamcinolone, genistein, raloxifene hydrochloride and tamoxifen citrate salt were from Santa Cruz Biotechnology (Santa Cruz, USA). Fugene HD transfection reagent was purchased from Roche (Basel, Switzerland). Reporter lysis buffer was from Promega (Hercules, CA). All other chemicals were of the highest quality commercially available.

Cell line

Human prostate carcinoma epithelial cells 22Rv1 (ECACC No. 105092802) were cultured in RPMI-1640 medium supplemented with 10% of fetal bovine serum, 100 U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

Reporter plasmid

Reporter plasmid p3ARR/ARE-luc2P/minP/hygro was designed as follows: three copies of ARR sequence (CAGGGATCAGGGAGTCTCACA) followed by a single copy of ARE sequence (TGCAGAACAGCAAGTGCTAGC) from promoter region of human prostate-specific antigen (PSA) gene were inserted into pGL4.27 [luc2P/minP/hygro] vector (Cat. No. E8451; Promega, Hercules, CA), using Kpn-1/Xho restriction enzymes.

Stable transfection of 22Rv1 cells

22Rv1 cells were transfected with reporter plasmid p3ARR/ARE-luc2P/minP/hygro (4 μg) using Fugene HD transfection reagent and seeded at density 8×10^5 cells in 60 mm culture dishes in 5 mL of the RPMI-1640 medium. Following 48 hours of incubation, the culture medium was replaced by the selection medium supplemented with hygromycin B (0.5 mg/mL). The selection medium was changed every 3–4 days for the period of 3 weeks until a polyclonal population was selected. Subsequently, the cells were transferred to 10 cm culture dishes at the density 300–700 cells per dish and cultured for additional 2 weeks in the selection medium until small colonies were visible. Thereafter, 23 colonies were sub-cloned to 24-well culture plate to obtain monoclonal populations. Clones 8 and 14 were selected for further characterization of resulting transgenic AIZ-AR cells. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref. 91997/ENV/10).

Cytotoxicity Assay

AIZ-AR cells were seeded in 96-well plates at density $5x10^4$ cells per well. Following 16 h of stabilization, cells were treated with tested compounds and vehicle (DMSO; 0.1% v/v). After 24 h, medium was replaced by charcoal stripped RPMI-1640 medium supplemented with 10% of MTT (10 mg/mL) and incubated for additional 2 hours. Following the incubation, MTT assay was measured spectrophotometrically at 540 nm using Tecan Infinite M2000 plate luminometer.

Inhibition of firefly luciferase

AIZ-AR cells were treated with 100 nM DHT for 24 hours. Cell lysates containing firefly luciferase, having a catalytic activity corresponding to model ligand-treated cells, were isolated. Tested compounds (10 μM) were added to cell lysates and luciferase activity was measured.

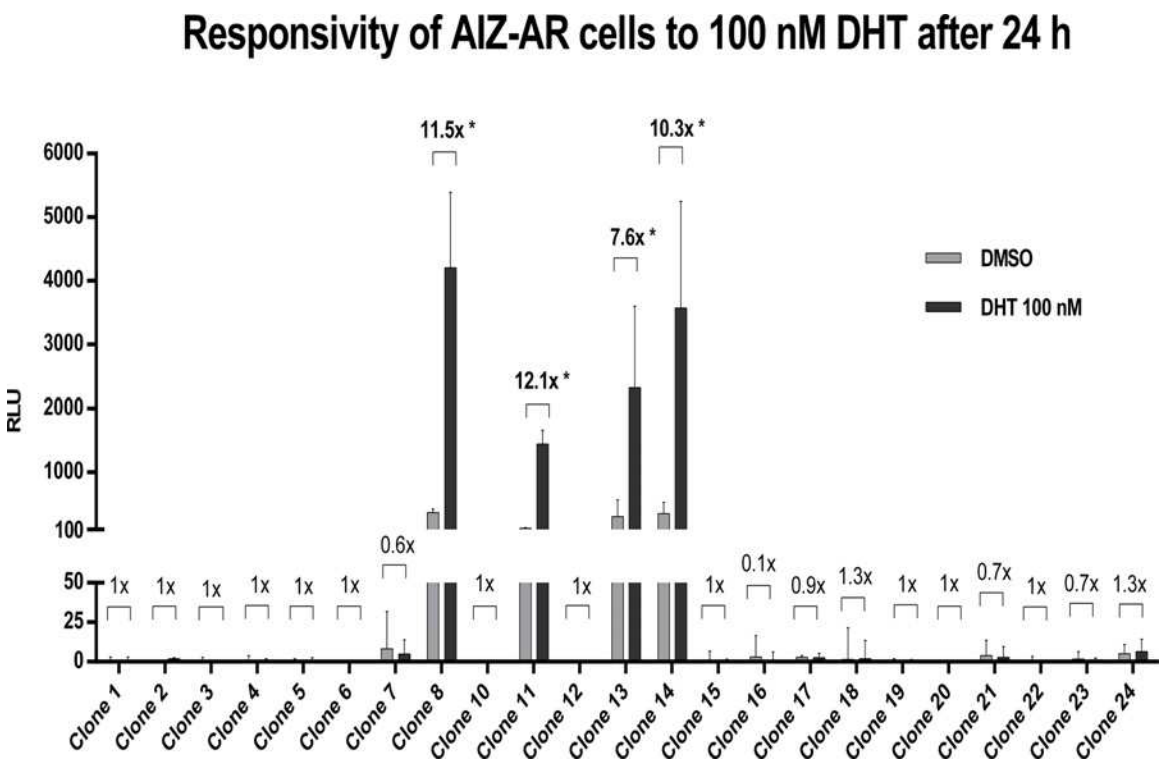

Fig 1. Responsivity of hygromycin-resistant AIZ-AR clones to 100 nM DHT after 24 hours. Cells were treated for 24 h with 5α-dihydrotestosterone (DHT; 100 nM) and vehicle (DMSO; 0.1% v/v). Luciferase activity was measured in cell lysates. Data are mean from triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and it is indicated above each clone in the bar graph. Similar data were obtained from three consecutive passages.

doi:10.1371/journal.pone.0121316.g001

Gene Reporter Assay

AIZ-AR cells were seeded in 96-well plates at density $5x10^4$ cells per well. Following 16 h of stabilization, cells were treated with tested compounds. After the treatments, cells were lysed and luciferase activity was measured in 96-well plate format using Tecan Infinite M2000 plate luminometer.

Statistical Analyses

Student's pair t-test as well as calculations of EC_{50} and IC_{50} values were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA (www.graphpad.com).

Results

Generation of stably transfected AIZ-AR clones

Human prostate carcinoma epithelial cell line 22Rv1 was transfected by lipofection with a reporter plasmid p3ARR/ARE-luc2P/minP/hygro. Stably transfected population of AIZ-AR cells were selected using hygromycin B, as described in Materials and Methods section. We obtained 23 hygromycin B-resistant clones that showed the same morphology as parental 22Rv1 cell line. We tested the responsiveness of clones to 5α-dihydrotestosterone (DHT), a model AR agonist. Cells were treated with DHT (100 nM) and vehicle (DMSO; 0.1% v/v) for 24 hours. Four clones displayed an induction of luciferase activity after the DHT treatment (clones 8, 11, 13 and 14) with fold inductions ranging from 7.6-fold to 12.1-fold (Fig. 1). Clones 8 and 14 were

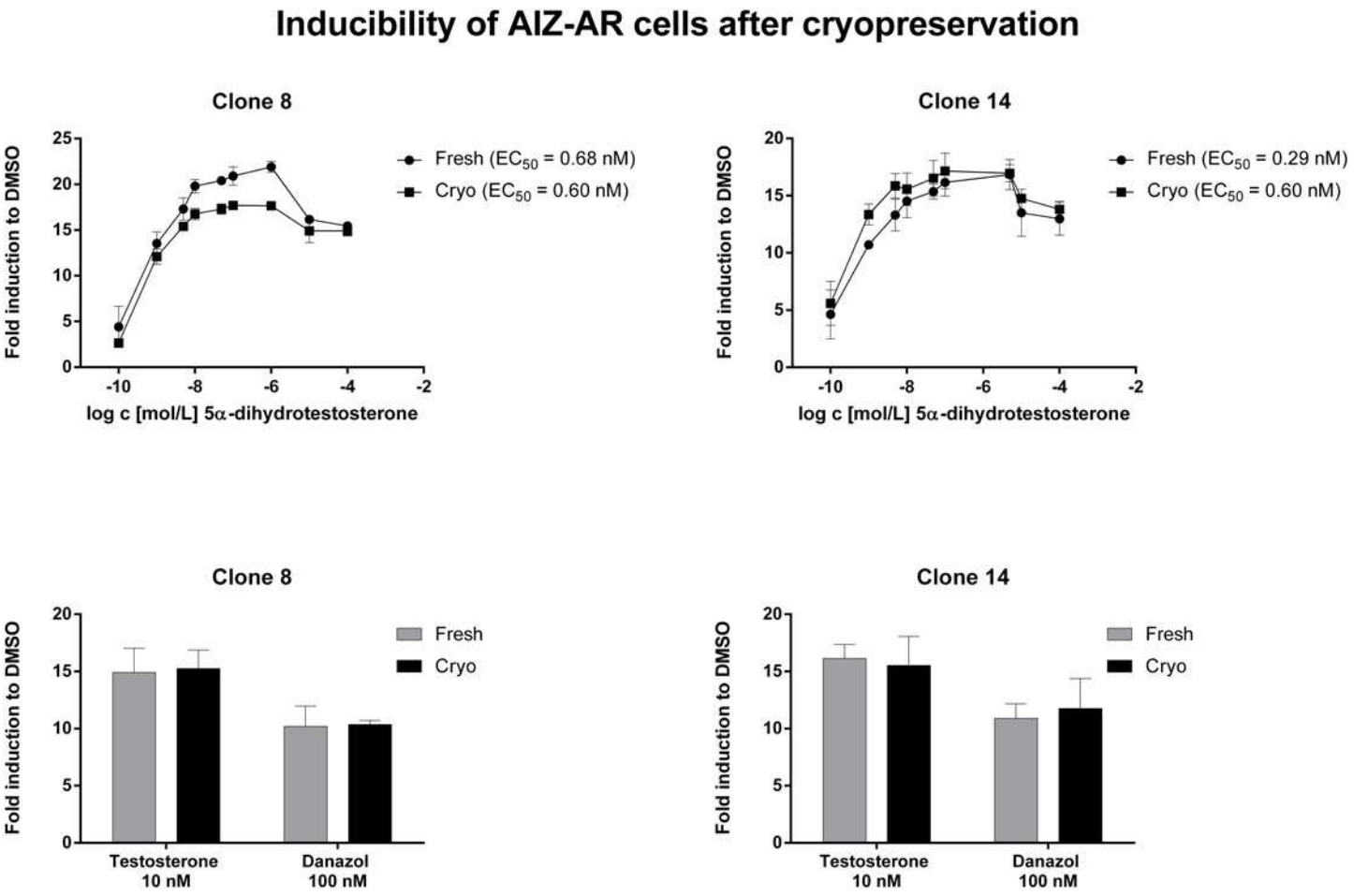

Fig 2. Induction of luciferase activity in AIZ-AR cells after cryopreservation. Cells were treated for 24 h with 5α-dihydrotestosterone (DHT; 0.1 nM—10 μM), testosterone (10 nM) and danazol (100 nM). Cells were lysed and luciferase activity was measured. Data are mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent experiments (three passages).

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selected for further characterization due to strongest obtained signal, i.e. relative luciferase units (RLU).

Maintenance of AIZ-AR cells functionality after cryopreservation

We tested the maintenance of luciferase activity induction in AIZ-AR cells after freeze-thaw cycle—cryopreservation. For this purpose, cells were frozen in fetal bovine serum and DMSO as cryo-protectant in ratio 9:1 and stored in -80°C for 1 week. After thawing, both fresh and cryopreserved cells were seeded in 96-well plates at density $5x10^4$ cells per well. Following 16 h of stabilization, cells were treated for 24 h with AR agonists including DHT (0.1 nM-100 μM), testosterone (10 nM) and danazol (100 nM). No significant difference was observed between cryopreserved and fresh cells in terms of fold inductions and EC_{50} values (Fig. 2). Therefore, AIZ-AR cell line can be considered to remain fully functional after cryopreservation.

Long-term maintenance of luciferase inducibility in AIZ-AR cells

We tested the ability of both clones of AIZ-AR cells to respond to DHT in long-term period. We checked response of cells to DHT (100 nM; 24 h) after each passage of the cells. The

Table 1. Maintenance of responsivness of AIZ-AR cells to 5α-dihydrotestosterone.

Days in culture	Clone 8			Clone 14		
	Passage	RLU	FOLD	Passage	RLU	FOLD
8	4	3368.8	10.4	3	2383.6	10.9
10	5	5039.6	12.4	4	4757.6	10.1
15	7	2489.2	11.2	6	2133.0	11.8
22	9	1759.2	11.1	8	n.d.	n.d.
25	10	2787.5	20.5	9	2297.8	20.5
30	12	2517.2	22.4	11	2185.0	20.7
32	13	3396.4	29.2	12	2127.2	23.2
37	15	3976.5	21.1	14	2121.4	23.9
39	16	4506.3	16.9	15	4517.0	16.8
44	18	4269.3	6.5	17	3470.3	5.7
46	19	4799.0	13.8	18	4023.5	14.8
51	21	3918.0	20.6	20	4011.0	17.3
53	22	6534.5	21.9	21	5746.5	20.3
58	24	5244.3	18.6	23	4787.0	14.4
60	25	2241.8	20.5	24	2044.8	16.6
65	27	3225.8	18.7	26	3499.0	20.3
67	28	4043.5	18.5	27	2485.5	15.5

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induction of luciferase activity by DHT was stable for more than 2 months of AIZ-AR cells in culture, which corresponds to 27–28 passages. Even though there was some variability between passages, there was no systematic decline or decrease in luciferase induction in both absolute RLU values and fold induction magnitude (Table 1).

Time-course analyses of responsiveness of AIZ-AR cells to DHT treatment

In the first series of experiments, AIZ-AR cells were incubated with DHT (100 nM) for 24 h, 48 h and 72 h. Absolute luciferase activity (RLU) progressively grew with increasing time of incubation and ranged between 4000 and 8000 RLU. However, fold induction over the vehicle-treated cells remained nearly constant, regardless the time of incubation (Fig. 3A). Therefore, incubation of AIZ-AR cells for 24 h is optimal, and prolonged incubation time does not bring additional value. Since evaluation of cytotoxic compounds in cell culture could fail after 24 h of incubation due to the loss of cell viability, we analyzed luciferase induction by DHT in short time periods, in order to find minimal incubation period for reliable identification of AR agonists. For this purpose, we incubated AIZ-AR cells with DHT (100 nM) for 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h and 24 h. We observed time-dependent increase of both RLU and fold induction after the treatment with DHT. Plateau in fold induction was attained approximately after 16 h of incubation. Luciferase activity around 1000 RLU and induction about 6-fold was attained after 8 hours of incubation with DHT, implying the possibility to test cytotoxic compounds in 8 h time period (Fig. 3B).

Dose-response analyses in AIZ-AR cells treated with a panel of steroids

To assess selectivity of new AIZ-AR cell line towards androgens, we performed dose-response agonist and antagonist analyses using 23 different endogenous and synthetic steroids. Prior to

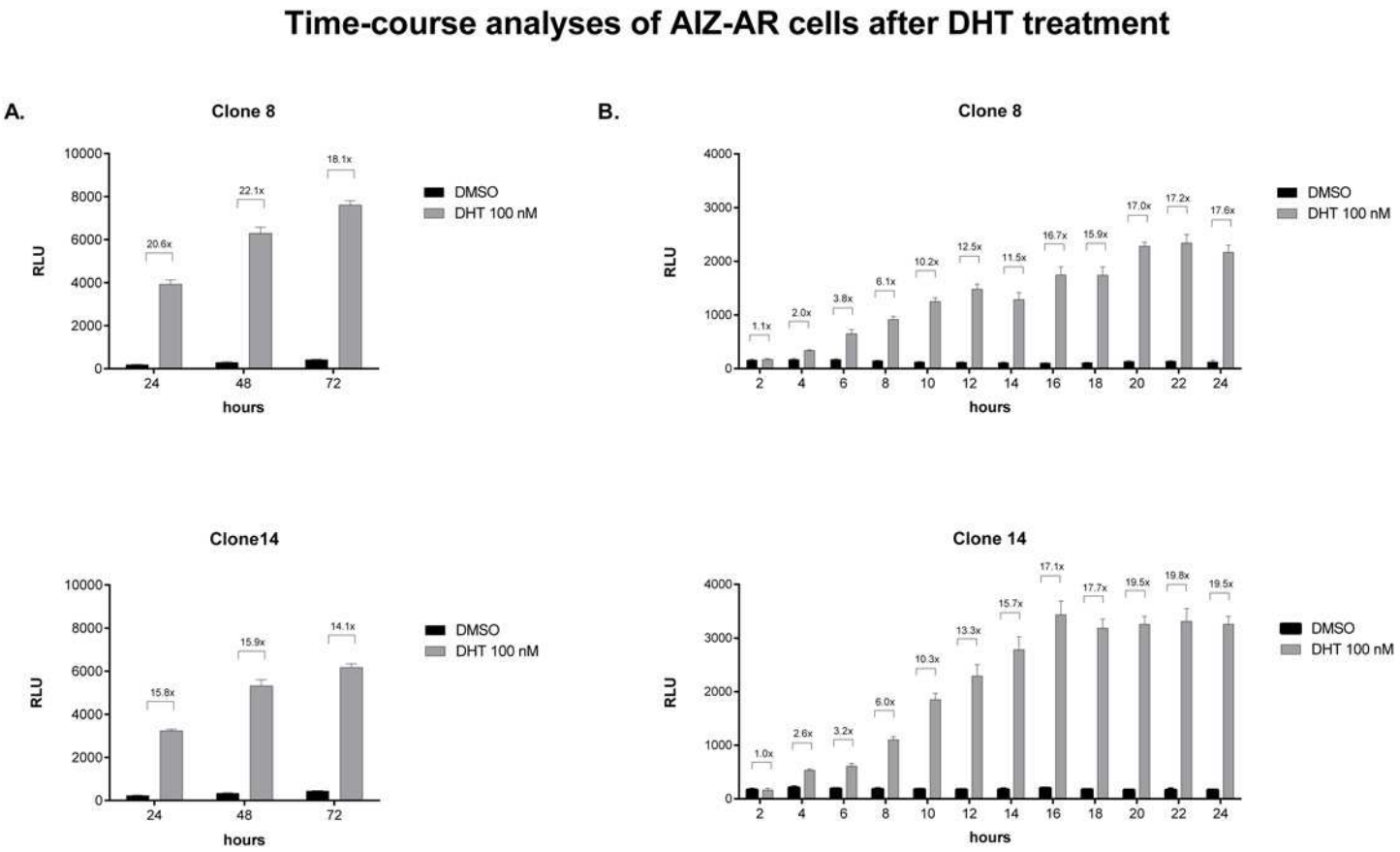

Fig 3. Time-course analyses in AIZ-AR cells after DHT treatment. Cells were treated with 5α-dihydrotestosterone (DHT; 100 nM) and vehicle (DMSO; 0.1% v/v) for time periods from 2 h to 72 h. After the incubations, luciferase activity was measured in cell lysates. Data are mean of triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated and it is shown above the bars in the graph. Similar data were obtained from two consecutive passages. Panel A: Long-term time-course analyses; incubation times: 24 h, 48 h and 72 h. Panel B: Short-term time-course analyses; incubation times: 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h and 24 h.

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the experiments, we have tested: (i) Cytotoxicity of tested compounds (up to 10 μM) using standard MTT test as described in the methods. Of 23 substances, significant cytotoxicity after 24 h was observed for spironolactone, mifepristone, raloxifene hydrochloride, 4-hydroxytamoxifen and tamoxifen citrate, with IC_{50} values ranging from 1.2 μM to 5.4 μM (Table 2); (ii) Inhibition of firefly luciferase catalytic activity, as described in methods. With exception of genistein, none of tested steroids significantly influenced luciferase activity (data not shown). Overall, effects of tested compound on cell viability and luciferase activity should be taken in account when interpreting the data from gene reporter assays.

Dose-response analyses assays were performed in two different experimental layouts. In agonist mode, cells were treated with increasing concentrations of tested compounds and EC_{50} (half maximal effective concentration) values were calculated. In antagonist mode, cells were treated with increasing concentrations of tested compounds in combination with model AR agonists DHT (100 nM) or testosterone (10 nM). Where appropriate, IC_{50} (half maximal inhibitory concentration) values were calculated.

In agonist mode, androgens testosterone, DHT and danazol produced typical dose-dependent sigmoid curves. Antiandrogens cyproterone acetate and spironolactone displayed

Table 2. Viability of AIZ-AR cells after treatment with 23 steroid compounds. Data were calculated from triplicate measurements and are expressed as mean ± SD. Analyses were performed in two independent experiments.

