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IN VITRO BIOLOGICAL ACTIVITY OF SELECTED COMPLEXES OF TRANSITION METALS

Ph.D. Thesis

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I declare that the Ph.D. thesis is an original work, based on research carried out by me from September 2013 – September 2017 at the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc. The co-authors are in agreement regarding the inclusion of the published results. All the references cited in this study are listed in the "References" section.

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Abstract

Metal compounds have an invaluable and vital role in medicinal chemistry. The D-block elements form a unique group, referred to as transition metals, in the periodic table. These elements use the *d* orbitals of their valence layer, which are gradually filled, to form chemical bonds. These elements have different oxidation states and could bind to different molecules or anions (referred to as ligands), to form coordination compounds. Metal atoms are often coordinated by negatively charged biomolecules (e.g. proteins and nucleic acids). Moreover, significant progress has reportedly been observed in the use of transition metal complexes for the treatment of human diseases, including carcinomas, lymphomas, anti-inflammatory, anti-diabetic, anti-infective, and neurological disorders. The current study enabled us to understand the mechanism of action and pharmacological effect of selected coordination compounds. Copper(II)/gold(I) mixed-ligand complexes containing quinolinone and *N*-donor heterocyclic ligands and/or triphenylphosphine and deprotonated forms of *O*-substituted derivatives 9-deazahypoxanthine in their structure were studied, respectively. Their effects on the transcriptional activity of receptors for steroid hormones (androgen receptor, glucocorticoid receptor), xenoreceptors (aryl hydrocarbon receptor, pregnane X receptor) and nuclear receptors (thyroid receptor, vitamin D receptor) were evaluated by employing the gene reporter assay, electrophoretic mobility shift assay (EMSA), qRT-PCR, and simple western blotting. The results revealed that gold(I) mixed-ligand complexes of 9-deazahypoxanthine exhibited pleiotropic effects against the panel of tested receptors, and that mixed-ligand copper(II) complexes activated the aryl hydrocarbon receptor AhR, and induced CYP1A gene expression in human hepatocytes and human cancer cell lines.

Keywords	Cytochrome P450, copper(II) complexes, gold(I) complexes, human hepatocytes, nuclear receptors, xenobiotics
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Abstrakt

Sloučeniny kovů mají neocenitelnou a zásadní úlohu v lékařské chemii. Prvky *d-bloku* reprezentují v periodickém systému unikátní skupinu označovanou jako přechodné kovy. Tyto prvky využívají k tvorbě chemických vazeb postupného zaplňování *d* orbitalů jejich valenční vrstvy. Kromě toho se vyskytují v různých oxidačních stavech a mohou na sebe vázat různé molekuly nebo anionty (označované jako ligandy) za vzniku koordinačních sloučenin. Atomy kovu jsou často koordinovány záporně nabitými biomolekulami (např. proteiny a nukleovými kyselinami). Výzkumné studie navíc ukázaly významný pokrok ve využití komplexů přechodných kovů pro léčbu lidských onemocnění, jako jsou karcinomy, lymfomy, protizánětlivé, anti-diabetické, anti-infekční a neurologické onemocnění. Důvodem pro vypracování současné studie bylo porozumění mechanismu účinku a popis farmakologické účinnosti vybraných koordinačních sloučenin. Z tohoto důvodu byly studovány měďnaté/zlatné komplexy obsahující ve své struktuře chinolinon a *N*-donorové heterocyklické ligandy a/nebo trifenylfosfin a deprotonovanou formu *O*-substituovaných derivátů 9-deazahypoxantinu. Hodnotily se jejich účinky na transkripční aktivitu receptorů pro steroidní hormony (androgenní receptor, glukokortikoidní receptor), xenoreceptorů (aryl uhlovodíkový receptor, pregnanový X receptor) a jaderných receptorů (thyroidní receptor, receptor pro vitamin D), za využití reportérové eseje a gelové retardační analýzy (EMSA), qRT-PCR a western blotu. Hlavní zjištěné poznatky jsou takové, že zlatné komplexy s deriváty 9-deazahypoxantinu vykazují pleiotropní účinky vůči panelu testovaných receptorů a že měďnaté komplexy aktivují aryl uhlovodíkový receptor a indukují expresi genu CYP1A v lidských hepatocytech a lidských nádorových buněčných liniích.