Compound	log c/ % of viability					
	-10	-9	-8	-7	-6	-5
5α-dihydrotestosterone	100.0	107.5 ± 4.6	108.7 ± 2.1	114.4 ± 5.7	107.7 ± 2.6	104.1 ± 2.0
Testosterone	100.0	108.6 ± 1.0	108.0 ± 4.8	102.9 ± 5.6	105.4 ± 6.9	105.3 ± 1.0
Danazol	100.0	106.5 ± 1.5	108.1 ± 6.9	109.0 ± 10.6	108.2 ± 11.6	113.6 ± 12.1
Cyproterone acetate	100.0	105.3 ± 1.7	104.7 ± 2.0	105.8 ± 5.7	99.4 ± 7.8	89.5 ± 6.6
Spironolactone	100.0	107.7 ± 1.5	111.8 ± 5.7	112.6 ± 10.0	98.9 ± 5.5	58.9 ± 2.5
Dexamethasone	100.0	99.8 ± 3.9	114.0 ± 2.0	112.7 ± 1.1	111.4 ± 3.4	108.3 ± 2.6
Beclomethasone	100.0	99.9 ± 3.9	106.5 ± 6.1	109.2 ± 5.7	98.4 ± 0.9	92.3 ± 2.1
Betamethasone	100.0	104.9 ± 4.0	110.0 ± 7.3	109.1 ± 7.9	108.0 ± 14.6	101.3 ± 9.9
Cortisol	100.0	109.1 ± 4.1	106.4 ± 9.1	107.3 ± 13.2	107.4 ± 12.3	105.4 ± 10.4
Triamcinolone	100.0	101.0 ± 4.2	100.0 ± 8.2	105.2 ± 11.4	103.2 ± 11.0	101.2 ± 5.4
Prednisolone	100.0	100.7 ± 0.8	103.8 ± 3.7	106.9 ± 5.2	104.2 ± 5.2	101.0 ± 8.3
Methyl-prednisolone	100.0	106.8 ± 3.1	111.4 ± 9.4	104.8 ± 8.9	104.6 ± 14.3	104.9 ± 16.0
Mifepristone	100.0	104.0 ± 2.8	102.2 ± 9.7	103.9 ± 18.2	94.7 ± 9.5	41.7 ± 1.1
Corticosterone	100.0	104.0 ± 0.0	107.3 ± 2.5	110.8 ± 5.7	109.2 ± 6.5	105.2 ± 3.4
Aldosterone	100.0	110.4 ± 1.4	99.4 ± 1.0	99.0 ± 1.4	98.6 ± 0.8	97.0 ± 5.4
17α-progesterone	100.0	98.2 ± 2.6	95.0 ± 4.0	95.0 ± 4.0	92.0 ± 3.7	92.0 ± 3.7
Progesterone	100.0	98.7 ± 7.3	98.7 ± 12.2	99.8 ± 14.1	98.1 ± 13.9	91.0 ± 9.5
17β-estradiole	100.0	106.6 ± 4.3	110.5 ± 3.2	110.2 ± 6.2	110.2 ± 6.2	116.4 ± 16.4
Genistein	100.0	96.5 ± 2.4	96.7 ± 3.3	102.9 ± 7.5	102.9 ± 7.5	119.9 ± 6.4
Diethylstilbestrol	100.0	100.7 ± 5.1	102.1 ± 7.6	101.7 ± 5.1	98.0 ± 4.3	99.9 ± 7.0
4-hydroxytamoxifen	100.0	101.8 ± 1.0	100.0 ± 5.4	103.6 ± 4.4	105.9 ± 3.8	9.2 ± 7.7
Raloxifene hydrochloride	100.0	102.9 ± 8.5	99.8 ± 4.3	105.7 ± 6.8	95.9 ± 10.3	7.1 ± 0.2
Tamoxifen citrate	100.0	102.7 ± 4.3	107.0 ± 4.6	105.5 ± 3.8	88.5 ± 2.4	1.7 ± 0.8

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partial agonist and antagonist patterns, respectively (Fig. 4; Table 3). Mineralocorticoid aldosterone and estrogens (with exception of 17β-estradiol) did not significantly increased luciferase activity. Progesterone, 17β-estradiol and some glucocorticoids, in particular triamcinolone, corticosterone and cortisol, also induced luciferase activity, but their potencies were 2–3 orders of magnitude weaker as compared to androgens (Fig. 4; Table 3).

In antagonist mode, antiandrogens cyproterone acetate and spironolactone dose-dependently inhibited testosterone- and/or DHT-induced luciferase activity. Interestingly, the IC_{50} values for these antiandrogens were approximately 10 times higher in DHT-treated cells as compared to testosterone-treated cells (Fig. 5, Fig. 6; Table 3). Corticoids (except mifepristone, which caused dose-dependent inhibition) yielded an additive effect in concentrations up to 10^{-6} M, resulting in augmentation of luciferase activity in comparison to agonist itself (DHT or testosterone). Interestingly, in concentration of 10^{-5} M, luciferase activity remained augmented when DHT was used as agonist, while it dropped to 60%- 120% of initial agonist-induced activity, when testosterone was used as agonist (Fig. 5, Fig. 6; Table 3).

Inhibitory effects of some estrogens and gestagenes were rather due to their intrinsic cytotoxicity than due to receptor antagonism itself. Interestingly, genistein strongly augmented agonist-induced luciferase activity, with massive drop at 10^{-5} M, which is probably due to the luciferase inhibition. Collectively, AIZ-AR cell line allows effective detection of compounds

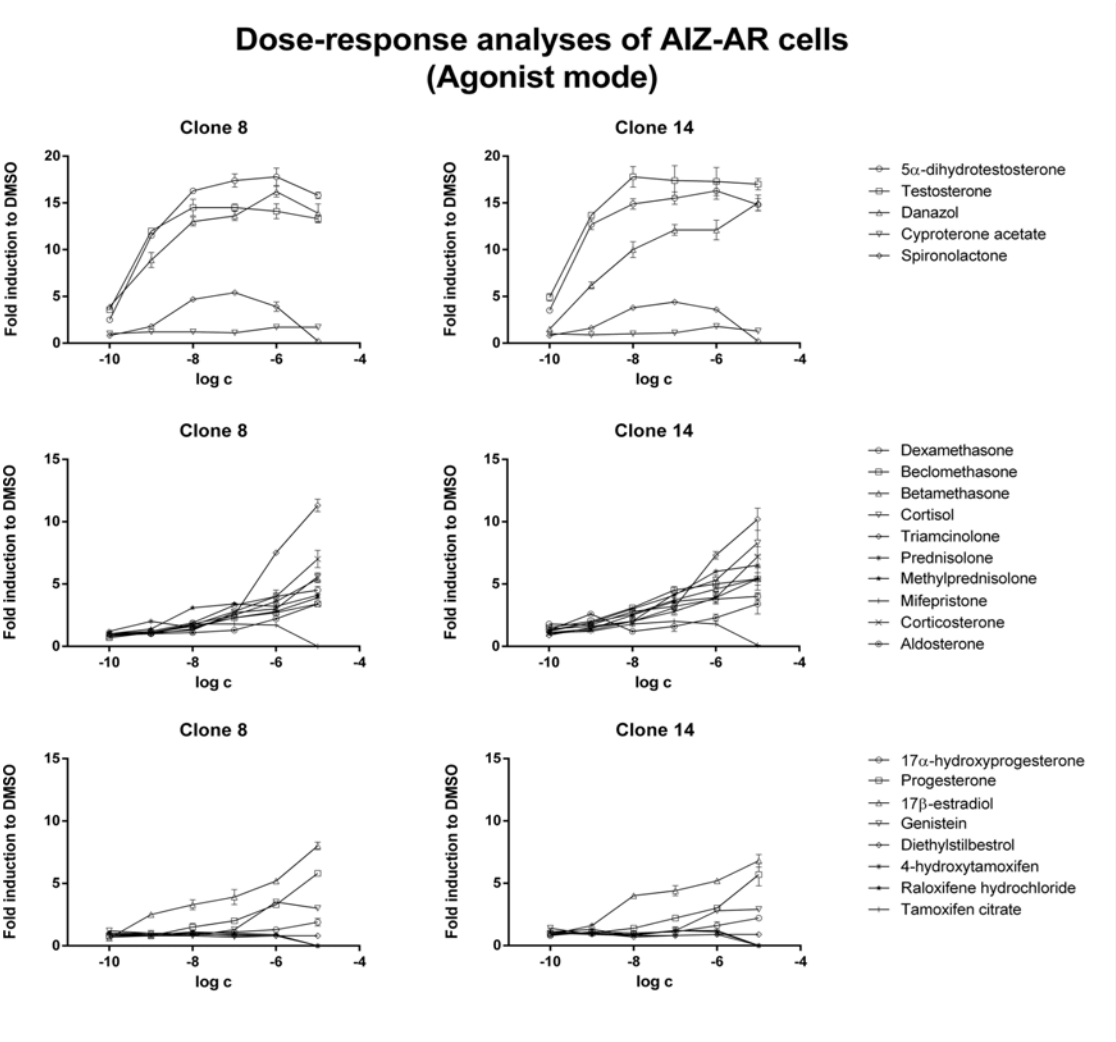

Fig 4. Dose-response analyses in AIZ-AR cells after treatment with steroid compounds—agonist mode. Cells were treated for 24 h with various endogenous and synthetic steroids. Cells were lysed and luciferase activity was measured. Data are mean of triplicate measurements and are expressed as a fold induction over DMSO-treated cells. Similar data were obtained from three consecutive cell passages. Upper plots—androgens, middle plots—corticoids, lower plots—gestagens and estrogens.

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with androgenic activity. Some cross-reactivity with glucocorticoids occurs in concentrations of 2–3 orders of magnitude higher as compared to androgens. In antagonist experimentation, different profiles depending on the agonist used, possible cytotoxicity and luciferase inhibition must be taken in account when interpreting the data.

Discussion

In the current paper, we present a novel stably transfected human gene reporter cell line for assessment of human AR transcriptional activity. Human AIZ-AR cell line expresses endogenous functional human AR and it was transfected with reporter plasmid containing sequence of androgen response element from promoter of human prostate-specific antigen (PSA). AIZ-AR cell line provides a tool for high-throughput and sensitive identification and characterization

Table 3. Characteristics of AIZ-AR cells in comparison with published data. EC_{50}—half maximal effective concentration; IC_{50}—half maximal inhibitory concentration; n.c.—not calculated; n.d.—not determined.

Compound	Log EC_{50}		Log IC_{50} (DHT 100nM)		Log IC_{50} (Testosterone 10 nM)		Literature Log EC_{50}	
	Clone 8	Clone 14	Clone 8	Clone 14	Clone 8	Clone 14	Sedlak *et al.*	Wilkinson *et al.*
5α-dihydrotestosterone	-9.16 ± 0.09	-9.19 ± 0.10	-	-	> -5.00	> -5.00	-11.38 ± 0.21	-9.52
Testosterone	-8.92 ± 0.33	-9.10 ± 0.09	> -5.00	> -5.00	-	-	-10.00 ± 0.26	-11.37
Danazol	-8.29 ± 0.34	-8.13 ± 0.12	> -5.00	> -5.00	> -5.00	> -5.00	-9.38 ± 0.17	-9.47
Cyproterone acetate	-6.69 ± 0.17	-6.65 ± 0.51	-6.75 ± 0.05	-6.62 ± 0.02	-7.50 ± 0.04	-7.52 ± 0.06	-8.02 ± 0.19	> -5.00
Spironolactone	-8.80 ± 0.41	-8.36 ± 0.26	-6.37 ± 0.10	-6.17 ± 0.19	-6.72 ± 0.12	-6.65 ± 0.04	-7.52 ± 0.12	> -5.00
Dexamethasone	-7.58 ± 0.31	-7.59 ± 0.27	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
Beclomethasone	-7.59 ± 0.41	-7.70 ± 0.10	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Betamethasone	-6.62 ± 0.04	-7.15 ± 0.26	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Cortisol	-6.35 ± 0.11	-6.49 ± 0.04	n.c.	n.c.	n.c.	n.c.	> -5.00	-8.22
Triamcinolone	-6.37 ± 0.13	-6.56 ± 0.19	n.c.	n.c.	n.c.	n.c.	> -5.00	n.d.
Prednisolone	-6.61 ± 0.09	-7.10 ± 0.38	n.c.	n.c.	n.c.	n.c.	n.d.	n.d.
Methyl-prednisolone	-7.60 ± 0.43	-7.48 ± 0.30	n.c.	n.c.	n.c.	n.c.	n.d.	-8.75
Mifepristone	-5.81 ± 0.05	-5.86 ± 0.06	-6.15 ± 0.10	-6.08 ± 0.03	-6.88 ± 0.20	-6.95 ± 0.21	-7.89 ± 0.13	> -5.00
Corticosterone	-6.22 ± 0.08	-6.25 ± 0.11	n.c.	n.c.	n.c.	n.c.	> -5.00	-8.22
Aldosterone	-6.03 ± 0.04	-6.11 ± 0.02	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
17α-progesterone	-6.45 ± 0.16	-6.29 ± 0.03	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	n.d.
Progesterone	-6.13 ± 0.08	-6.22 ± 0.12	> -5.00	> -5.00	> -5.00	> -5.00	-7.85 ± 0.11	-7.55
17β-estradiole	-7.19 ± 0.20	-7.54 ± 0.01	> -5.00	> -5.00	> -5.00	> -5.00	-7.30 ± 0.25	> -5.00
Genistein	-6.62 ± 0.42	-6.79 ± 0.23	n.c.	n.c.	n.c.	n.c.	> -5.00	> -5.00
Diethylstilbestrol	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
4-hydroxytamoxifen	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
Raloxifene hydrochloride	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00
Tamoxifen citrate	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	> -5.00	n.d.

Data were calculated from triplicate measuments and are expressed as mean ± SD. Analyses were performed in three independent experiments for each clone.

doi:10.1371/journal.pone.0121316.t003

of compounds with androgenic and anti-androgenic activity. Cell line AIZ-AR allows detection of androgens as soon as after 8 hours of incubation, and it remains fully functional for more than 28 passages and over 67 days in culture as well as after freeze/thaw cycle.

Reliable, rapid, sensitive, selective and high throughput tools for assessment of transcriptional activities of nuclear receptors, steroid receptors and xenoreceptors, are needed for various purposes. Drugs targeting androgen receptor are widely used in human pharmacotherapy, e.g. non-steroid antiandrogen flutamide for the treatment of prostate cancer. Therefore, *in vitro* tool for identification and characterization of synthetic androgens and antiandrogens in the process of drug design and development is of value. Since androgen receptor active substances influence hormonal homeostasis, they are referred as to endocrine disruptors. Indeed, there are numerous reports on the use of gene reporter assays in environmental [12], cosmetics [13] or food safety applications [14].

Experimental models differ in their complexity and species-specificity, which has an impact on the reliable and credible transfer of the data to human pharmacology and toxicology. Besides the properties indicated above, the major strengths of AIZ-AR cell line presented here

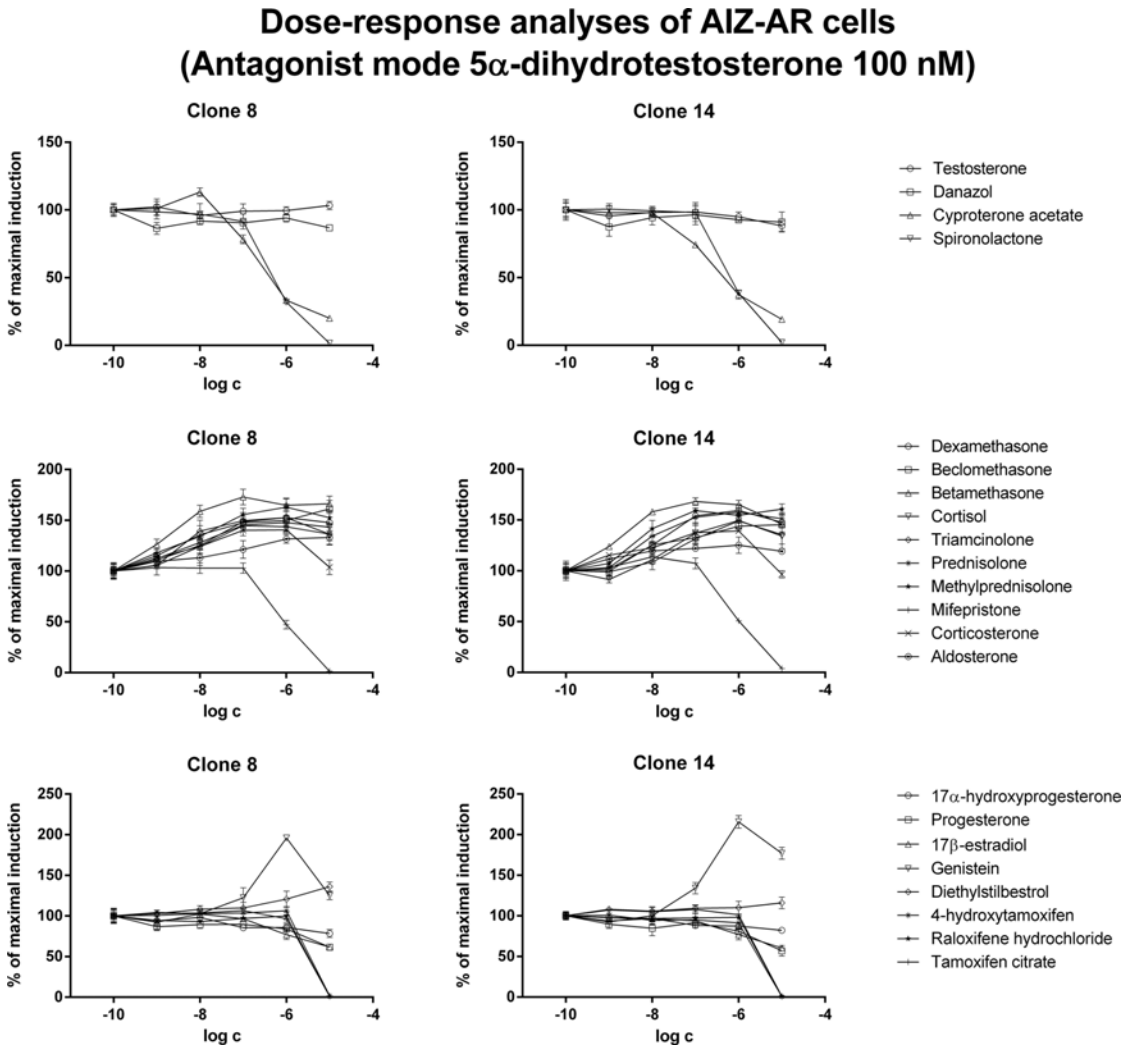

Fig 5. Dose-response analyses in AIZ-AR cells after treatment with steroid compounds—antagonist mode with dihydrotestosterone. Cells were treated for 24 h with various endogenous and synthetic steroids in the presence of 5α-dihydrotestosterone (DHT; 100 nM). Cells were lysed and luciferase activity was measured. Data are mean of triplicate measurements and are expressed as a fold induction over DMSO-treated cells. Similar data were obtained from three consecutive cell passages. Upper plots—androgens, middle plots—corticoids, lower plots—gestagens and estrogens.

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are: (i) AIZ-AR cell line is an exclusively human system; i.e. human maternal cell line, containing endogenous human receptor AR, stably transfected with reporter gene driven by binding sequence from human gene. (ii) AIZ-AR cell line conserves cell signaling stoichiometry; since AIZ-AR cell line contains endogenous human AR, without extra co-transfected AR vector, the stoichiometric ratio between the AR receptor protein and other transcriptional regulators reflects natural situation rather than artificial one with over-expressed AR. The characteristics given above clearly demonstrate significant advancements and added value for AIZ-AR cell line, as compared to yet developed cell lines. Indeed, existing experimental models, such as human AR-LUX [9] and MDA-kb2 lines [10] were transfected with reporters containing rodent promoters but not human ones. In addition cell lines human PALM [8] and AR CALUX [11] are transfected with exogenous AR, therefore, over-expressing AR vector.

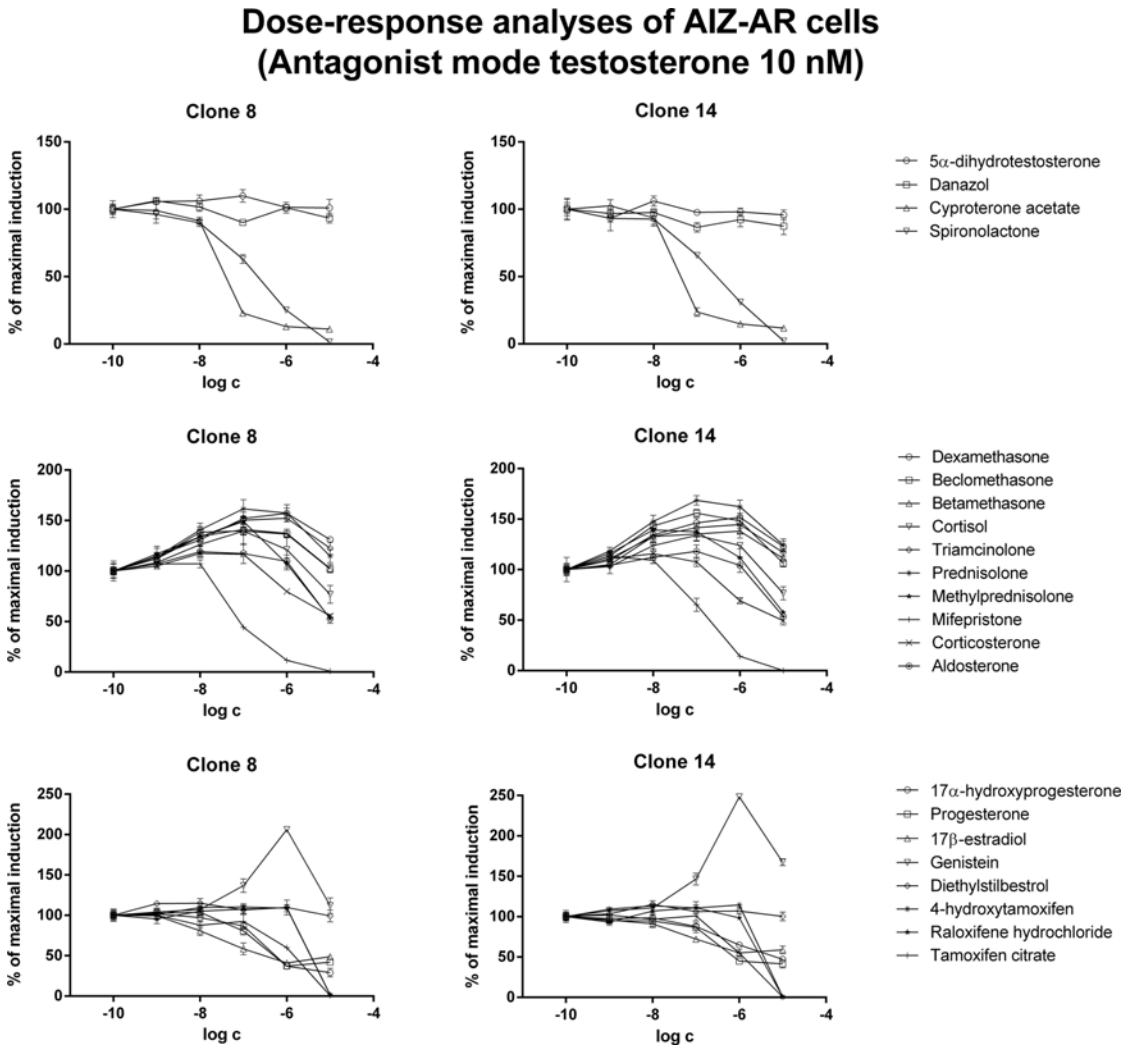

Fig 6. Dose-response analyses in AIZ-AR cells after treatment with steroid compounds—antagonist mode with testosterone. Cells were treated for 24 h with various endogenous and synthetic steroids in the presence of testosterone (10 nM). Cells were lysed and luciferase activity was measured. Data are mean of triplicate measurements and are expressed as a fold induction over DMSO-treated cells. Similar data were obtained from three consecutive cell passages. Upper plots—androgens, middle plots—corticoids, lower plots—gestagens and estrogens.

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Author Contributions

Conceived and designed the experiments: IB AN ZD. Performed the experiments: IB AN. Analyzed the data: IB AN ZD. Contributed reagents/materials/analysis tools: ZD. Wrote the paper: IB ZD.

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APPENDIX II:

Bartonkova I, Novotna A, Dvorak Z (2016): Profiling of vitamin D metabolic intermediates towards VDR using novel stable gene reporter cell lines IZ-VDRE and IZ-CYP24. *Chem Res Toxicol*. 29. 1211 - 1222 [IF_{2015} 3.025]

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Profiling of Vitamin D Metabolic Intermediates toward VDR Using Novel Stable Gene Reporter Cell Lines IZ-VDRE and IZ-CYP24

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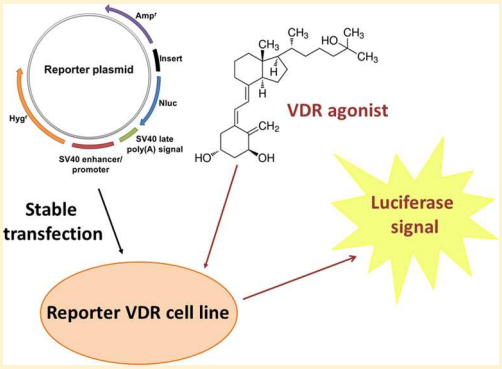
ABSTRACT: Variety of xenobiotics, including therapeutically used vitamin D analogues or environmental and alimentary endocrine disruptors, may interfere with vitamin D receptor (VDR) signaling, with serious physiological or pathophysiological consequences. Therefore, it is of topical interest to have reliable and efficient *in vitro* screening tools for the identification of agonists and activators of human VDR. We present here two novel stably transfected human reporter cell lines allowing rapid, high-throughput, and selective identification of VDR agonists and activators. Human colon adenocarcinoma cells LS180 were stably transfected with reporter plasmids CYP24_minP-pNL2.1[Nluc/Hygro] (IZ-CYP24 cells contain the -326/-46 sequence from the human CYP24A1 promoter) or VDREI3_SV40-pNL2.1[Nluc/Hygro] (IZ-VDRE cells contain three copies of vitamin D response elements VDRE-I from the human *CYP24A1* promoter). Both cell lines remained fully functional for over two months in the culture and also after cryopreservation. Luciferase inductions ranged from 10-fold to 25-fold (RLU 10^6-10^7) and from 30-fold to 80-fold (RLU 10^3-10^4) in IZ-VDRE and IZ-CYP24 cells, respectively. Time-course analyses revealed that detection of VDR activators is possible as soon as after 8 h of incubation. Cell lines were highly selective toward VDR agonists, displaying no cross-activation by retinoids, thyroids, and steroids. As a proof of concept, we used IZ-VDRE and IZ-CYP24 cells for profiling analogues of vitamin D, and intermediates in vitamin D2 and vitamin D3 metabolic pathways against VDR transcriptional activity. The data obtained revealed significant activation of VDR not only by obligatory ligands calcitriol and ergocalcitriol but also by their precursors and degradation products.



INTRODUCTION

The vitamin D receptor (VDR, NR1I1) is a member of the nuclear hormone receptor superfamily, and it acts as a ligand-activated transcriptional factor. Liganded and unliganded VDRs carry out distinct functions; both types of functions require heterodimerization with retinoid X receptors (RXR, NR2B1). The regulation of VDR nuclear import by RXR is essential for ligand-independent functions of VDR.[1] The VDR-RXR heterodimers bind specific DNA sequences designated as vitamin D response elements (VDREs), which are hexameric half-sites separated by 3 nucleotides, also known as the DR3 motif.[2] Vitamin D response elements were found in promoter sequences of several human genes, e.g., osteocalcin,[3] ILGF binding protein,[4] PTH,[5] 25-hydroxyvitamin D_3-24-hydroxylase (*CYP24*),[6,7] etc. There are two major forms of vitamin D, i.e., vitamin D2 and vitamin D3, which differ in side-chain structure. The endogenous ligands of VDR, vitamin D2 and vitamin D3, undergo a tightly regulated metabolic activation and inactivation. The main source of vitamin D in humans is a UV light-induced photolytic conversion of its precursor, ergosterol or 7-dehydrocholesterol, to previtamin D followed by its isomerization to vitamin D2 or vitamin D3, i.e., ergocalciferol or cholecalciferol, respectively.[8,9] The next step is a formation of 25-hydroxyvitamin D2 (ergocalcidiol) or 25-hydroxyvitamin D3 (calcifediol), followed by hydroxylation to 1α,25-dihydroxyvitamin D2 (ergocalcitriol) or 1α,25-dihydroxyvitamin D3 (calcitriol), which are the active forms of vitamin D. Simultaneously, 24,25-dihydroxyvitamin D2 and 24,25-dihydroxyvitamin D3 (secalciferol), byproducts with negative feedback function, are produced. A common and ultimate degradation product for vitamin D2 and vitamin D3 pathways is water-soluble calcitroic acid.[10] Vitamin D is essential for calcium homeostasis and bone metabolism. It plays roles in the induction of cell differentiation, inhibition of cell proliferation, and modulation of the immune system. Any decline from the proper homeostasis of vitamin D results in the pathophysiological status of the organism. At least three areas are covered in this respect: (i) pathophysiology. Vitamin D deficiency was historically associated with childhood rickets and adult osteomalacia, but it is also linked to autoimmune disorders such as multiple sclerosis or psoriasis, chronic systemic illnesses including cancer as well as cardiovascular and neurodegenerative disease.[11] Various synthetic analogues of vitamin D were developed for the treatment of hyperproliferative diseases, such as psoriasis,[12] osteoporosis,[13] or secondary hyperparathyroidism.[14] (ii) Endocrine disruption by chemicals: steroid and nuclear receptors, including biosynthetic pathways for their ligands, are the main targets for the so-called endocrine disrupting chemicals.[15] These compounds are common environmental pollutants and food contaminants. (iii) Drug interactions: there

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exist multiple cross-talks between cell signaling by nuclear receptors and by transcriptional regulation of xenobiotics metabolizing enzymes. For instance, an activation of PXR or CAR by xenobiotics results in drug-induced osteomalacia, probably involving the perturbation of the vitamin D metabolic pathway at the transcriptional level.[16,17]

Given the reasons described above, the rationale for the current study was to develop and characterize a cell-based experimental model allowing the assessment of VDR transcriptional activity for various applications including drug-development, food safety, and environmental analyses. Different approaches were used, including the measurement of the expression of VDR target genes or transient transfection gene reporter assays, but the effectivity and robustness of such techniques is limited. Therefore, we employed the strategy of cell lines harboring endogenously expressed and functional VDR with a stably transfected gene reporter. In the current work, we present two novel stably transfected human reporter cell lines IZ-VDRE and IZ-CYP24 allowing rapid, sensitive, high-throughput, and selective identification of VDR agonists and activators. Cell lines were derived from human colon adenocarcinoma cells LS180 stably transfected with nanoluciferase reporter plasmids. Cell lines remained fully functional for over two months in the culture and also after cryopreservation. As a proof of concept, we used IZ-VDRE and IZ-CYP24 cells for profiling analogues of vitamin D, and intermediates in vitamin D2 and vitamin D3 metabolic pathways against VDR transcriptional activity. The data obtained revealed significant activation of VDR not only by obligatory ligands calcitriol and ergocalcitriol but also by their precursors and degradation products. Overall, developed cell lines could be an appropriate tool for toxicological, environmental, and food safety applications. Given the involvement of VDR in prostate cancer and tumor growth, the cell lines are also a suitable model exploitable in fundamental research in human oncology and endocrinology.[18]

MATERIALS AND METHODS

Chemicals and Reagents. DMSO, 5α-dihydrotestosterone, dexamethasone, aldosterone, progesterone, 17β-estradiol 3,3′,5-triiodo-L-thyronine, and hygromycin B were purchased from Sigma-Aldrich (Prague, Czech Republic). All-*trans*-retinoic acid, 9-*cis*-retinoic acid, 7-dehydrocholesterol, vitamin D3 (cholecalciferol), EB 1089 (seocalcitol), 1α,25-dihydroxyvitamin D3 (calcitriol), 1α,25-dihydroxyvitamin D2 (ergocalcitriol), ZK 159222, and 22-oxacalcitriol were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calcitroic acid, 25-hydroxyvitamin D3 (calcifediol), 1α-hydroxyvitamin D3 (alfacalcidol), 24*R*,25-dihydroxyvitamin D3 (secalciferol), tacalcitol monohydrate, ergosterol, vitamin D2 (ergocalciferol), 25-hydroxyvitamin D2 (ergocalcidiol), and 1α-hydroxyvitamin D2 (doxercalciferol) were from Toronto Research Centre Inc. (Toronto, Canada). Fugene HD transfection reagent was purchased from Roche (Basel, Switzerland). Reporter lysis buffer was from Promega (Hercules, CA, USA). All other chemicals were of the highest quality commercially available.

Cell Line. Human Caucasian colon adenocarcinoma cell line LS 180 (ECACC No. 87021202) was purchased from European Collection of Cell Cultures (*ECACC*) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum, 100 U/mL streptomycin, 100 μg/mL penicilin, 4 mM L-glutamine, 1% nonessential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Construction of Reporter Plasmids. Reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] was designed as follows:[16] a partial sequence (−326/−46) from the promoter region of human *CYP24A1* gene containing two VDREs, VDRE-I (−174/−151), and VDRE-II (−194/−174), followed by a minimal promoter sequence (41 bp) as a control of luciferase expression was inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and *Hin*dIII restriction enzymes. Reporter plasmid VDREI3_SV40-pNL2.1-[Nluc/Hygro] was designed as follows: three copies of VDRE-I (−174/−151) from the promoter region of human *CYP24A1* gene followed by a basic SV40 promoter with a deleted 5′ enhancer region as a control of luciferase expression was inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and *Hin*dIII restriction enzymes.

Development of Stably Transfected Cell Lines. Human Caucasian colon adenocarcinoma cell line LS 180 was transfected with reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] (for IZ-CYP24 cell line) or VDREI3_SV40-pNL2.1[Nluc/Hygro] (for IZ-VDRE cell line), using the Fugene HD transfection reagent at a ratio of 3:1 (reagent/DNA) and seeded at a density of 1×10^6 cells in 60 mm culture dishes in 5 mL of the DMEM culture medium. After 24 h of incubation, the selection medium supplemented with hygromycin B at a concentration of 0.25 mg/mL was applied. The selection medium was replaced every 3−4 days for 3 weeks until polyclonal populations of stably transfected cells were selected.

Polyclonal populations with the highest induction of luciferase activity by 1α,25-dihydrovitamin D3 were selected for development of monoclonal populations. For this purpose, cells were seeded in 100 mm culture dishes at a density of 100−1000 cells per dish and cultured for an additional 4 weeks in the selection medium until small colonies were visible. Thereafter, individual colonies were subcloned in 24-well plates and cultured for an additional 3 weeks in the selection medium to obtain monoclonal populations of stably transfected cells. One clone of each monoclonal population of IZ-CYP24 and IZ-VDRE cells was selected for further characterization. The use of GMO at Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref 91997/ENV/10).

Cytotoxicity Assay (MTT Test). IZ-CYP24 and IZ-VDRE cells were seeded in 96-well plates at density 25×10^3 cells per well in charcoal stripped DMEM. Following 24 h of stabilization, cells were treated with increasing concentrations of tested compounds (from 100 pM to 10 μM) and vehicle (DMSO; 0.1% v/v). After 24 h of incubation, the medium was replaced with medium supplemented with 10% of MTT (3 mg/mL) and incubated for an additional 30 min, and the MTT assay was measured spectrophotometrically at 540 nm using a Tecan Infinite M2000 plate luminometer.

Inhibition of Luciferase Assay. Cell lysates containing nanoluciferase were mixed with tested compounds in the highest tested concentration (10 μM), and luciferase activity was measured. Decline in luciferase activity less than 15% was considered as not impeding with the assay.

Gene Reporter Assay. IZ-CYP24 and IZ-VDRE cells were seeded in 96-well plates at a density of 25×10^3 cells per well in charcoal stripped DMEM. After 16 h of stabilization, cells were treated with tested compounds (from 100 pM to 10 μM) and vehicle (DMSO; 0.1% v/v). Following the incubation, cells were lysed, and luciferase activity was measured in 96-well plate format using a Tecan Infinite M2000 plate luminometer.

Statistical Analyses. Student's pair *t* test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA (www.graphpad.com)).

RESULTS

Generation of Stably Transfected Cell Lines IZ-CYP24 and IZ-VDRE. Human Caucasian colon adenocarcinoma cell line LS 180 was transfected using lipofection with reporter plasmid CYP24_minP-pNL2.1 [Nluc/Hygro] or VDREI3_SV40-pNL2.1 [Nluc/Hygro] to generate IZ-CYP24 and IZ-VDRE cells, respectively. Stably transfected monoclonal populations of IZ-CYP24 and IZ-VDRE cells were generated as described in Materials and Methods section. We obtained 11 and 30 hygromycin B-resistant clones of IZ-CYP24 and IZ-VDRE cells, respectively, displaying the same morphology as the parental LS 180 cell line. Responsiveness of clones to calcitriol (50 nM; 24 h),

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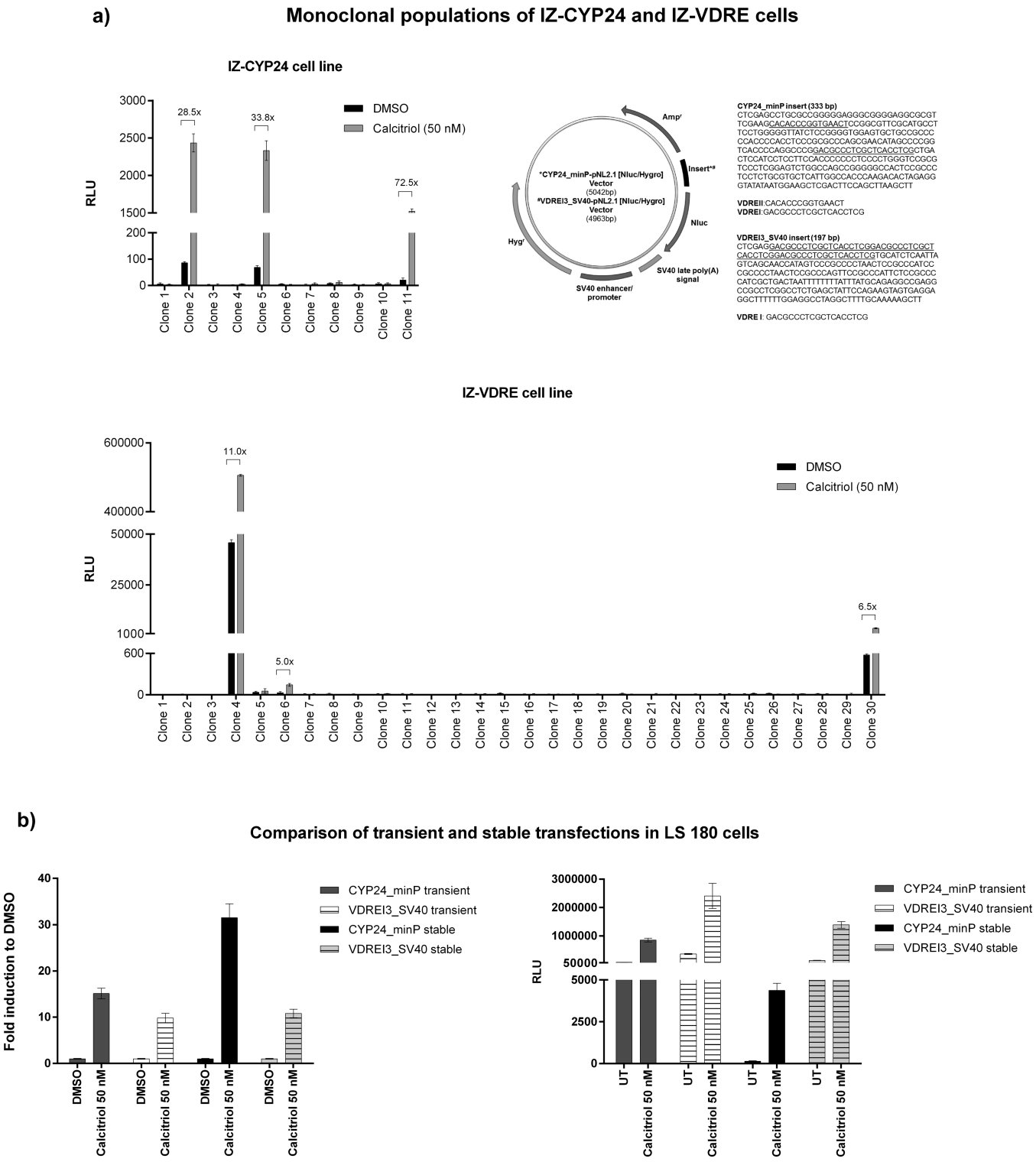

Figure 1. Panel A: Monoclonal populations of IZ-CYP24 and IZ-VDRE cells. Cells were treated for 24 h with calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v). Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated, and it is indicated above calcitriol-inducible clones in the bar graphs. Similar data were obtained from 3 consecutive passages. Upper left panel, IZ-CYP24 cell line; upper right panel, scheme of reporter plasmids used for stable transfection; lower panel, IZ-VDRE cell line. Panel B: Comparison of transient and stable transfections of LS 180 cells. Cells were treated for 24 h with calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v). Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as relative luciferase units (RLU) and as fold induction over control cells. Similar data were obtained from 3 independent transfections.

as a model VDR agonist, was tested. We obtained three calcitriol-inducible clones of each cell line, displaying fold inductions in the range from 29-fold to 73-fold (RLU 10^3−10^4) and 5-fold to 11-fold (RLU 10^6−10^7) for IZ-CYP24 and IZ-VDRE cells, respectively (Figure 1A). On the basis of the values of RLU and fold-inductions, clone 2 and clone 4 were selected for detailed characterization of IZ-CYP24 and IZ-VDRE cells, respectively. Comparative experiments in transiently and stably transfected

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LS 180 cells revealed that stable transfection has either no effect (IZ-VDRE) or improves (IZ-CYP24) the responsivity of cells to calcitriol. The sensitivity of IZ-VDRE cells was similar to that in LS 180 cells transiently transfected with the VDREI3_SV40 plasmid. In contrast, sensitivity of IZ-CYP24 cells drastically decreased in comparison with that of cells transiently transfected with the CYP24_minP plasmid (Figure 1B).

Functionality of IZ-CYP24 and IZ-VDRE Cells after Cryopreservation. We tested whether IZ-CYP24 and IZ-VDRE cell lines maintain luciferase induction by VDR agonists after cryopreservation (freeze−thaw cycle). Cells were frozen down-to −80 °C in 2 mL cryotubes, using the mixture of fetal bovine serum and DMSO (ratio 9:1) as a cryoprotectant, and stored at −80 °C for 2 weeks. Both thawed and freshly passaged IZ-CYP24 and IZ-VDRE cells were seeded at 96-well plates, stabilized for 16 h, and then incubated with increasing concentrations of calcitriol and cholecalciferol (from 0.1 nM to 10 μM). Although a slight drop in fold inductions was observed in cells after cryopreservation as compared to fresh ones, potency (EC_{50} values) was not altered (Figure 2). Therefore, IZ-CYP24 and IZ-VDRE cell lines can be considered to remain fully functional after cryopreservation.

Long-term maintenance of luciferase inducibility in IZ-CYP24 and IZ-VDRE cell lines. The maintenance of luciferase induction by calcitriol for the long-term was continuously tested. For this purpose, cells were incubated with 50 nM calcitriol for 24 h after each passage. We show that both cell lines remained functional for more than 78 days, which corresponds to 32 passages (Table 1).

Time-Course Analyses of Luciferase Induction in IZ-CYP24 and IZ-VDRE Cell Lines. We analyzed luciferase induction by calcitriol in long and short time periods, in order to find minimal and optimal incubation periods for reliable identification of VDR agonists and activators. Cell lines IZ-CYP24 and IZ-VDRE were seeded in 96-well plates, and following 16 h of stabilization, cells were incubated with 50 nM calcitriol for 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h, 24 h, 48 h, and 72 h.

In long incubation times (24 h, 48 h, and 72 h), we observed a progressive increase of luciferase signal ranging approximately from 4×10^3 to 1.4×10^4 RLU and from 1.3×10^6 to 7.1×10^6 RLU in IZ-CYP24 and IZ-VDRE cells, respectively (Figure 3). Also, fold inductions by calcitriol over DMSO-treated cells increased with time of incubation, from 32-fold to 58-fold and from 11-fold to 27-fold in IZ-CYP24 and IZ-VDRE cells, respectively (Figure 3). In both cell lines, the strength of the signal (RLU) and magnitude of induction were high enough to allow performing reliable luciferase assays already after 24 h. Therefore, the incubation period of 24 h was applied in subsequent experiments, and it was considered as the optimal time of incubation.