Klíčová slova	Cytochrom P450, měďnaté komplexy, zlatné komplexy, lidské hepatocyty, jaderné receptory, xenobiotika
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Abbreviations

AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
ATP	Adenosine triphosphate
bphen	Bathophenanthroline
CAR	Constitutive androstane receptor
cDNA	Complementary double-stranded deoxyribonucleic acid
CYP	Cytochrome P450
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	Cytochrome P450, family 1, subfamily A, polypeptide 2
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
CYP2A6	Cytochrome P450, family 2, subfamily A, polypeptide 6
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9
CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
CYPOR	NADPH: cytochrome P450 reductase
DBD	Deoxyribonucleic acid (DNA) binding domain
DDI	Drug-drug interactions
DEX	Dexamethasone
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DMF	*N,N´*-Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DR4	Direct repeat separated by four nucleotides
DTT	1,4-dithiothreitol
ECACC	The European Collection of Authenticated Cell Cultures
EC_{50}	Half-maximal effective concentration
EM	Extensive metaboliser
EMSA	Electrophoretic mobility shift assay
ER	Oestrogen receptor
ERα	Oestrogen receptor alpha
FAD	Flavin adenine dinucleotide

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIT	Gastrointestinal tract
GR	Glucocorticoid receptor
HAH	Halogenic aromatic hydrocarbon
HeLa	Human Negroid cervix epitheloid carcinoma cells
HEP	Human hepatocytes
HepG2	Human Caucasian hepatocellular carcinoma cells
HHx	Human hepatocyte (x = patient´s number)
HNF	Hepatocyte nuclear factor
Hqui1	2-phenyl-3-hydroxy-4(1*H*)-quinolinone
Hqui2	2-(4-amino-3,5-dichlorophenyl)- N-propyl-3-hydroxy-4(1*H*)-quinolinone-7-carboxamide
HSP	Heat-shock protein
IC_{50}	Half-maximal inhibitory concentration
IM	Intermediate metabolizer
LBD	Ligand-binding domain
LS180	Human Caucasian colon adenocarcinoma cells
LXR	Liver X receptor
MCF7	Human Caucasian breast adenocarcinoma cells
mphen	5-methyl-1,10-phenanthroline
mRNA	Messenger single-stranded ribonucleic acid
M-MuLV	Moloney Murine Leukemia Virus
MR	Mineralocorticoid receptor
MT	Metallothionein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NR	Nuclear receptor
OATP	Organic anion transporting polypeptides
OCT	Organic cation transporters
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
P-gp	P-glycoprotein

phen	1,10-phenanthroline
PM	Poor metabolizer
PNP	Purine nucleoside phosphorylase
PPAR	Peroxisome proliferator-activated receptor
PPh_3	Triphenylphosphine
PR	Progesterone receptor
PXR	Pregnane X receptor
qRT-PCR	Real-time quantitative polymerase chain reaction
RAR	Retinoic acid receptors
RE	Response element
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 medium
RVT	Resveratrol
RXR	Retinoid X receptors
SOD	Superoxide dismutase
SPOT14	Thyroid hormone responsive
SXR	Steroid and xenobiotic receptor
T3	3,3´,5-triiodo-L-thyronine
TAT	Tyrosine aminotransferase
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TR	Thyroid hormone receptor
TRE	Thyroid hormone response elements
TrxR	Thioredoxin reductase
TX100	Triton X100
UM	Ultra-rapid metaboliser
UPL	Universal Probe Library
VD3	25-hydroxyvitamin D3
VDR	Vitamin D receptor
XMEs	Xenobiotic-metabolizing enzymes
22Rv1	Human prostate carcinoma epithelial cells
3-MC	3-methylcholantrene

1 INTRODUCTION

Humans are permanently exposed to a variety of exogenous chemicals (xenobiotics), such as food additives, metals, pesticides, alkaloids, environmental pollutants, and drugs. If the human body is exposed to xenobiotics, biochemical regulatory pathways might be disrupted, which could lead to an imbalance in the organism, resulting in the onset of disease. Physiological (sex, age, nutrition, pregnancy, and polymorphisms) and pathophysiological (inflammation, infection, environmental pollutants, oxidative stress, and hypoxia) factors also play a crucial role in the regulation of xenobiotic metabolism. Therefore, it is important to understand the molecular basis of interactions between xenobiotics and the signalling pathways involved in eubiotic and xenobiotic metabolism.

Cytochromes P450 (CYPs) are enzymes that play a pivotal role in oxidative xenobiotic biotransformation. CYPs could be transcriptionally activated by various xenobiotics and endogenous substrates through receptor-dependent mechanisms. CYP enzyme inhibition is a principal mechanism that enables metabolism-based drug-drug interactions (DDI) to occur. The use of many chemotherapeutic drugs could cause DDI to occur owing to their ability to either inhibit or induce the CYP enzyme system. Genetic polymorphisms and epigenetic changes in CYP genes might be responsible for inter-individual and inter-ethnic variations in disease susceptibility and the therapeutic efficacy of drugs.

Organometallic compounds have received a great amount of attention in the past three decades because they are potential chemotherapeutic agents (Brabec and Novakova, 2006; Liu and Sadler, 2011). Moreover, it has been unambiguously demonstrated that these compounds might also exhibit anti-inflammatory, anti-microbial (Berners-Price and Filipovska, 2011), or anti-viral activity (e.g. anti-HIV) (Lewis et al., 2011) in both *in vitro* and *in vivo* systems, depending on the central atom (metal) and ligand type.

The discovery of the anti-proliferative properties of *cis*-diammine-dichloridoplatinum(II), *cis*-$[Pt(NH_3)_2Cl_2]$, commonly known as cisplatin, in the late 1960s had sparked an interest in metal-based anticancer drugs (Rosenberg et al., 1967). Currently, this platinum-based chemotherapeutic agent remains the most widely used drug (Kelland and Farrell, 2000) in oncology patients. Though it is primarily used in the treatment of cancer (e.g. cancer of the ovaries or testicles), it is also used against other types of tumours (sarcomas, lymphomas) (Zhang and Lippard, 2003). Unfortunately, the use of cisplatin is limited because of a number of drawbacks (e.g. nephrotoxicity,

neurotoxicity, and myelosuppression) (Wang and Lippard, 2005). Additionally, there is a risk of the patient developing drug resistance to cisplatin. Consequently, tremendous efforts have been devoted to the research and development of new anti-tumour active coordination compounds with improved and/or selective pharmacological properties (Alessio, 2011). A number of transition metal complexes have been tested to estimate their biological activity, particularly complexes of platinum, but also complexes of copper, zinc, iron, cobalt, and nickel, etc. However, only a few cisplatin derivatives have been approved for treatment (Tiekink and Gielen, 2005). Therefore, designing new metal complexes is one possible way to discover new biologically active coordinating compounds. Numerous studies have shown that coordination with a suitable metal can cause a change or increase in the biological activity of the complex, as compared to that of parental molecule (Vanco et al., 2017).

The present study focused on gold(I) and copper(II) mixed-ligand complexes. Gold(I), the central atom, was coordinated with an *O*-substituted derivative of 9-deazahypoxanthine. In addition, a triphenylphosphine molecule was introduced into the structure. The central atom of copper(II) was coordinated with bidentate *N*-donor heterocyclic ligands, quinolinone, and quinolinone-7-carboxamide derivatives. Their complexes were also extensively studied, particularly with respect to cancer therapy (Tardito and Marchio, 2009). Their activities of several biologically active complexes of transition metals at nuclear receptors and the expression of their target genes were investigated.

The activation of nuclear receptors by xenobiotics and the subsequent induction of xenobiotic-metabolizing enzymes (XMEs) together affect human health and might result in the following: (i) endocrine disruption and/or perturbation of intermediary metabolism (Heindel et al., 2015); drug-drug and food-drug interactions (change in drug metabolism) (Chen et al., 2014; Margina et al., 2015); (iii) bioactivation of relatively harmless compounds (e.g. pro-carcinogens) to yield highly toxic substances (e.g. carcinogens).

2 AIMS

The current study aimed to investigate the effects of the selected copper(II) and gold(I) mixed-ligand complexes on the signalling pathways of human nuclear receptors, steroid receptors, and xenoreceptors, evaluate the following:

1. The transcriptional activities of androgen receptor, glucocorticoid receptor, receptor for thyroid hormone, vitamin D receptor, aryl hydrocarbon receptor, and pregnane X receptor in human cancer cell lines, by means of reporter gene assays and EMSA.
2. The expression of target genes for the above-listed receptors at the mRNA and protein levels in primary cultures of human hepatocytes and human cancer cell lines. These genes included *CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP3A4*, tyrosine aminotransferase (*TAT*) and *SPOT14*.