For the reasons of intrinsic cytotoxicity of many compounds, it is desirable to determine the minimal incubation time needed for reliable detection of VDR activators. Therefore, short time experiments were also carried out. We observed the time-dependent increase of both RLU and fold induction in both cell lines incubated with calcitriol. Differential induction of luciferase was observed in either cell line (compared at 24 h of incubation), i.e., high RLU (10^6) and moderate magnitude of induction (11-fold) in IZ-VDRE cells versus moderate RLU (10^3) and higher magnitude of induction (32-fold) in IZ-CYP24 cells. Therefore, the minimal incubation times were estimated to be 6 h−8 and 8 h−10 h for IZ-CYP24 and IZ-VDRE cells, respectively. Overall, incubation for 8 h can be considered as

Table 1. Long-Term Maintenance of the Responsivness of IZ-CYP24 and 1Z-VDRE Cells to Calcitriol

	IZ-CYP24 cell line			1Z-VDRE cell line		
days in culture	passage	RLU	FOLD	passage	RLU	FOLD
7	4	6984	31.0	3	505902	11.0
20	8	1930	21.7	8	217855	8.9
22	9	2438	28.5	9	458518	10.1
27	11	2977	33.4	11	440674	13.1
29	12	5350	33.0	12	925705	13.4
34	14	1687	32.6	14	513340	9.5
36	15	2820	38.0	15	875467	11.4
41	17	2300	42.8	17	554728	7.4
48	20	1563	34.1	19	747650	7.2
50	21	3204	36.7	20	787154	10.6
55	23	1897	29.4	22	499042	8.9
57	24	1620	33.4	23	1307103	8.0
63	26	1452	22.2	25	652532	10.8
69	28	966	30.8	26	544402	10.9
71	29	1078	30.2	28	386239	9.0
76	31	552	27.6	29	771601	10.2
78	32	830	26.5	31	34961 1	11.3

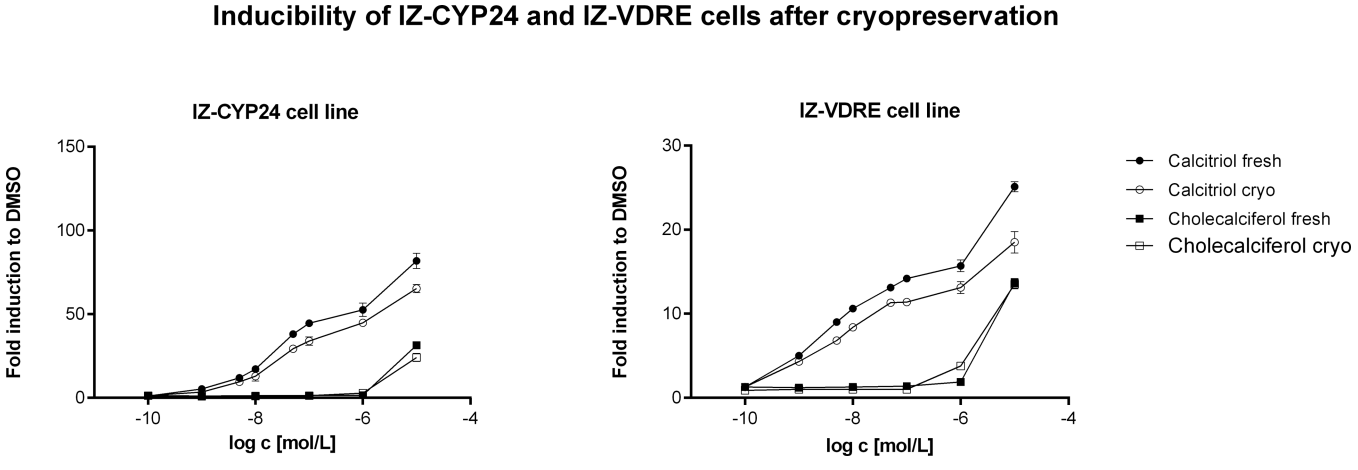

Figure 2. Inducibility of IZ-CYP24 and IZ-VDRE cells after cryopreservation. Fresh cells and cells after the freeze/thaw cycle were treated for 24 h with calcitriol and cholecalciferol (0.1 nM−10 μM). Cells were lysed, and luciferase activity was measured. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three different cell passages.

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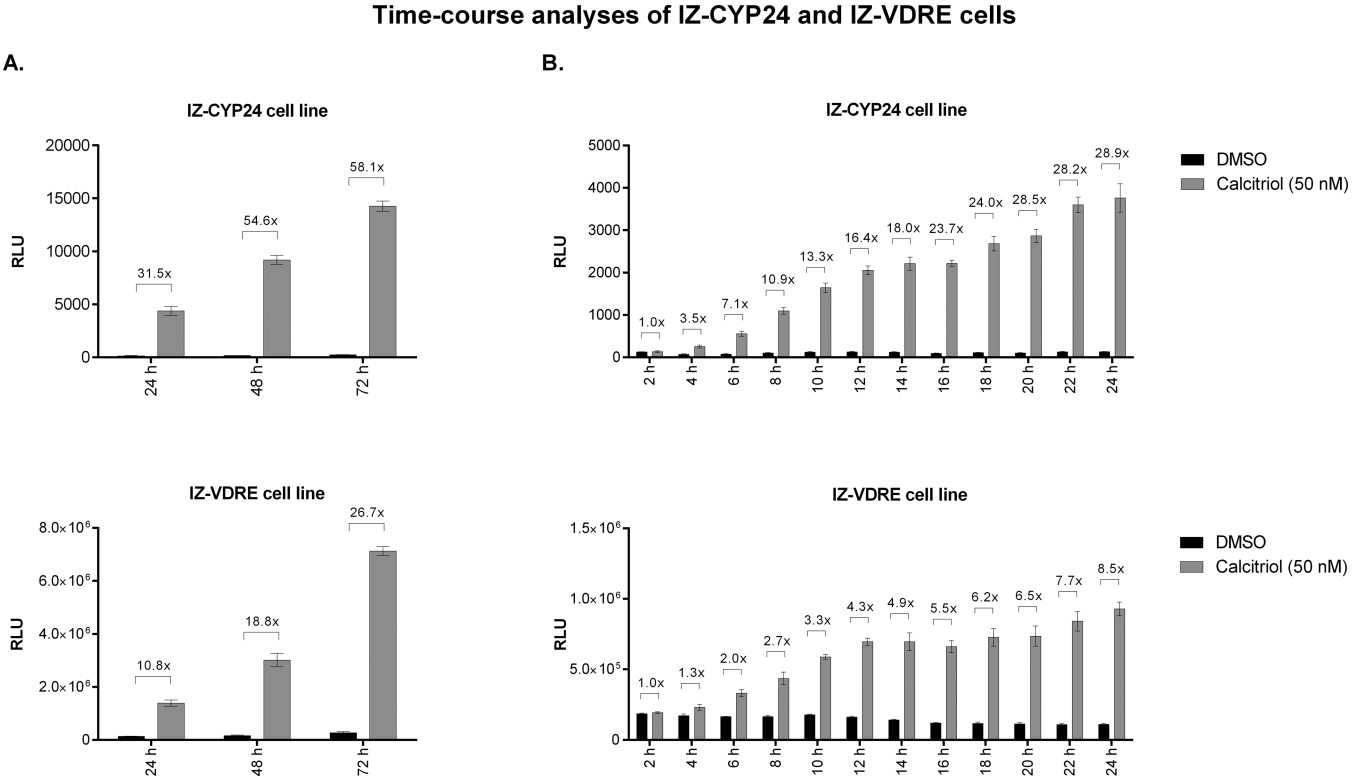

Figure 3. Time-course analyses of luciferase induction in IZ-CYP24 and IZ-VDRE cells. Cells were incubated with calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v) for time periods ranging from 2 to 72 h. After the incubations, luciferase activity was measured in cell lysates. Data are the mean of triplicate measurements and are expressed as relative luciferase units (RLU). Fold induction over DMSO-treated cells was calculated, and it is shown above the bars in the graph. Similar data were obtained from two consecutive passages. Panel A: Long-term analyses; incubation times, 24, 48, and 72 h. Panel B: Short-term analyses; incubation times, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h, and 24 h.

Table 2. Control Means and Standard Deviations for IZ-CYP24 and IZ-VDRE Cell Lines[a]

	IZ-CYP24 cell line							accepted criteria[19]
	maximum signal		minimum signal					SW
n	mean	SD	mean	SD	AVR	SW	Z′ factor	recommended: SW > 2
8	4195.5	388.4	144.7	29.3	0.31	7.20	0.69	acceptable: SW < 1
8	4209.5	491.1	210.3	40.4	0.40	4.90	0.60	unnacceptable: SW < 1
8	2394.4	202.3	177.3	32.2	0.32	7.48	0.68	Z′-factor
8	3835.1	336.4	146.9	24.2	0.29	7.75	0.71	excellent: Z′ > 0.5
8	3210.9	257.8	81.9	9.3	0.26	9.03	0.74	doable: 0 < Z′ < 0.5
8	1894.5	101.6	68.3	11.8	0.19	14.63	0.81	yes/no assay: Z′ = 0
8	1352.7	155.9	68.8	16.7	0.40	4.91	0.60	unaccaptable: Z′ < 0
12	16784.4	1383.0	493.9	32.1	0.26	8.71	0.74	AVR
12	10397.2	637.9	297.5	57.3	0.21	12.56	0.79	recommended: AVR < 0.6
12	14583.6	1704.6	367.7	83.2	0.38	5.19	0.62	unaccaptable: AVR > 0.6
	IZ-VDRE cell line							
	maximum signal		minimum signal					
n	mean	SD	mean	SD	AVR	SW	Z′ factor	
8	2188403.4	161344.4	306926.1	13926.0	0.28	8.40	0.72	
8	1511659.3	65439.6	212762.6	8612.4	0.17	16.45	0.83	
8	901158.6	73182.4	118792.0	11637.2	0.33	7.21	0.67	
8	946285.6	28440.9	69581.2	2103.1	0.10	27.60	0.90	
8	504835.8	13683.8	54551.7	2205.5	0.11	29.42	0.89	
8	521925.5	38139.3	76799.6	3366.2	0.28	8.41	0.72	
12	2407244.0	231765.3	229513.8	21037.0	0.35	6.12	0.65	
12	2263459.0	62808.0	220615.8	14784.5	0.11	28.82	0.89	
12	1454875.0	134865.5	176279.1	26582.6	0.38	5.89	0.62	
12	1369790.0	153760.6	125924.0	12592.8	0.40	4.84	0.60	

[a]Values of assay variability ratio (AVR), signal window (SW) and Z′ factor were calculated according to Iversen et al.[19] In the table, *n* represents the number of samples; calculations were performed from 10 individual experiments.

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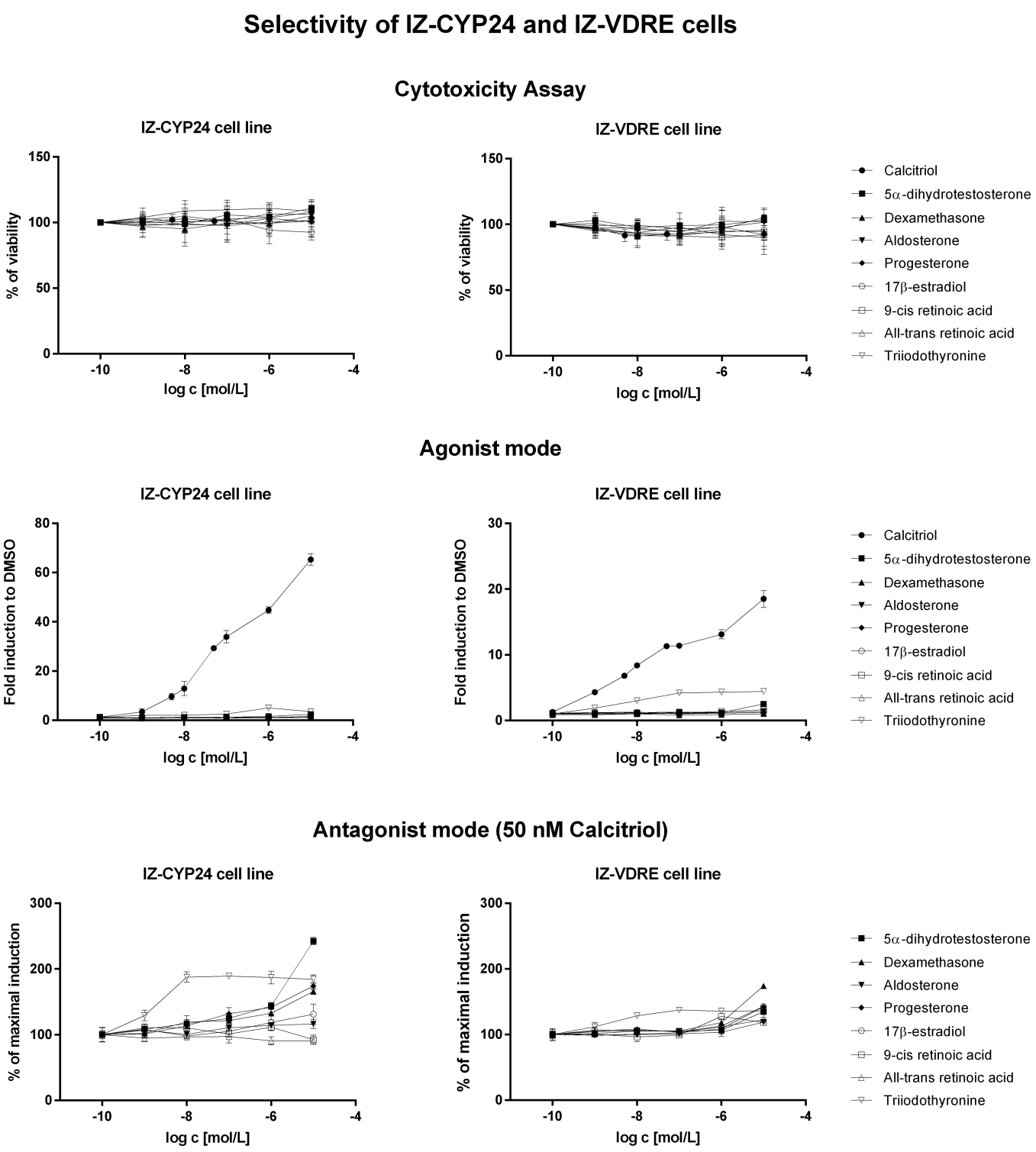

Figure 4. Selectivity of IZ-CYP24 and IZ-VDRE cells. Cells were treated for 24 h with model endogenous ligands of selected nuclear receptors in the concentration range from 0.1 nM to 10 μM in the presence (antagonist mode) or absence (cytotoxicity assay, agonist mode) of calcitriol (50 nM); and vehicle (DMSO; 0.1% v/v). Upper panel: Cytotoxicity assay. The MTT test was performed, and absorbance was measured at 540 nm. The data are mean the from experiments from three consecutive passages of cells and are expressed as a percentage of the viability of control cells. Middle panel: Agonist mode. Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent experiments cell passages. Lower panel: Antagonist mode. Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as a percentage of maximal induction attained by calcitriol. Similar data were obtained from three independent cell passages.

minimal for both cell lines to reliably identify VDR activators (Figure 3).

Assay Performance Indicators. For both cell lines, IZ-CYP24 and IZ-VDRE, assay performace measures were calculated according to Iversen et al.[19] Minimum signal and maximum signal were RLU acquired from cells incubated for 24 h with vehicle and 50 nM calcitriol, respectively. For each cell line, 10 individual experiments comprising 8 or 12 samples,

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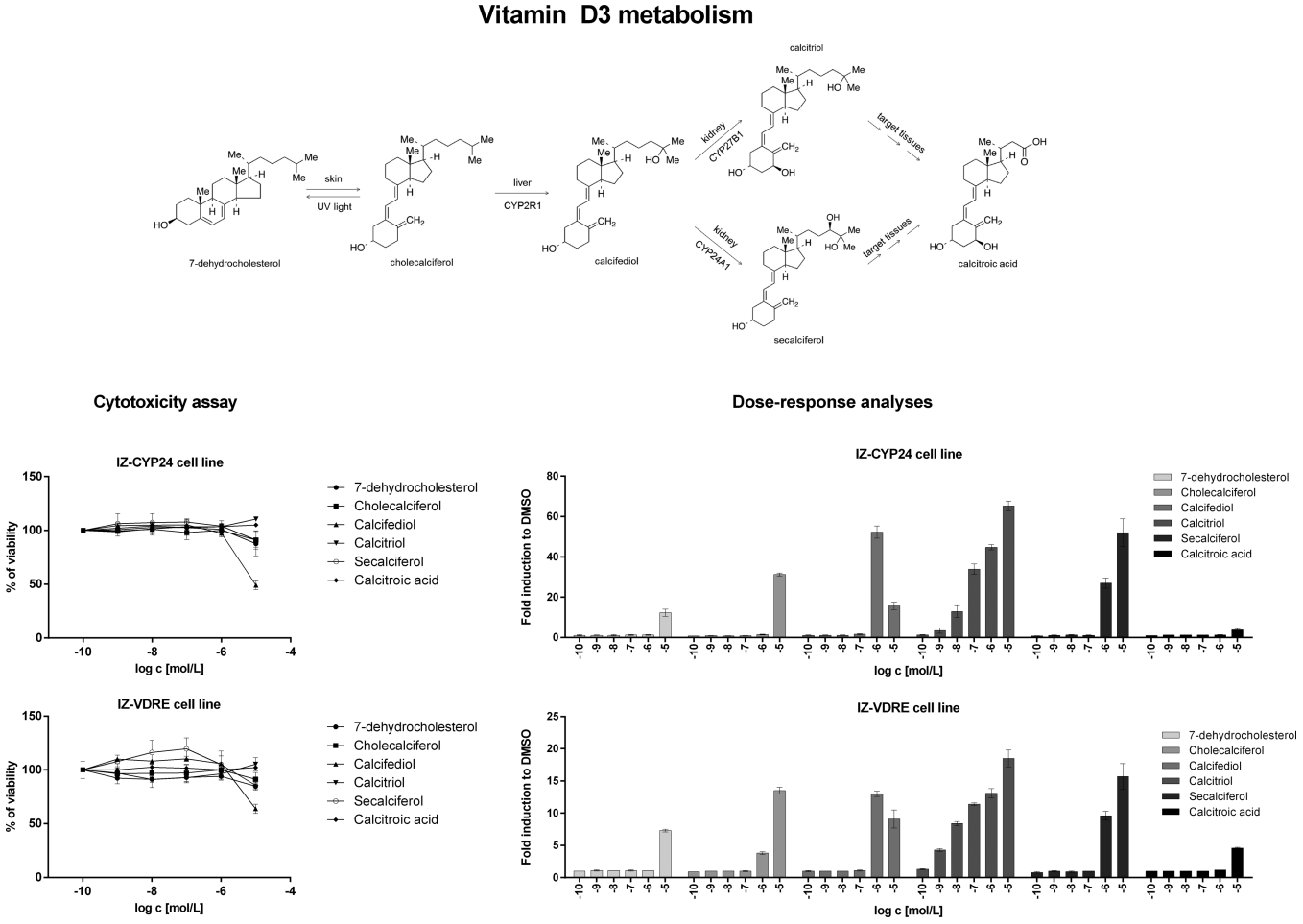

Figure 5. Profiling the vitamin D3 metabolic pathway. Cells were treated for 24 h with intermediates from the vitamin D3 pathway in the concentration range from 0.1 nM to 10 μM and vehicle (DMSO; 0.1% v/v). Upper panel: A scheme of vitamin D3 metabolism. Lower left panel: Cytotoxicity assay. The MTT test was performed, and absorbance was measured at 540 nm. The data are the mean from experiments from three consecutive passages of cells and are expressed as a percentage of the viability of control cells. Lower right panel: Dose−response analyses. Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

assay variability ratio (AVR), signal window (SW), and Z′-factor were calculated (Table 2). The values of SW in IZ-CYP24 and IZ-VDRE cells ranged from 4.9 to 14.6 and from 4.8 to 29.4, with averages of 8.2 and 14.3, respectively, which is in both cases considerably higher than the recommended value (SW > 2). The values of Z′-factor in IZ-CYP24 and IZ-VDRE cells ranged from 0.60 to 0.81 and from 0.60 to 0.90, with an average of 0.70 and 0.75, respectively. Since assays with values of Z′-factor exceeding 0.5 are considered "excellent", both cell lines easily passed this criterion very well. Since assay variability ratio is reliant on the Z′-factor, both cell lines manifested AVR < 0.60, which is the recommended value.

Selectivity of IZ-CYP24 and IZ-VDRE Cell Lines for VDR Activators. In the next series of functional tests, we assessed the selectivity of IZ-CYP24 and IZ-VDRE cells against VDR ligands and activators. For this purpose, we incubated cells with model ligands of glucocorticoid receptor (GR; dexamethasone), androgen receptor (AR; 5α-dihydrotestosterone), mineralocorticoid receptor (MR; aldosterone), progesterone receptor (PR; progesterone), estrogen receptor (ER; 17β-estradiol), retinoid X receptor (RXR; 9-*cis* retinoic acid), retinoic acid receptor (RAR; all-*trans* retinoic acid), and thyroid hormone receptor (TR; 3,3′,5-triiodo-L-thyronine). Prior to dose−response analyses, the MTT test and luciferase inhibition assay were performed as described in the Materials and Methods section. In the tested concentration range, neither cytotoxicity (Figure 4, upper plots) nor inhibition of nanoluciferase (data not shown) was observed for any compound.

Dose−response analyses were performed in two different experimental layouts. In agonist mode, cells were incubated with increasing concentrations of tested compounds. In the antagonist mode, a combination of 50 nM calcitriol and increasing concentrations of tested compounds was applied. We observed no induction of luciferase activity after the incubation of any cell line with dexamethasone, aldosterone, progesterone, 17β-estradiol, 5α-dihydrotestosterone, 9-*cis*-retinoic acid or all-*trans* retinoic acid. 3,3′,5-Triiodo-L-thyronine displayed dose−response effects on luciferase activity, with inductions about 5-fold in both cell lines, which corresponds to approximately 15% and 50% of those by 50 nM calcitriol in IZ-CYP24 and IZ-VDRE cells, respectively. Such behavior indicates the binding of TR to a sequence different from VDREs in reporter plasmids used for stable transfection.

In antagonist mode, aldosterone, 17β-estradiol, 9-*cis*-retinoic acid, and all-*trans* retinoic acid did not significantly influence calcitriol-mediated luciferase induction in any cell line.

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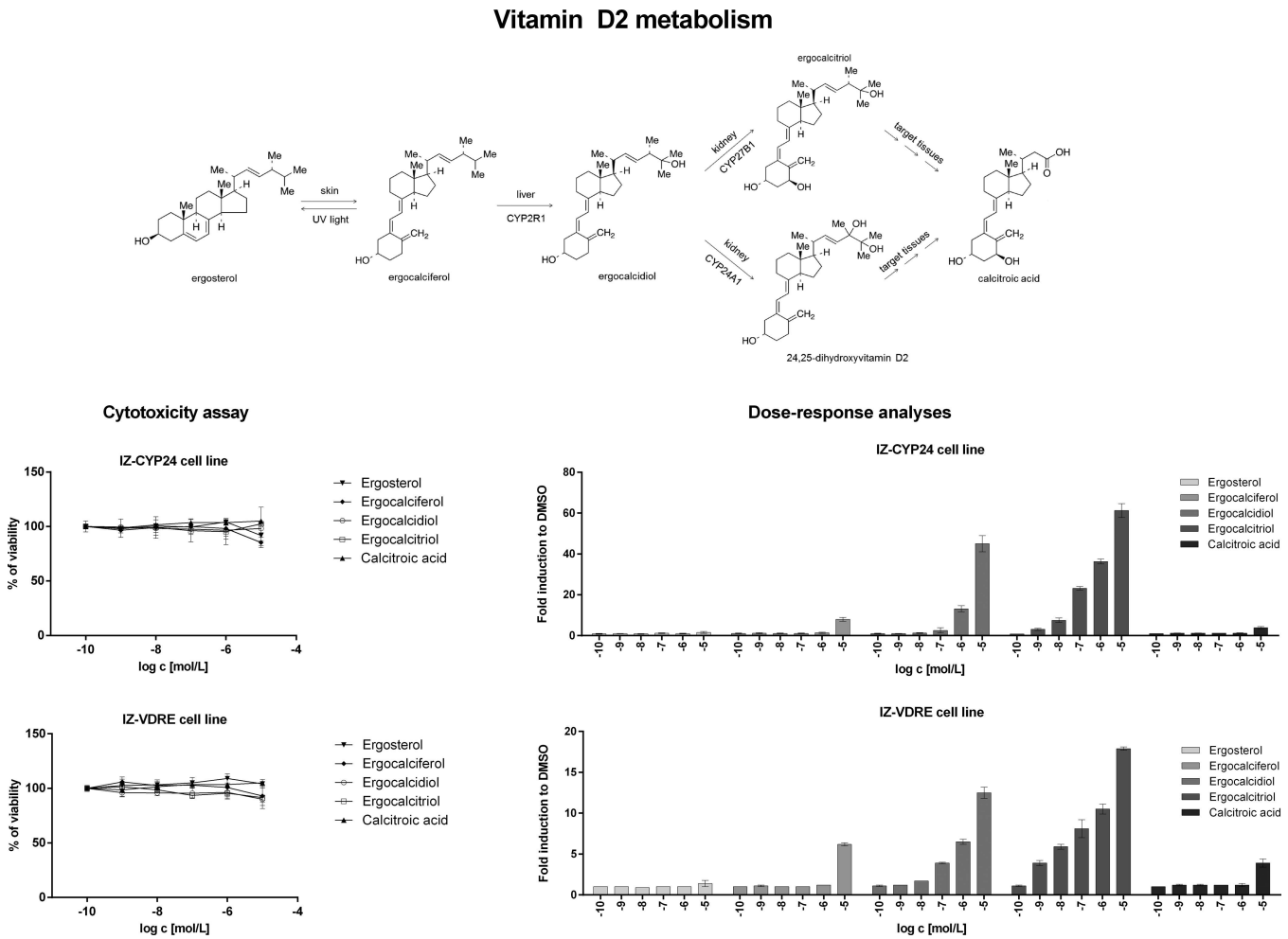

Figure 6. Profiling the vitamin D2 metabolic pathway. Cells were treated for 24 h with intermediates from the vitamin D3 pathway in the concentration range from 0.1 nM to 10 μM and vehicle (DMSO; 0.1% v/v). Upper panel: A scheme of vitamin D2 metabolism. Lower left panel: Cytotoxicity assay. The MTT test was performed, and absorbance was measured at 540 nm. The data are the mean from experiments from three consecutive passages of cells and are expressed as a percentage of the viability of control cells. Lower right panel: Dose−response analyses. Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

Dexamethasone, 5α-dihydrotestosterone, and progesterone slightly augmented calcitriol-mediated luciferase induction in IZ-CYP24 cells, but the potentiation occurred only at supraphysiological concentrations of hormones (10^{-5}−10^{-6} M). In contrast, these effects were not observed in IZ-VDRE cells, with the exception of a slight increase by 10 μM dexamethasone. 3,3′,5-Triiodo-L-thyronine yielded a dose-dependent additive effect in concentrations from 10^{-9} to 10^{-5} M (Figure 4; lower panels) in both cell lines. Taken together, both cell lines allow the detection of VDR activators, with relatively high selectivity. Some cross-reactivity with thyroid hormone was observed in both lines; therefore, it must be taken into account while interpreting the experimental data.

Profiling Vitamin D3 and Vitamin D2 Intermediates in IZ-CYP24 and IZ-VDRE Cells. As a proof of concept, we used IZ-VDRE and IZ-CYP24 cells for profiling analogues of vitamin D, and intermediates in vitamin D2 and vitamin D3 metabolic pathways against VDR transcriptional activity. MTT assay and luciferase inhibition assay were performed prior to dose−response analyses. Out of 10 vitamin D2 and vitamin D3 intermediates, significant cytotoxicity was observed for 10 μM calcifediol in both cell lines, which was also accompanied by a drop in luciferase activity (Figure 5, Figure 6, and Table 3). No inhibition of luciferase catalytic activity was observed for any of tested compounds (data not shown).

Within the vitamin D3 pathway, we performed dose−response analyses for precursors (7-dehydrocholesterol; cholecalciferol; calcifediol), the obligatory VDR ligand (calcitriol) and its byproduct (secalciferol), and the common ultimate degradation product (calcitroic acid). The effects of individual compounds were similar in both cell lines under investigation. All tested substances increased luciferase activity but with different dose−response profiles, potency, and efficacy. Early metabolic precursors 7-dehydroxycholesterol and cholecalciferol activated VDR only at 10 μM concentrations, with efficacy between 20% and 70% as compared to that of calcitriol. Late precursor calcifediol activated VDR with EC_{50} between 10^{-7} M and 10^{-6} M, and with efficacy similar to that by calcitriol. The most potent and efficacious activator and ligand for VDR was calcitriol, yielding EC_{50} 7 nM and 36 nM in IZ-VDRE and IZ-CYP24 cells, respectively. Dose−response effects of tested compounds were somewhat two-staged, when after reaching a plateau between 10^{-7} M and 10^{-6} M, an additional increase in luciferase activity was observed by 10^{-5} M concentration (probably off-target

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Table 3. Vitamin D Intermediates Used for Dose−Response Analyses of IZ-CYP24 and 12-VDRE Cells

substance	synonym	chemical name	CAS no.
7-dehydrocholesterol	provitamin D3	3β-hydroxyholesta-5,7-cdiene	434-16-2
22-oxacalcitriol	maxacalcitol	9,10-seco-22-oxacholesta-5,7,10(19)-triene-1α,3β,25-triol	103909-75-7
alfacalcidol	1α-hydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3-diol	41294-56-8
calcifediol	25-hydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-3,25-diol	19356-17-3
calcitriol	1α,25-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol	32222-06-3
calcitroic acid	1α-hydroxycalcioic acid	1,3-dihydroxy-24-nor-9,10-secochola-5,7,10(19)-trien-23-oic Acid	71204-89-2
calcipotriol	calcipotriene	24-cyclopropyl-9,10-secoc hola-5,7,10(19),22-tetraene-1,3,24-triol	112965-21-6
cholecalciferol	vitamin D3	9,10-secocholesta-5,7,10(19)-trien-3-ol	67-97-0
doxercalciferol	1α-hydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-1,3-diol	54573-75-0
EB 1089	seocalcitol	24a,26a,27a-trihomo-9,10-secocholesta-5,7,10(19),22,24-pentaene-1,3,25-triol	134404-52-7
ergocalcidiol	25-hydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-3,25-diol	21343-40-8
ergocalciferol	vitamin D2	9,10-secoergosta-5,7,10(19),22-tetraen-3-ol	50-14-6
ergocalcitriol	1α,25-dihydroxyvitamin D2	9,10-secoergosta-5,7,10(19),22-tetraene-1,3,25-triol	60133-18-8
ergosterol	provitamin D2	3β-hydroxyergosta-5,7,22-triene	57-87-4
secalciferol	24*R*,25-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol	55721-11-4
tacalcitol	1α,24*R*-dihydroxyvitamin D3	9,10-secocholesta-5,7,10(19)-triene-1,3,24-triol	57333-96-7

effects). A byproduct arising from calcifediol 24-hydroxylation, secalciferol, dose-dependently activated VDR, with EC_{50} approximately 150 nM and with efficacy similar to that by calcitriol. A common degradation product calcitroic acid activated VDR only in 10 μM concentration and with negligible efficacy (Table 4).

Table 4. Half Maximal Effective Concentrations (EC_{50}) for Tested Vitamin D Precursors, Metabolites, and Analogues[a]

	EC_{50}			
compound	*n*	IZ-CYP24	*n*	IZ-VDRE
7-dehydrocholesterol	3	>1000 nM	3	>1000 nM
22-oxacalcitriol	4	24.6 ± 3.3 nM	4	8.3 ± 2.1 nM
alfacalcidol	3	126.6 ± 5.3 nM	3	88.5 ± 1.1 nM
calcifediol	3	154.5 ± 16.0 nM	3	153.8 ± 13.8 nM
calcitriol	3	36.2 ± 6.7 nM	3	7.2 ± 1.6 nM
calcitroic acid	3	>1000 nM	3	>1000 nM
calcipotriol	4	5.3 ± 1.9 nM	4	2.3 ± 0.7 nM
cholecalciferol	3	136.8 ± 26.3 nM	3	143.5 ± 47.4 nM
doxercalciferol	3	130.1 ± 15.3 nM	3	87.1 ± 2.5 nM
EB 1089	4	0.9 ± 0.1 nM	4	0.3 ± 0.1 nM
ergocalcidiol	3	171.2 ± 12.1 nM	3	172.7 ± 41.9 nM
ergocalciferol	3	316.3 ± 1.6 nM	3	368.7 ± 76.0 nM
ergocalcitriol	3	53.4 ± 5.8 nM	3	11.4 ± 3.6 nM
ergosterol	3	n.d.	3	n.d.
secalciferol	3	166.1 ± 15.2 nM	3	140.8 ± 45.8 nM
tacalcitol	4	14.0 ± 5.8 nM	4	2.7 ± 0.2 nM
ZK 159222	3	85.5 ± 12.6 nM	3	49.7 ± 25.4 nM

[a]Data are expressed as the mean ± SD, and *n* represents the number of experiments. Values of EC_{50} were calculated using 1 μM as the highest concentration.

Within the vitamin D2 pathway, we performed dose−response analyses for precursors (ergosterol; ergocalciferol; ergocalcidiol), the obligatory VDR ligand (ergocalcitriol), and common ultimate degradation product (calcitroic acid). Early precursor ergosterol did not activate VDR at the entire range of tested concentrations, while the later precursor ergocalciferol did increase luciferase activity only in 10 μM concentration in both cell lines. The canonical ligand and activator of VDR, ergocalcitriol, dose-dependently activated VDR yielding EC_{50} of 11 nM and 53 nM in IZ-VDRE and IZ-CYP24 cells, respectively. Its progenitor ergocalciferol activated VDR with similar potency and efficacy (Figure 6; Table 4).

Profiling Vitamin D Analogues in IZ-CYP24 and IZ-VDRE Cell Lines. We measured dose−response effects of 7 commercially available vitamin D2 and vitamin D3 analogues, out of which 6 were agonists and 1 was an antagonist, in IZ-CYP24 and IZ-VDRE cell lines. Moderate cytotoxicity was observed for 10 μM alfacalcidol and doxercalciferol in both lines (Figure 7), while all other compounds were not cytotoxic. All tested VDR agonists elicited a dose−response increase of luciferase activity in both cell lines, and the maximal fold-inductions were similar to that by calcitriol. The values of EC_{50} decreased in order: alfacalcidiol = doxecalciferol > calcitriol ∼22-oxacalcitriol > calcipotriol ∼ tacalcitol > EB1089 (<1 nM). Interestingly, when assaying VDR antagonist ZK159222 in combination with 50 nM calcitriol, the IC_{50} of 0.41 ± 0.12 μM was observed in IZ-CYP24 cells, while it was not attained in IZ-VDRE cells even at 10 μM concentration.

DISCUSSION

In the current article, we describe the construction and characterization of novel stably transfected reporter cell lines IZ-CYP24 and IZ-VDRE suitable for screening and studies of VDR activators or antagonists. The strategy was the stable transfection of human colon adenocarcinoma cells LS180 that endogenously express functional VDR with two different nanoluciferase reporter plasmids: (i) the cell line IZ-CYP24 was transfected with a reporter construct containing a partial sequence (−326/−46) from the promoter region of human *CYP24A1* gene that includes two VDR responsive elements VDRE-I (−174/−151) and VDRE-II (−194/−174). In functional tests when 50 nM calcitriol was applied, IZ-CYP24 cells yielded approximately 30-fold induction, with RLU in the order of magnitude 10^3−10^4. Since IZ-CYP24 cells contain a substantial part of the *CYP24A1* promoter, they are a potential tool for fundamental studies of *CYP24A1* transcriptional regulation. (ii) The cell line IZ-VDRE was transfected with plasmid containing three copies of VDRE-I from the human *CYP24A1* promoter. Incubation of the IZ-VDRE line with calcitriol yielded approximately 10-fold induction, with RLU in the order of magnitude 10^6−10^7. Such a massive luminescent signal predestines IZ-VDRE cells as a very sensitive and high-throughput tool for screening purposes. Both cell lines were used in 96-well plate format, with sufficiently high signals. The optimal time of incubation for evaluation of VDR activators is deemed to be 24 h,

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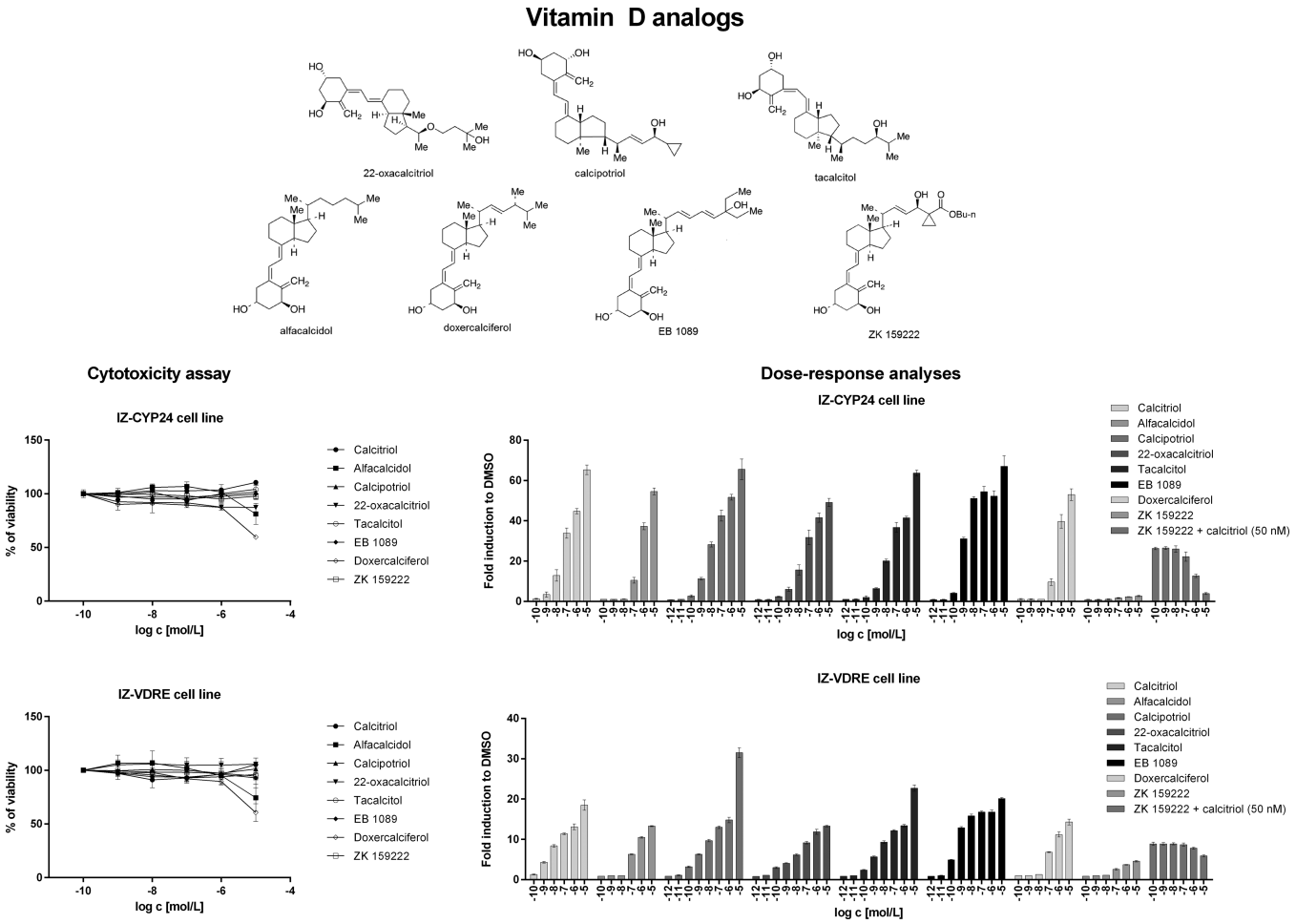

Figure 7. Profiling vitamin D analogues. Cells were treated for 24 h with vitamin D analogues in the concentration range from 1 pM to 10 μM in the presence or absence of calcitriol (50 nM) and vehicle (DMSO; 0.1% v/v). Upper panel: Chemical structures of vitamin D analogues. Lower left panel: Cytotoxicity assay. The MTT test was performed, and absorbance was measured at 540 nm. The data are the mean from experiments from three consecutive passages of cells and are expressed as a percentage of the viability of control cells. Lower right panel: Dose−response analyses. Luciferase activity was measured in cell lysates. Data are the mean from triplicate measurements and are expressed as fold induction over DMSO-treated cells. Similar data were obtained from three independent cell passages.

while the minimal time needed to reach reliable signal and fold induction was deemed to be 8 h, allowing the testing of cytotoxic compounds. An important aspect of laboratory-based experiments is the comfort and effectivity of manipulating cell lines. Both IZ-CYP24 and IZ-VDRE remained functional for more than 78 days in culture, which corresponds to 32 passages. Full responsiveness to calcitriol was retained by both cell lines also following the freeze/thaw cycle. Selectivity of IZ-CYP24 and IZ-VDRE for activators of VDR was checked by incubation with ligands for steroid hormone receptors (GR, MR, AR, PR, and ER) and nuclear receptors (RAR, RXR, and TR). We did not observe any influence of aldosterone, dexamethasone, progesterone, 5α-dihydrotestosterone, 17β-estradiol, 9-*cis* retinoic acid, and all-*trans*-retinoic acid on basal and calcitriol-inducible VDR activity in either cell line, implying no false positive data by mineralocorticoids, glucocorticoids, gestagenes, androgens, estrogens, retinoids, and rexinoids. Very weak augmentation of calcitriol-mediated luciferase induction by dexamethasone, 5α-dihydrotestosterone, and progesterone was observed in IZ-CYP24 cells but not in IZ-VDRE cells. This phenomenon may reveal some roles of GR, AR, and PR in the regulation of *CYP24A1*; however, given supra-physiological concentrations of the hormones (10^{-5} M−10^{-6} M) that caused an effect, nongenomic mechanisms are likely. Some cross-reactivity with the thyroid hormone was observed in both lines, which is consistent with up-regulation of *p*-glycoprotein by levothyroxine in LS 180 and Caco-2 human intestinal cell lines.[20] Therefore, it must be taken into account while interpreting the experimental data.

Dose−response analyses in both cell lines indicated that VDR ligands cause a somewhat two-stage increase in luciferase activity. For instance, calcitriol produced a typical sigmoid curve with EC_{50} 7 nM and 36 nM in IZ-VDRE and IZ-CYP24 cells, respectively. The curve got saturated between 10^{-7} M and 10^{-6} M of calcitriol, and further increase of calcitriol concentration up to 10^{-5} M caused an additional rise in luciferase activity. Such behavior may reveal nongenomic effects of VDR ligands in supra-physiological concentrations (approximately 1000-times higher than EC_{50}). The values of EC_{50} for calcitriol obtained by the LanthaScreen TR-FRET VDR coactivator assay and the GeneBLAzer VDR assay from Invitrogen were 0.75 nM and 0.50 nM, respectively, which is approximately 10 times lower as compared to the cell lines described here. Indigo Biosciences human VDR Reporter Assay System, using nonhuman cells, yielded EC_{50} = 3.5 nM for calcitriol, which is close to 7 nM obtained in IZ-VDRE cells. In transiently transfected CHO-K1

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cells, EC_{50} for calcitriol was 1.7 nM.[21] However, the same authors, using the same method reported EC_{50} of calcitriol to be 15 nM.[22] Collectively, there is a substantial variability between EC_{50} values obtained by various methods.