3 THEORETICAL PART

3.1 TRANSITION METALS

The d-block elements, called transition metals, are located between the s-block and p-block elements. The presence of the valence *d*-orbital (mostly only partially occupied) in these elements sets them apart from the rest of the elements in the periodic table. In general, d-block elements have similar physical and chemical properties, which are conferred by the configuration of electrons in their orbits. In addition, the *d*-elements have a metallic character, thus, they are good conductors of electricity and heat. Transition metals possess a higher density, melting, and boiling point, and they are solid and hard at room temperature, with the exception of mercury. They possess a characteristic ability to exist in many different oxidation states, which could be very important in the formation of metal-based drugs. This property is attributable to the presence of multiple valence electrons, which could be arranged in many similar energy states in *d*-orbits. Their extraordinary ability to form highly stable compounds, results from the presence of vacant valence *d*-orbitals (Housecroft and Sharpe, 2012).

3.1.1 Copper

Copper is a transition metal located at the 4th period and 11th group in the periodic table. Copper occurs in different structural forms, depending on the oxidation state and coordination number of a particular coordination compound. Copper forms a number of compounds (e.g. oxides, sulphides, halogens, salts); it is predominant in compounds of metals with the oxidation numbers I and II. The oxidation numbers III, IV, and V, and negative oxidation states are less common and these are especially stabilized in complexes (Greenwood and Earnshaw, 1997).

Copper is an essential trace element that is indispensable for many living organisms. The human body receives 2 - 5 mg of copper per day via food, and the total amount of copper in the body of an adult is approximately 150 mg (Linder and Goode, 1991). It is important to consume a daily dose of copper, because it plays an irreplaceable role in the proper functioning of organs and metabolic processes. Albumin, transcuprein, and ceruloplasmin are among the major copper transporter proteins. Fifty percent (ca. 1.5 mg) of copper obtained from food is absorbed in the stomach and the upper part of the small intestine; the unused amount of metal is removed via stools. Absorbed copper is transported via albumin to the liver, where it is

taken up by liver cells and partially excreted into the bile (Silva and Williams, 2001). Consequently, it is transmitted to peripheral tissues that are mainly bound to the protein ceruloplasmin and, to a lesser extent, to albumin. The transport of copper is compromised in individuals with Wilson's disease (Gitlin, 2003). The liver contains 10% of the total copper content in the body. An excess of copper is regulated by alterations in both the absorptive efficiency and biliary excretion in the gut. If the intake of copper is too low or high, the absorption efficiency is regulated up and down, respectively, but it is predominantly controlled via endogenous excretion (Harvey et al., 2003). However, any alteration in the metabolism of copper results in toxic symptoms (Wang and Guo, 2006). Copper can accumulate in the liver and brain, leading to their dysfunction (Strausak et al., 2001).

Copper is an important constituent of many proteins and metalloenzymes (see Table 1) (Alessio, 2011). It is important for building and maintaining the skeletal system and for the activity of the nervous system. Furthermore, copper is also included in many other biological processes such as iron absorption and transport, haematopoiesis, and cholesterol and glucose metabolism. The total amount of copper in the body is regulated by metallothioneins (MTs). They are low-molecular weight proteins rich in the amino acid cysteine, which easily binds metals through -SH groups. In metalloenzymes, copper participates in vital oxidation-reduction (redox) processes via redox cycling between the oxidation states I and II (Linder and Goode, 1991). The potential toxicity of this metal arises from its ability to generate reactive oxygen species (ROS), which are capable of binding to biomolecules such as lipids, proteins, and nucleic acids (Jomova and Valko, 2011). Cu, Zn-superoxide dismutase (Cu, Zn-SOD) plays a key role in the protection of cells and the entire human body against ROS (e.g. hydroxyl radical, singlet oxygen, hydrogen peroxide radicals). Cu, Zn-SOD catalyses the conversion of the cytotoxic superoxide anion to hydrogen peroxide and oxygen.

The increased occurrence of superoxide radicals (oxidative stress) has been observed in cancer cells. The specific activity of superoxide dismutase enzymes has been shown to be reduced in these cells (Oberley and Buettner, 1979). In general, oxidative stress has been implicated in the occurrence of more than hundreds of diseases, including diabetes, and in neurodegenerative or inflammatory processes (Slemmer et al., 2008; Yorek, 2003). Some other diseases result from an imbalance of copper homeostasis, due to chronic copper deficiency or overload, and/or genetic predisposition (Shim and Harris, 2003). These include Menkes disease, Wilson disease

(DiDonato and Sarkar, 1997), Indian childhood cirrhosis (Tanner, 1998), and idiopathic copper toxicosis (Muller et al., 1998).