As a proof of concept, we used IZ-VDRE and IZ-CYP24 cells for profiling analogues of vitamin D and intermediates in vitamin D2 and vitamin D3 metabolic pathways against VDR transcriptional activity. The data obtained revealed significant activation of VDR not only by canonical ligands but also by their precursors and degradation products. However, for some VDR analogues and metabolic intermediates, significant activation of VDR was observed in very high, nonphysiological concentrations (>1 μM). Apart from the low efficacy of these compounds, other mechanisms may apply, including nongenomic ones.

We also calculated assay performace measures for both cell lines, according to Iversen et al.[19] The SW in IZ-CYP24 and IZ-VDRE cells was on average 8.2 and 14.3, respectively, which is considerably higher than the recommended value (SW > 2). The values of Z′-factor substantially exceeded 0.5, which is considered "excellent". Consistently, the AVR was lower than 0.60, which is the recommended value. Taken together, both cell lines very well passed all tested criteria for high throughput models and are comparable to those of other existing models. For instance, the LanthaScreen TR-FRET VDR coactivator assay has SW ∼ 5−6 and Z′-factor ∼0.75. Similarly, the GeneBLAzer VDR assay displayed Z′-factor ∼0.86 (www.invitrogen.com). A comprehensive study comparing different methods used for screening for FXR ligands showed very similar Z′-factors for time-resolved fluorescence TRF (Z′ = 0.60), TR-FRET (Z′ = 0.85), and ALPHAscreen (Z′ = 0.75).[23]

In conclusion, we developed two stably transfected gene reporter cell lines, which allow selective, sensitive, high-throughput, and rapid measurement of VDR transcriptional activity. A prospective application of the cell lines is deemed in toxicological, environmental, food-safety, pharmacological, and drug-development studies. Given the involvement of VDR in prostate cancer and tumor growth, the cell lines are also suitable models exploitable in fundamental research in human oncology and endocrinology.[18]

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

AR, androgen receptor; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; ER, estrogen receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; RLU, relative luciferase units; TR, thyroid hormone receptor; VDR, vitamin D receptor; VDRE, vitamin D response element

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PALACKY UNIVERSITY OLOMOUC

Faculty of Science

Department of Cell Biology and Genetics

CONSTRUCTION OF REPORTER CELL LINES FOR TOXICOLOGICAL AND ENVIRONMENTAL APPLICATIONS

Ph.D. Thesis Summary

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Mgr. Iveta Bartoňková

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The Ph.D. thesis is based on my own research carried out in the Department of Cell Biology and Genetics, Faculty of Science, Palacky University Olomouc, in the period from September 2013 to May 2017.

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SUMMARY

Endocrine disrupting chemicals (EDCs) are substances possessing the ability to interfere with endocrine signalling by altering steroid hormone homeostasis and the transcriptional activity of nuclear receptors. Examples of EDCs are agrochemicals, food additives and contaminants, and industrial and environmental pollutants. Given the impact of EDCs on human health and living organisms in general, it is relevant and interesting to develop reliable tools for the detection of endocrine disruptors.

In the present thesis, I describe the development and characterization of human stably transfected reporter cell lines for the assessment of androgen receptor (AR; AIZ-AR cell line) and vitamin D receptor (VDR; IZ-CYP24 and IZ-VDRE cell lines) transcriptional activities. These cell lines represent unique entirely human systems derived from human cancer cell lines expressing fully functional endogenous AR and/or VDR, respectively, that were transfected with reporter plasmids containing reporter sequences from promoter regions of human AR and/or VDR-responsive genes. These cell lines remained fully functional for over 2 months in the culture (this period corresponds to more than 25 cell passages) as well as after a freeze–thaw cycle. The reliable detection of ligands of human AR and VDR, respectively, is possible as soon as after 8 hours of the incubation. The assay was sensitive enough to allow for experiments in 96-well plates. The specificity of luciferase induction was proven by applying model ligands of other nuclear and steroid receptors: no significant off-activation was observed.

In conclusion, the human stably transfected reporter cell lines AIZ-AR, IZ-CYP24 and IZ-VDRE present rapid, reliable, effective, sensitive, selective and reproducible tools for identification of ligands and activators of human AR and VDR, respectively. These cell lines can potentially be used in pharmacological, environmental and toxicological studies.

SOUHRN

Endokrinní disruptory (EDCs) jsou látky mající schopnost interagovat se signálními dráhami endokrinního systému, jejíž hlavním mechanismem je změna transkripční aktivity jaderných receptorů. Příkladem těchto látek jsou nejrůznější pesticidy, potravinová aditiva a kontaminanty nebo látky znečišťující životní prostředí. Vzhledem ke vlivu endokrinních disruptorů na živé organismy včetně člověka představuje vývoj spolehlivých detekčních systémů jeden z hlavních směrů jejich studia.

V předkládané disertační práci jsem se zabývala konstrukcí a charakterizací lidských stabilně transfekovaných reportérových buněčných linií určených pro stanovení transkripční aktivity androgenního receptoru (buněčná linie AIZ-AR) a vitamin D receptoru (buněčné linie IZ-CYP24 a IZ-VDRE). Tyto buněčné linie představují unikátní lidský reportérový systém odvozený od lidských nádorových buněčných linií exprimujících plně funkční androgenní receptor/vitamin D receptor, které byly transfekovány reportérovými plasmidy obsahujícími reportérové sekvence odvozené z lidských AR/VDR responsivních genů. Všechny výše zmíněné buněčné linie byly plně funkční po více než 2 měsíce v buněčné kultuře, což odpovídá více než 25 pasážím, a stejně tak i pro kryoprezervaci. Spolehlivá detekce AR/VDR ligandů je možná již po osmi hodinách po aplikaci látek. Výše uvedené stabilní buněčné linie jsou natolik citlivé, že umožňují testování v 96-jamkovém formátu. Specifita buněčných linií byla ověřena testováním modelových ligandů ostatních steroidních a jaderných receptorů, přičemž nebyly pozorovány žádné signifikantní indukce luciferázové aktivity, které by potenciálně mohly ovlivnit interpretaci experimentálních dat.

Závěrem lze konstatovat, že lidské stabilně transfekované reportérové buněčné linie AIZ-AR, IZ-CYP24 a IZ-VDRE představují rychlý, spolehlivý, efektivní, selektivní a reprodukovatelný nástroj pro detekci ligandů a aktivátorů lidského androgenního receptoru, respektive vitamin D receptoru. Využití těchto linií spočívá ve farmakologických, environmentálních a toxikologických aplikacích.

CONTENTS

1 OBJECTIVES

The aim of this thesis was to develop stably transfected human reporter cell lines that could be used in toxicological, pharmacological and environmental studies. In particular

1. Construction and characterization of human reporter cell line for the assessment of human androgen receptor (AR) transcriptional activity.
2. Construction and characterization of human reporter cell line for the assessment of vitamin D receptor (VDR) transcriptional activity.

2 INTRODUCTION

Xenobiotics are compounds that are foreign to the body. They include drugs, food additives, agrochemicals, cosmetics, and environmental pollutants. A distinct class of these substances interfere with functions of nuclear receptors leading to perturbation in drug metabolism, cell signalling pathways, to the onset and progression of disease or pathological states, or even to cancerous growth. Because molecular basis of these interactions is to compromise or to disrupt the activity of hormones and endocrine signalling molecules, these xenobiotics are referred to as endocrine disruptors. These substances are often persistent in the environment (e.g. polychlorinated biphenyls, dioxins or polycyclic aromatic hydrocarbons) and they can be introduced into a food chain.

Different *in vitro* techniques have been used to evaluate the effects of xenobiotics on the transcriptional activity of nuclear receptors. Gene reporter assays were introduced in the 1980s as a rapid and reliable tool for environmental studies and drug discovery and a fundamental tool for pharmacological and toxicological research. This approach is based on a transfection of cells, either transient or stable, with a reporter vector containing a reporter gene under the control of a specific sequence termed a response element (RE). The nuclear receptor (NR) in its active (ligand-bound) form binds this RE, thus triggering the expression of the reporter gene.

In the present thesis, I describe the construction and characterization of novel stably transfected human reporter cell lines for the assessment of androgen receptor (AR; AIZ-AR cell line) and vitamin D receptor (VDR; IZ-CYP24 and IZ-VDRE cell lines)

transcriptional activities. These cell lines are unique entirely human *in vitro* systems expressing endogenous AR or VDR, respectively that were transfected with a reporter plasmid containing reporter sequences derived from the promoter region of human target genes. Therefore, these cell lines represent an optimal system for the detection of compounds that could disrupt physiological receptors-mediated functions in the human body. All cell lines remained fully functional for over 2 months in cell culture as well as after cryopreservation. Ligands of target receptors can be identified as soon as after 8 hours of treatment. The specificity of the induction of the luciferase signal was evaluated by the applying model ligands of other NRs; no significant off-response (false positive) that could lead to the misinterpretation of experimental data was identified. The assays were sensitive enough to allow tor experimentation in 96-well plate format.

Taken together, stably transfected reporter cell lines described in this thesis present rapid, high-throughput, reliable, robust, selective and sensitive tools for the identification of ligands of human AR and VDR, with possible future use in pharmacological, environmental and toxicological studies.

3 A GENE REPORTER ASSAY AND STABLE TRANSFECTION

The technique of the gene reporter assay has been introduced as a reliable *in vitro* tool for screening of transcriptional activation in general. Gene reporter assays are based on the introduction (transfection or transduction) of an expression vector and reporter plasmid into appropriate cells in culture *in vitro*, although *in vivo* applications are employed as well. The reporter plasmid is designed by combining an appropriate reporter gene under transcriptional control of a promoter sequence derived from the gene of interest. There are several requirements for an optimal reporter gene: it has to be easily and rapidly detectable, sensitive, non-toxic to host cells and not endogenously expressed in target cells. The first reporter gene to be used was chloramphenicol acetyltransferase (*CAT*), an *Escherichia coli* enzyme (Gorman *et al*., 1982). Since 1980 other reporter genes have been introduced, including various luciferases, β-galactosidase, β-glucuronidase, alkaline phosphatase (*AP*), secreted alkaline phosphatase (*SEAP*), and green fluorescent protein (*GFP*) (Schenborn *et* Groskreutz, 1999).

There are several different ways to deliver the reporter vector into target mammalian cells (Kim *et* Eberwine, 2010). Probably the most common chemical method of transfection is the lipofection/lipid-mediated gene delivery. The transfer of the reporter vector is mediated by liposomes made from phospholipid bilayer that can merge with the cell membrane. The process of lipofection is highly efficient and easy to use, but it is not applicable to all cell types. Another technique is based on calcium phosphate which is mixed with cultured cells, and the DNA is taken up by the cells by endocytosis. Non-chemical (physical) techniques include electroporation, magnetic nanoparticles, and direct injection of biolistic particles. In general, these techniques require a more specific material, proper equipment, and experimental skills. The third group of transfection methods is represented by virus-mediated transfection (or transduction), which is highly efficient and easy to use, but has many serious disadvantages that include potential insertional mutagenesis, limited DNA size or immunogenicity and cytotoxicity.

The significant drawback of the reporter assays, in particular when intended for large-scale experimentation, is the need for transfection of a plasmid. This problem can be overcome by means of the stable transfection approach, a process of permanent integration of a reporter vector into the cell. Because all the cells in the culture are stably transfected with a reporter (100% efficacy), this approach has several advantages (Novotna *et al*., 2011):

1. There is no need to transiently transfect the cells for each experiment: therefore, stable transfection is much less time- and material-consuming than transient transfection.
2. There is no need for internal control of transfection efficiency.
3. The assay is much more sensitive in comparison with transient transfection.

The process of stable transfection involves a systematic process of selection under the pressure of a selection antibiotic. The selection marker (gene of drug resistance) can be encoded either in the reporter plasmid or in a separate plasmid that needs to be co-transfected along with the reporter plasmid. The first population of antibiotic-resistant cells obtained by the selection process is called polyclonal population, because it is derived from the original transfected culture containing all the successfully transfected clones. Given that the individual clones in the polyclonal population may differ in cell morphology, functionality and responsiveness, the

generation of monoclonal populations (derived from a defined single clone) by limiting dilution should be the next step for the development of a stable cell line.

Recently, CRISPR/Cas9 genome editing was used successfully to generate stably transfected cell lines. The microbial CRISPR/Cas immune system is composed of clustered regularly interspaced short palindromic repeats (CRISPR) loci together with the CRISPR-associated (cas) genes and is responsible for cleavage of foreign genetic elements (Garneau *et al*., 2010). The Cas nucleases are targeted to specific DNA sequences by noncoding RNAs that aim the Cas nuclease to its target through Watson-Crick base pairing. After a simple modification in the sequence of guide RNA, this system can be easily used for gene editing in a huge variety of cell cultures and experimental systems. Lee *et al*. (2015) used this novel approach to stably transfect Chinese hamster ovary (CHO) cells, demonstrating precise insertion of a 3.7-kb gene expression vector at defined loci in CHO cells, followed by a simple selection process that resulted in homogeneous transgene expression throughout the cell culture.

In the past, diverse methods were used to evaluate the effect of exogenous compounds on the transcriptional activity of human AR. *In vivo* experiments were conducted in rats (Ostby *et al.*, 1999) or transient transfections procedures were used (Vinggaard *et al*., 1999, Vinggaard *et al.*, 2008). Aside from these costly and time-consuming approaches, several stably transfected cell lines were employed. The PALM cell line was developed by stable co-transfection of the PC-3 cell line with human AR and a reporter plasmid containing the firefly luciferase gene under the control of the androgen-dependent mouse mammary tumour virus (*MMTV*) promoter (Térouanne *et al*., 2000). Blankvoort *et al*. (2001) published stable reporter cell line AR-LUX derived from human breast cancer cell line T47D that was constructed using a reporter plasmid pBRARE2tataluc^{+} containing two AREs from rat probasin gene (Blankvoort *et al*., 2001). The response of the AR-LUX cell line to model androgen R1881 was maximal five-fold induction. The MDA-kb2 cell line was constructed by a transfection of human breast cancer cell line MDA-MB-453 with reporter vector MMTV.luciferase.neo (Wilson *et al*., 2002). The AR CALUX bioassay was developed by Sonneveld *et al*. (2005) from human osteosarcoma cell line U2-OS by co-transfection of reporter plasmid 3xHRE-TATA-Luc and expression plasmid pSG5-

neo-AR. This stably transfected cell line was tested for the responsiveness after DHT treatment, maintenance of inducibility and stability (Sonneveld *et al*., 2005).

Different strategies have been used for evaluation of VDR transcriptional activity, including commercially available systems. To date, they have been mostly based on transient transfection of human cancer cell lines with reporter constructs (Vrzal *et al*., 2011; Salimgareeva *et al*., 2014, Mano *et al*., 2015).

4 MATERIALS AND METHODS

4.1 Biological material

4.1.1 22Rv1 cell line

Human prostate carcinoma epithelial cell line 22Rv1 (ECACC No. 105092802) was purchased from the European Collection of Cell Cultures (ECACC) and cultured in RPMI-1640 medium supplemented with 10% of foetal bovine serum, 100 U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.1.2 LS180 cell line

Human Caucasian colon adenocarcinoma cell line LS180 (ECACC No. 87021202) was purchased from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco′s modified Eagle′s medium (DMEM) supplemented with 10% of foetal bovine serum, 100U/mL streptomycin, 100 μg/mL penicillin, 4 mM L-glutamine, 1% non-essential amino acids and 1 mM sodium pyruvate. The cells were maintained at 37 °C and 5% CO_2 in a humidified incubator.

4.2 Materials and reagents

Dulbecco′s Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI-1640), dimethyl sulfoxide (DMSO), hygromycin B, thiazolyl blue tetrazolium bromide (MTT), foetal bovine serum (FBS), charcoal stripped foetal bovine serum (CS-FBS), non-essential amino-acids, penicillin, streptomycin, L-glutamine, D-luciferin, coenzyme A, adenosine triphosphate (ATP), testosterone, spironolactone, cortisol, dexamethasone, beclomethasone, betamethasone, corticosterone, aldosterone, prednisolone, methylprednisolone, 17α progesterone, 3,3′,5-triiodo-L-thyronine, progesterone, 17β-estradiol, diethylstilbestrol and 4-

hydroxytamoxifen were purchased from Sigma-Aldrich (Prague, Czech Republic). Danazol, cyproterone acetate, mifepristone, triamcinolone, genistein, raloxifene hydrochloride, tamoxifen citrate salt, all-trans-retinoic acid, 9-cis-retinoic acid, 7-dehydrocholesterol, vitamin D3 (cholecalciferol), EB 1089 (seocalcitol), 1α,25-dihydroxyvitamin D3 (calcitriol), 1α,25-dihydroxyvitamin D2 (ergocalcitriol), ZK 159222 and 22-oxacalcitriol were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calcitroic acid, 25-hydroxyvitamin D3 (calcifediol), 1α-hydroxyvitamin D3 (alfacalcidol), 24R,25-dihydroxyvitamin D3 (secalciferol), tacalcitol monohydrate, ergosterol, vitamin D2 (ergocalciferol), 25-hydroxyvitamin D2 (ergocalcidiol) and 1α-hydroxyvitamin D2 (doxercalciferol) were purchased from Toronto Research Centre Inc. (Toronto, Canada). Fugene HD transfection reagent was purchased from Roche (Basel, Switzerland). Reporter lysis buffer and Nano-Glo Luciferase Assay System were from Promega (Hercules, CA, USA). All other reagents were of the highest quality commercially available.

4.2 Reporter plasmids

4.2.1 p3ARR/ARE-luc2P/minP/hygro

Reporter plasmid p3ARR/ARE-luc2P/minP/hygro was designed as follows: three copies of the ARR sequence followed by a single copy of ARE sequence from the promoter region of the human prostate-specific antigen (*PSA*) gene were inserted into the pGL4.27 [luc2P/minP/hygro] vector (Cat. No. E8451; Promega, Hercules, CA), using Kpn-1 and XhoI restriction enzymes.

3ARR/ARE insert (84 bp)

5′-CAGGGATCAGGGAGTCTCACACAGGGATCAGGGAGTCTCACACAGGGATCAGGGAGTCTCACAATGcAGAACAGCAAGTGCTAGC-3′

ARR: 5′-CAGGGATCAGGGAGTCTCACA-3′

ARE: 5′-TGCAGAACAGCAAGTGCTAGC-3′

4.2.2 CYP24_minP-pNL2.1[Nluc/Hygro]

Reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] was designed as follows: a partial sequence (-326/-46) from the promoter region of the human *CYP24A1* gene containing two VDREs, VDRE-I (-174/-151) and VDRE-II (-194/-174), followed by a minimal promoter sequence (41 bp) as a control of luciferase expression was

inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and HindIII restriction enzymes.

CYP24_minP insert (333 bp)

5′-CTCGAGCCTGCGCCGGGGGAGGGCGGGGAGGCGCGTTCGAAGCACACCCG
GTGAACTCCGGCGTTCGCATGCCTTCCTGGGGGTTATCTCCGGGGTGGAGTGC
TGCCGCCCCCACCCCACCTCCCGCGCCCAGCGAACATAGCCCCGGTCACCCC
AGGCCCGGACGCCCTCGCTCACCTCGCTGACTCCATCCTCCTTCCACCCCCCC
TCCCCTGGGTCCGCGTCCCTCGGAGTCTGGCCAGCCGGGGGCCACTCCGCCC
TCCTCTGCGTGCTCATTGGCCACCCAAGACACTAGAGGGTATATAATGGAAGCT
CGACTTCCAGCTTAAGCTT-3′

VDRE-I: 5′-GACGCCCTCGCTCACCTCG-3′

VDRE-II: 5′-CACACCCGGTGAACT-3′

4.2.3 VDREI3_SV40-pNL2.1[Nluc/Hygro]

Reporter plasmid VDREI3_SV40-pNL2.1[Nluc/Hygro] was designed as follows: three copies of VDRE-I (-174/-151) from the promoter region of the human *CYP24A1* gene followed by the basic SV40 promoter with a deleted 5′-enhancer region as a control of luciferase expression was inserted into pNL2.1[Nluc/Hygro] (Cat. No. N1061; Promega, Hercules, CA, USA) using XhoI and HindIII restriction enzymes.

VDREI3_SV40 insert (197 bp)

5′-CTCGAGGACGCCCTCGCTCACCTCGGACGCCCTCGCTCACCTCGGACGCCC
TCGCTCACCTCGTGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTC
CGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATCGC
TGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTAT
TCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTT-3′

VDRE-I: 5′-GACGCCCTCGCTCACCTCG-3′

4.3 Generation of stably transfected cell lines

Human prostate carcinoma epithelial cell line 22Rv1 was transfected with reporter plasmid p3ARR/ARE-luc2P/minP/hygro (for AIZ-AR cell line). Human colon adenocarcinoma cell line LS180 was transfected with reporter plasmids CYP24_minP-pNL2.1[Nluc/Hygro] (for IZ-CYP24 cell line) or VDREI3_SV40-

pNL2.1[Nluc/Hygro] (for IZ-VDRE cell line). All the transfection procedures involved the Fugene HD transfection reagent at a ratio 3:1 (reagent/DNA) and cells were seeded in 60-mm culture dishes at a density of 8×10^5 (22Rv1 cell line) and 10^6 (LS180 cell line), respectively. Following stabilization, the culture medium was replaced by the selection medium containing selection antibiotic hygromycin B at concentration 0.5 mg/mL (22Rv1 cell line) or 0.25 mg/mL (LS180 cell line). The selection medium was changed every 3 or 4 days for the period of 3 weeks until polyclonal populations of stably transfected cells were obtained.

The polyclonal populations with the highest induction of luciferase signal after treatment with model agonists of AR (100 nM 5α-dihydrotestosterone, DHT) and VDR (50 nM 1α,25-dihydroxyvitamin D3, calcitriol) were selected for the production of monoclonal populations. For this purpose, cells were seeded in 100-mm culture dishes at a density of 300–700 (22Rv1 cell line) and 100–1000 (LS180 cell line) cells per culture dish and were cultured for additional 2 or 3 weeks, respectively, until small individual colonies of cells became visible. Thereafter, individual colonies were sub-cloned in 24-well plates and cultured for additional 3 weeks in the selection medium to obtain monoclonal populations of stably transfected cells. Two clones of the AIZ-AR cell line and one clone of each of cell lines IZ-CYP24 and IZ-VDRE were selected for detailed characterization. The use of GMO in the Faculty of Science, Palacky University Olomouc was approved by the Ministry of the Environment of the Czech Republic (ref. 91997/ENV/10).

4.4 The cytotoxicity assay (MTT assay)

Cells were seeded in 96-well plates at a density of 5×10^4 (AIZ-AR cell line) and 2.5×10^4 (IZ-CYP24 and IZ-VDRE cell lines) cells per well in charcoal-stripped culture medium. After 24 h of stabilization, the cells were treated with increasing concentrations of a tested compound (in the range 1 pM to 10 μM), vehicle (DMSO; 0.1% v/v) and Triton X-100 (2% v/v) as a positive control. After 24 h of incubation, the medium was replaced with a medium supplemented with 10% of MTT at a final concentration of 1 mg/mL (AIZ-AR cell line) or 0.3 mg/mL (IZ-CYP24 and IZ-VDRE cell lines) and incubated in 37 °C for additional 2 h or 30 min, respectively. At the end of incubation, purple formazan was dissolved in DMSO. The MTT assay results were measured spectrophotometrically at 540 nm using a Tecan Infinite M2000 plate luminometer.

4.5 The luciferase inhibition assay

Cells were incubated with model agonists for 24 h. Cell lysates containing high catalytic activity of firefly luciferase (AIZ-AR cell line) or Nano luciferase (IZ-CYP24 and IZ-VDRE cell lines) were obtained. The tested compounds at the highest concentration used during the experiments were added to the cell lysates and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.6 The gene reporter assay

Cells were seeded in 96-well plates at a density of 5×10^4 (AIZ-AR cell line) and 2.5×10^4 (IZ-CYP24 and IZ-VDRE cell lines) cells per well in a charcoal-stripped culture medium. Following 24 h of stabilization, the cells were incubated with the tested compounds. After the incubation, the cells were lysed and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.7 Statistical analyses

Student′s paired t-test as well as calculations of EC_{50} and IC_{50} values were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA (www.graphpad.com).

5 RESULTS AND DISCUSSION

5.1 Construction and characterization of stably transfected reporter cell line AIZ-AR

Human stably transfected reporter cell line AIZ-AR was derived from human prostate carcinoma epithelial cell line 22Rv1 by transfection with a reporter plasmid containing three AREs followed by an ARR sequence from the promoter region of the human prostate specific androgen (*PSA*) gene. Hygromycin B-resistant clones (23 clones), displaying the same morphology as the parental 22Rv1 cell line were obtained after the selection process. All the selected clones were tested for responsiveness to a model agonist of AR, 5α-dihydrotestosterone (DHT). Four of the 23 clones showed induction of luciferase between 1500 and 4000 relative luciferase units (RLU) and fold induction between 7.6 and 12.1 (Figure 1). Based on the strength of the signal, clones 8 and 14 were selected for further characterization and termed as the AIZ-AR cell line.

The AIZ-AR cell line remained fully functional for over 67 days in culture (this duration corresponds to 28 passages) as well as after a freeze–thaw cycle (Table 1, Figure 2). Time-course analyses revealed the possibility of identification of AR ligands as soon as after 8 hours of the incubation (Figure 3). Dose-response analyses with 23 steroid compounds were performed to assess specificity of the AIZ-AR cell line. Luciferase activity of the AIZ-AR cell line was induced by androgens, but not by oestrogens or mineralocorticoids. Glucocorticoids and progesterone induced the luciferase signal, but with 2–3 orders of magnitude higher in comparison with androgens (Figures 4–6). Sensitivity of the AIZ-AR cell line allowed for experiments in 96-well plates.

In comparison with previously published experimental models, the AIZ-AR cell line possesses several advantages:

1. The AIZ-AR cell line is an entirely human system: human prostate carcinoma epithelial cell line 22Rv1 expressing fully functional endogenous human AR was transfected with a reporter vector that is under control of AREs of the *PSA* gene.
2. The AIZ-AR cell line preserved the signalling stoichiometry. Because the expression of AR is endogenous in this cell line and no extra AR was cotransfected, the ratio between AR protein and regulatory proteins of transcriptional machinery remains unchanged.

The characteristic properties indicated above clearly describe the added value of AIZ-AR cell line as compared to previously published stably transfected cell lines. MDA-kb2 and AR-LUX cell lines both contain a reporter vector under the control of rodent promoter sequences, not human ones (Blankvoort *et al*., 2001; Wilson *et al*., 2002). In addition, human cell lines PALM and AR CALUX were co-transfected with expression vectors for AR (Térouanne *et al*., 2000; Sonneveld *et al*., 2005): therefore, the stoichiometry of cell signalling was significantly changed due to overexpression of AR.

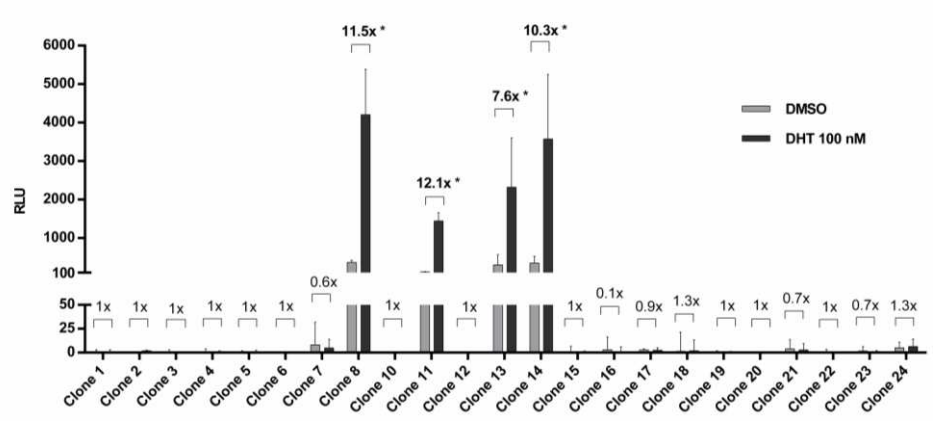

Figure 1: Responsivity of hygromycin B-resistant AIZ-AR clones to 100 nM DHT after 24 h.

Table 1: Long-term maintenance of responsiveness of AIZ-AR cells to 100 nM 5α-dihydrotestosterone.

Days in culture	Clone 8			Clone 14		
	Passage	RLU	FOLD	Passage	RLU	FOLD
8	4	3368.8	10.4	3	2383.6	10.9
15	7	2489.2	11.2	6	2133.0	11.8
22	9	1759.2	11.1	8	n.d.	n.d.
30	12	2517.2	22.4	11	2185.0	20.7
37	15	3976.5	21.1	14	2121.4	23.9
44	18	4269.3	6.5	17	3470.3	5.7
51	21	3918.0	20.6	20	4011.0	17.3
58	24	5244.3	18.6	23	4787.0	14.4
65	27	3225.8	18.7	26	3499.0	20.3
67	28	4043.5	18.5	27	2485.5	15.5

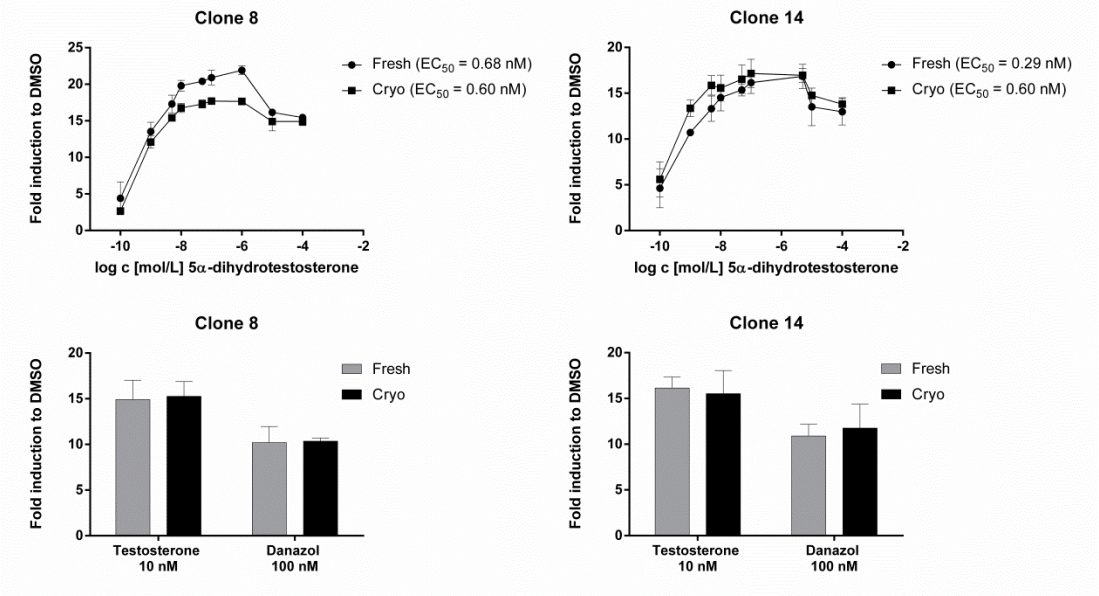

Figure 2: Luciferase inducibility in AIZ-AR cells after cryopreservation.

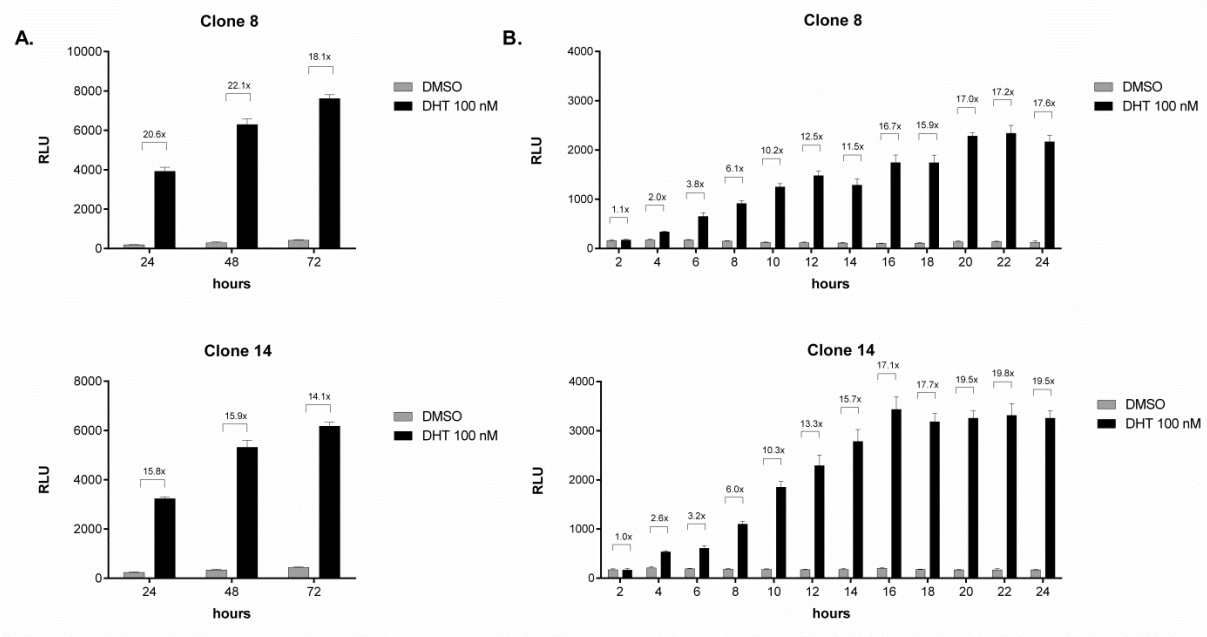

Figure 3: Time-course analyses of AIZ-AR cells after DHT treatment.

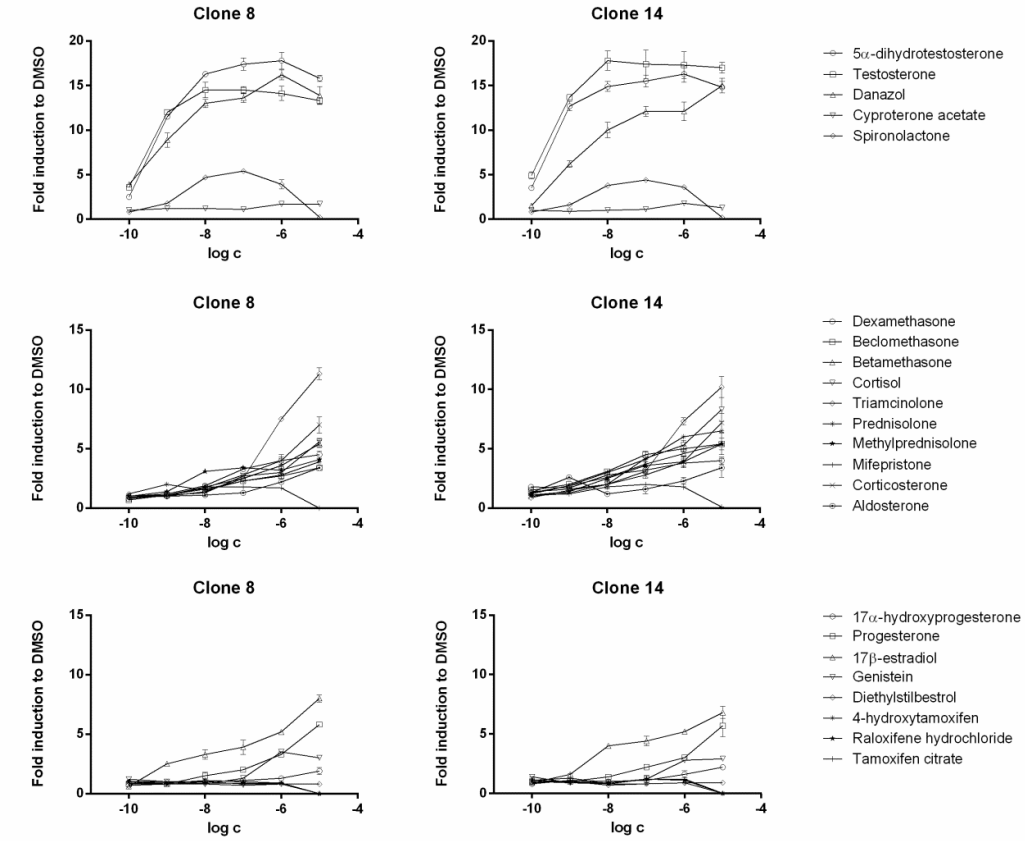

Figure 4: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: agonist mode.

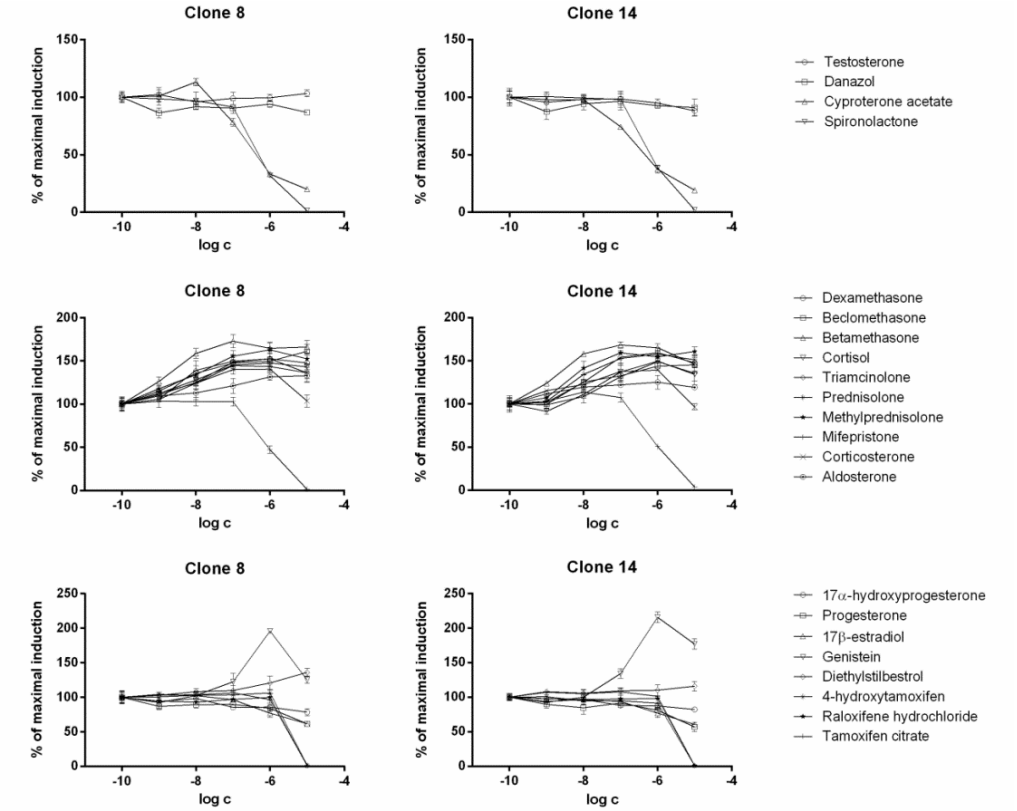

Figure 5: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: antagonist mode with 5α-dihydrotestosterone.

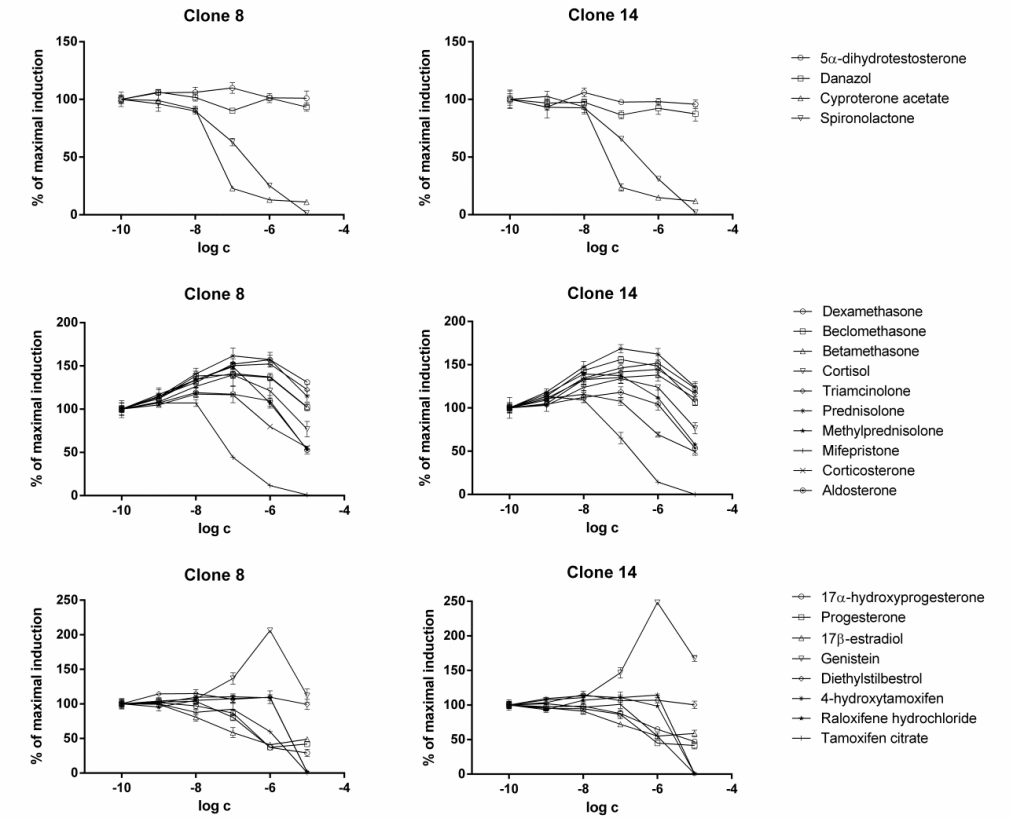

Figure 6: Dose-response analyses of AIZ-AR cells after treatment with steroid compounds: antagonist mode with testosterone.

5.2 Construction and characterization of stably transfected reporter cell lines IZ-CYP24 and IZ-VDRE

In the second part of this thesis, I describe the construction and characterization of two novel stably transfected reporter cell lines for the evaluation of human VDR ligands. Human Caucasian colon adenocarcinoma cell line LS180 was transfected using lipofection with reporter plasmid CYP24_minP-pNL2.1[Nluc/Hygro] or VDREI3_SV40-pNL2.1[Nluc/Hygro] to develop stably transfected IZ-CYP24 or IZ-VDRE cell line, respectively. 11 and 30 hygromycin B-resistant clones of IZ-CYP24 and IZ-VDRE cells were obtained in the selection process, respectively. All hygromycin B-resistant monoclonal populations were tested for responsiveness to model VDR agonist 1α,25-dihydroxyvitamin D3 (calcitriol). Three clones of each cell line inducible by calcitriol were acquired, displaying fold induction over DMSO-treated cells in the range 29- to 73-fold (10^3-10^4 RLU) and 5- to 11-fold (10^6-10^7 RLU) for the IZ-CYP24 and IZ-VDRE cell lines, respectively (Figure 7). Based on RLU values and fold induction levels, clones 2 and 4 were selected for detailed characterization of IZ-CYP24 and IZ-VDRE cells, respectively.

Both IZ-CYP24 and IZ-VDRE cell lines remained fully functional for more than 78 days in cell culture (corresponding to 32 passages) (Table 2).During the characterization, the IZ-CYP24 cell line yielded 30-fold induction: on the order of magnitude 10^3–10^4 RLU after treatment with 50 nM calcitriol. IZ-VDRE cells showed ~10-fold induction: on the order 10^6–10^7 RLU after the application of 50 nM calcitriol. This extremely high luciferase signal makes the IZ-VDRE cell line a good tool for sensitive and high-throughput screening for VDR activators and antagonists. Both cell lines were used in the 96-well plate format throughout the whole characterization process, with sufficiently strong signals. The responsiveness to calcitriol was fully retained after a freeze–thaw cycle (Figure 8). Time-course analyses revealed 24 h of incubation with the tested compounds to be optimal time for the identification of VDR ligands, while the minimal time to obtain a sufficient luciferase signal seems to be 8 hours after the treatment, allowing for the testing of cytotoxic compounds (Figure 9).

Selectivity of IZ-CYP24 and IZ-VDRE cells was tested by incubation with agonists of NRs (RAR, RXR, and TR) and steroid receptors (GR, MR, PR, ER, and AR). No induction of luciferase activity was observed after the treatment with aldosterone,

dexamethasone, progesterone, 5α-dihydrotestosterone, 17β-estradiol, 9-cis retinoic acid, or all-trans retinoic acid, either in the presence (antagonist mode) or absence (agonist mode) of 50 nM calcitriol. This finding indicates no cross-talk with GR, MR, PR, AR, ER, RXR, or RAR in either cell line, implying no false positive results (Figure 10). Slight augmentation of the calcitriol-induced luciferase signal was observed after the treatment with dexamethasone, progesterone and 5α-dihydrotestosterone in the IZ-CYP24 cell line, but not in the IZ-VDRE cell line, suggesting possible roles of GR, PR, and AR in the regulation of *CYP24A1* gene expression. By contrast, non-genomic mechanisms are likely due to supra-physiological concentrations (10^{-5}–10^{-6} M) of hormones used during the treatments. In both cell lines, moderate cross-reactivity with thyroids was observed. This finding is consistent with induction of p-glycoprotein by levothyroxine in human intestinal cell lines LS180 and Caco-2 (Mitin *et al*., 2004). This observation must be taken into consideration while interpreting the experimental results.

As proof of concept, the IZ-CYP24 and IZ-VDRE cell lines were used for profiling of vitamin D analogues and intermediates in vitamin D2 and vitamin D3 metabolic pathways in relation to VDR transcriptional activity. Our data revealed induction of luciferase activity not only by obligatory ligands, but also by their precursors and degradation products (Figures 11–13). Nevertheless, some intermediates and analogues significantly induced VDR at a supra-physiological concentration (above 10^{-5} M). A non-genomic mechanism may contribute to this phenomenon, apart from low efficacy of the tested compounds.

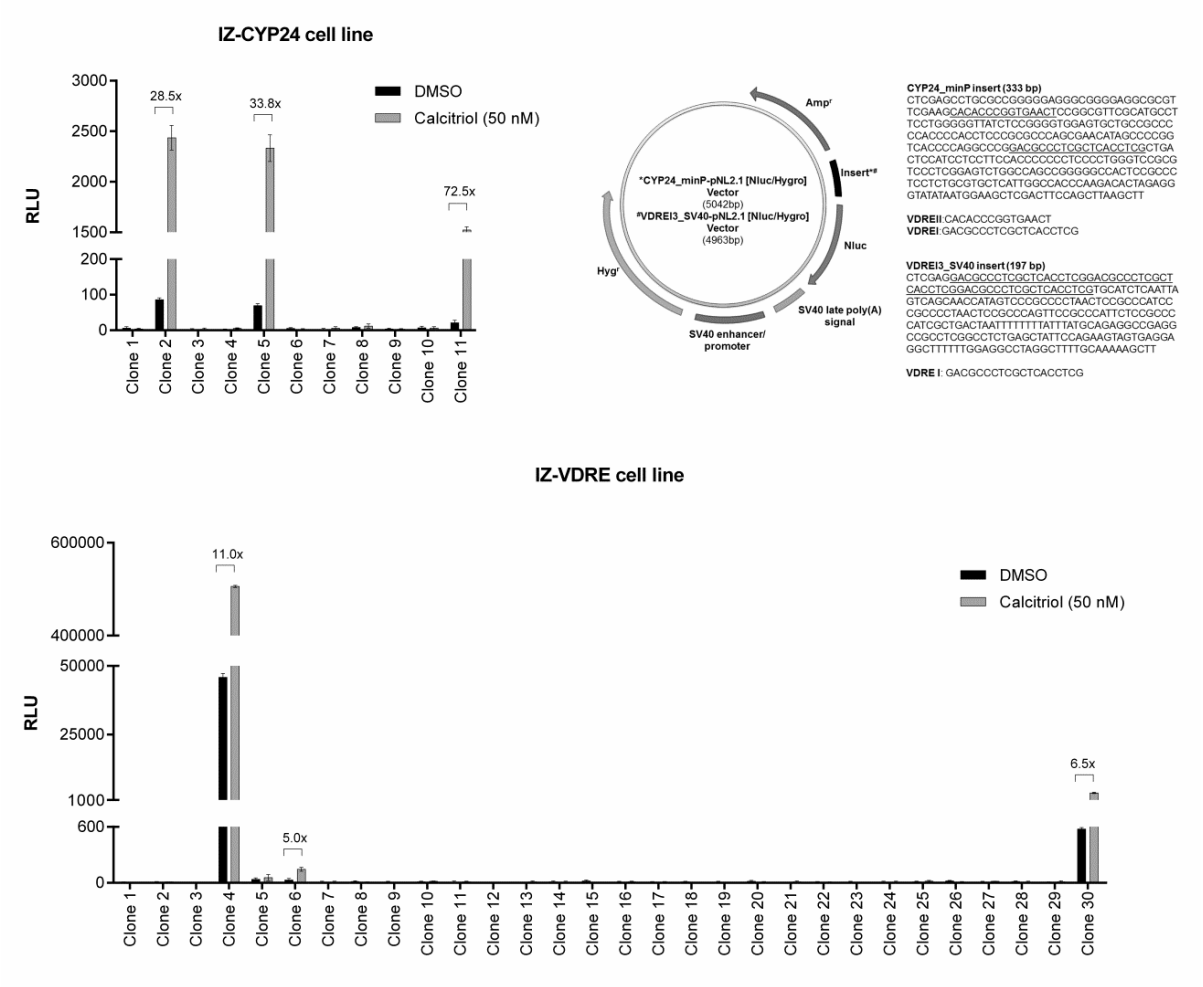

Figure 7: Monoclonal populations of IZ-CYP24 and IZ-VDRE cell lines.

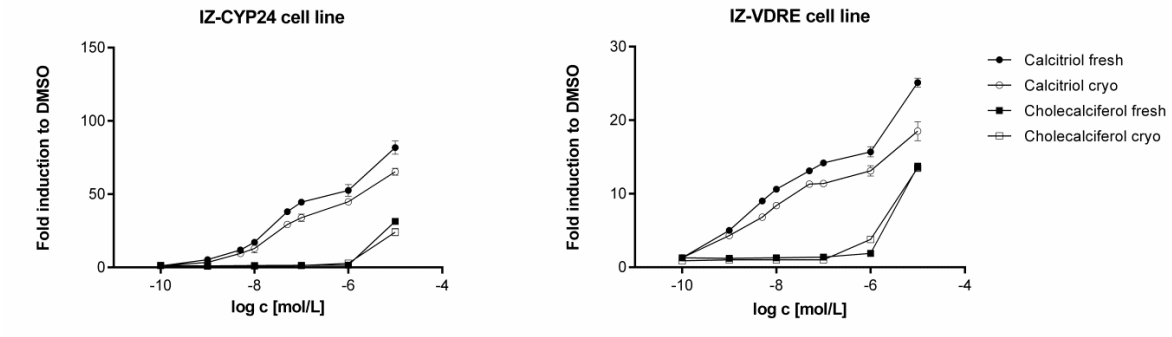

Figure 8: Inducibility of IZ-CYP24 and IZ-VDRE cells after cryopreservation.

Table 2: Long-term maintenance of the responsiveness of IZ-CYP24 and IZ-VDRE cell lines to calcitriol.

Days in culture	IZ-CYP24			IZ-VDRE		
	Passage	RLU	FOLD	Passage	RLU	FOLD
7	4	6984	31.0	3	505902	11.0
20	8	1930	21.7	8	217855	8.9
27	11	2977	33.4	11	440674	13.1
34	14	1687	32.6	14	513340	9.5
41	17	2300	42.8	17	554728	7.4
50	21	3204	36.7	20	787154	10.6
57	24	1620	33.4	23	1307103	8.0
63	26	1452	22.2	25	652532	10.8
71	29	1078	30.2	28	386239	9.0
78	32	830	26.5	31	349611	11.3

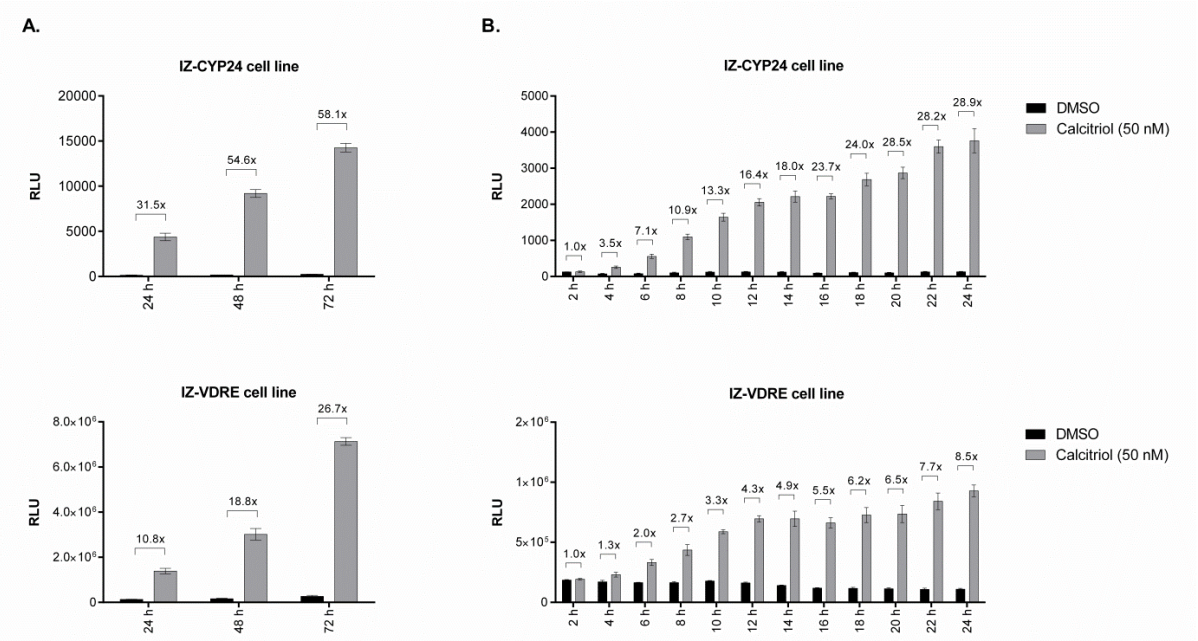

Figure 9: Time-course analyses of luciferase induction in the IZ-CYP24 and IZ-VDRE cell lines.

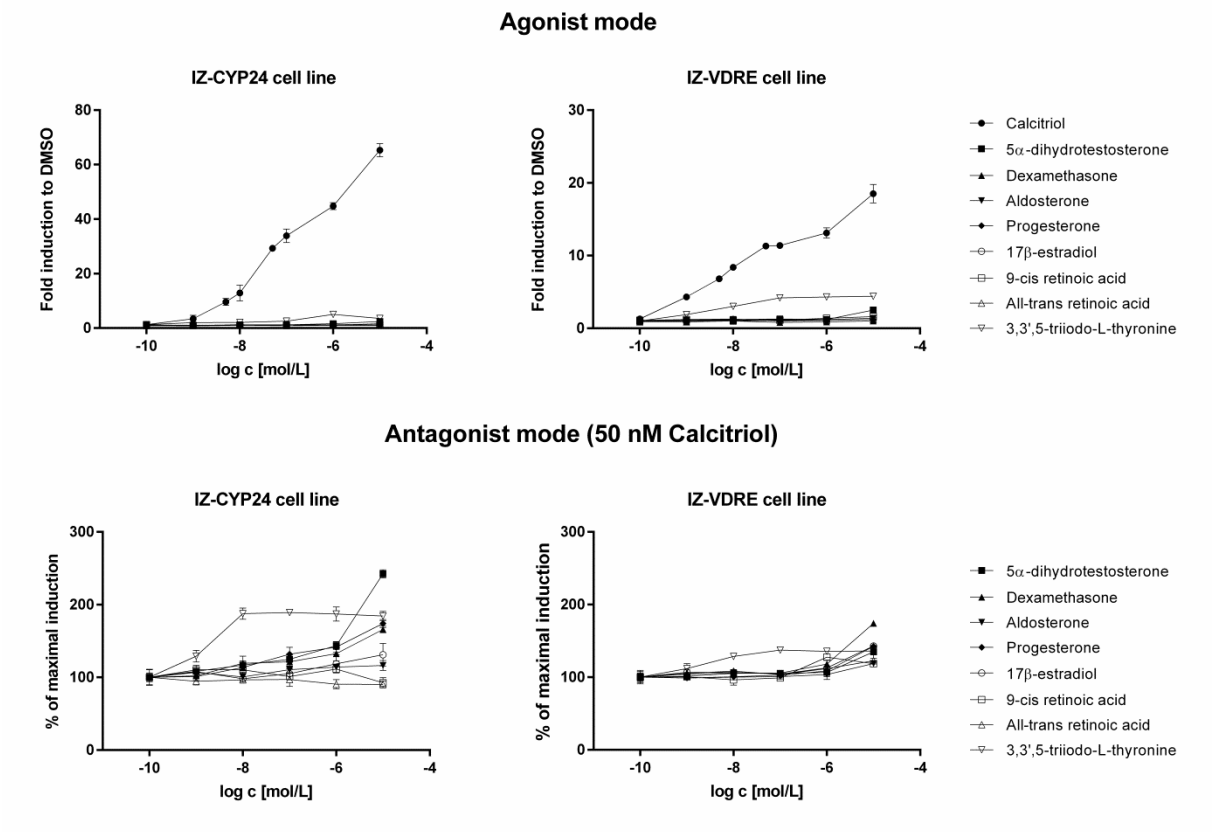

Figure 10: Selectivity of IZ-CYP24 and IZ-VDRE cells.

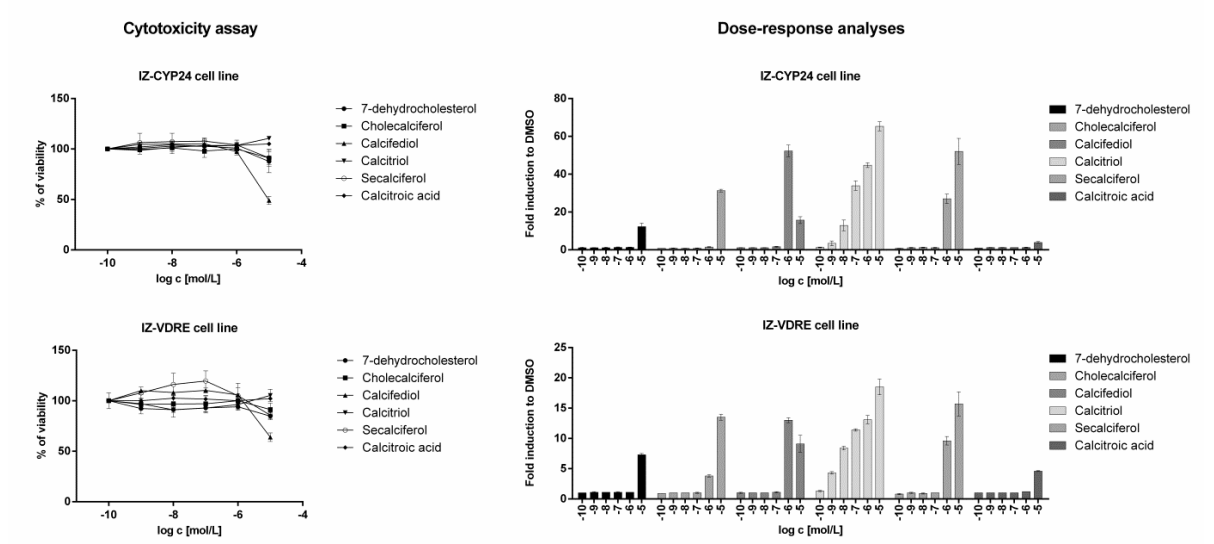

Figure 11: Profiling the vitamin D3 metabolic pathway.

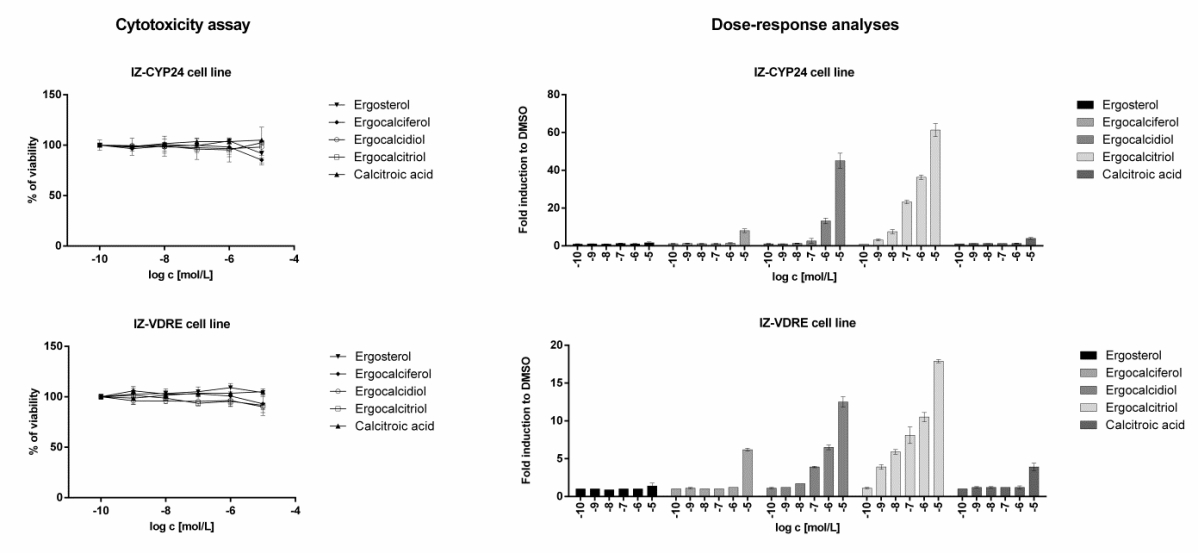

Figure 12: Profiling the vitamin D2 metabolic pathway.

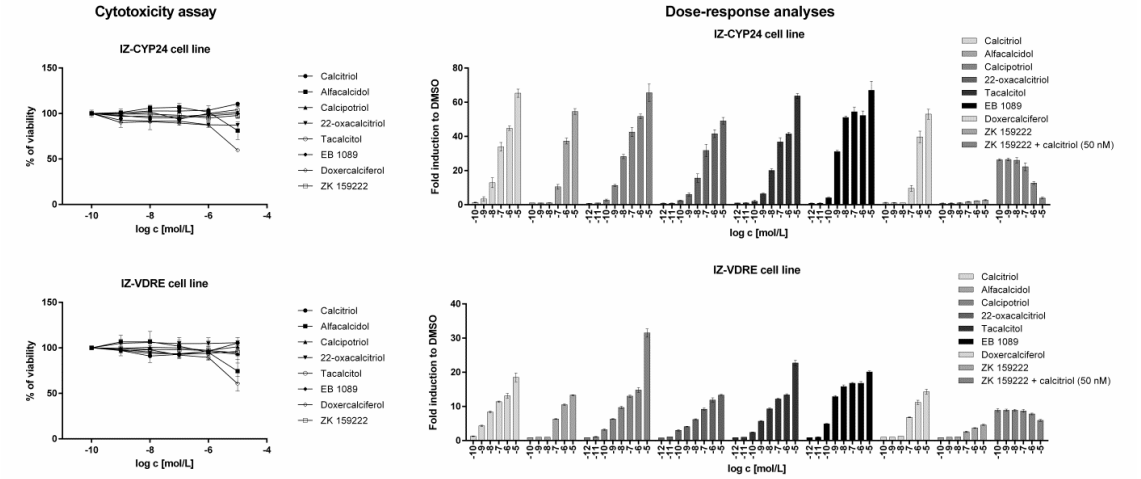

Figure 13: Profiling of vitamin D analogues.

6 CONCLUSION

In the presented thesis, I developed and characterized unique novel human stably transfected reporter cell lines for evaluation of the transcriptional activity AR (AIZ-AR cell line) and VDR (IZ-CYP24 and IZ-VDRE cell lines). These cell lines represent a rapid, high-throughput, selective, sensitive, reproducible and reliable tool for the identification of AR and VDR ligands. The possible use of these cell lines in toxicological, environmental, food safety, pharmacological and drug-discovery applications is anticipated.

Lastly, AIZ-AR, IZ-CYP24 and IZ-VDRE cell lines are now commercially distributed by the Canadian company *Applied Biological Materials (ABM) Inc.*

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- **Bartonkova, I**, Grycova, A, Dvorak, Z (2016): Profiling of vitamin D metabolic intermediates toward VDR using novel stable gene reporter cell lines IZ-VDRE and IZ-CYP24. *Chem Res Toxicol* 29, 1211-1222. [IF_{2015} 3.025]. Contributions to the published work: participated in research design, performed data analysis, conducted experiments, wrote or contributed to the manuscript.

Conference reports:

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