Table 1: Copper-dependent and copper-transporting proteins (Balamurugan and Schaffner, 2006).

Protein/Enzyme	Location	Function
Cu/Zn superoxide dismutase	Cytosol	Antioxidant defence (superoxide dismutation)
Cytochrome *c* oxidase	Mitochondria	Mitochondrial respiration
Ceruloplasmin	Plasma	Iron and copper transport (ferrioxidase)
Lysyl oxidase	Elastin and collagen	Connective tissue formation
Dopamine-b-hydroxylase	Storage vesicle	Catecholamine production
Tyrosinase	Storage vesicle	Melanin formation
Peptidylglycineα-amidating mono-oxygenase	Storage vesicle	Peptide amidation (activation of peptides)
Metallothionein	Liver and kidneys	Storage and chaperone

3.1.2 Copper(II) – quinolinone complexes

Quinolinones (4-quinolinones) are a class of synthetic heterocyclic compounds containing a quinolin-4(1*H*)-one skeleton (Figure 1A), and are known for their biological activities. The skeleton acts as the structural backbone of many drugs used in pharmacotherapy against a number of diseases (Andriole, 2005). In general, quinolinones could be divided based on their chemical structure as follows: quinolones and chemically non-quinolin-4-one derivatives. An example of a non-quinolin-4-one derivative is, nalidixic acid (Figure 1B), which is a therapeutic agent for urinary tract infections and, was first prepared in 1963 (Barlow, 1963). Since then, quinolinones have been used to treat a variety of inflammatory diseases (respiratory infections, soft tissue infections, bone-joint infections, typhoid fever, sexually transmitted diseases, prostatitis, etc.), apart from psychosis and cancer (Andriole, 2005). Another representative is a quinolinone analogue vesnarinone (quinolin-2-one), which causes an off-target anticancer effect during the treatment of congestive heart failure

(Sato et al., 1995). The development of new therapeutic agents has led to the discovery of the antibacterial effects of fluoroquinolones (Dembry et al., 1999).

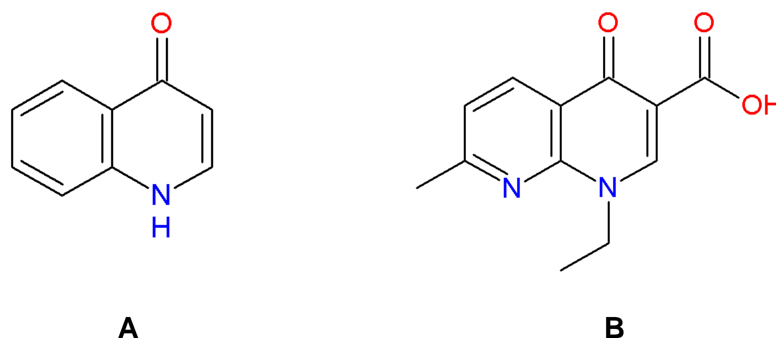


Figure 1:
The structural formulas of the quinolin-4(1H)-one skeleton (A) and nalidixic acid (B) (ACD/ChemSketch).

In the past, many alkaloids having a quinolin-4-one skeleton have been isolated and described independently from research regarding synthetic analogues. Many of them exhibit bactericidal and bacteriostatic effects. They are used as anti-fungal, anti-inflammatory, anti-tubercular, anti-cancer, anti-neoplastic, anti-ischemic, anti-allergic, anti-hypertensive and anti-ulcerative agents (Creaven et al., 2010; Kalkhambkar et al., 2008; Xu et al., 2006). Furthermore, the quinolinone derivatives have been reported to possess a pro-apoptotic activity (Claassen et al., 2009). All these derivatives have a common mechanism of action, i.e. they target DNA-gyrases. These enzymes (topoisomerase II and IV) are involved in DNA replication, and quinolones act as potent topoisomerase II inhibitors (Pommier et al., 2010).

The present study deals with quinolin-4-one in the form of copper(II) mixed-ligand complexes. Copper-containing compounds with a wide range of ligands are a frequent subject of research, because of their various biological effects. As mentioned above, copper is a biogenic element that is indispensable for the human organism, and therefore appears to be suitable for the preparation of biologically active non-platinum compounds. Copper complexes are an object of interest because of their demonstrated anti-oxidant (Vanco et al., 2004), anti-diabetic (Murata et al., 1998), anti-inflammatory (Weder et al., 2002), anti-microbial (Psomas et al., 2006), anti-parasitic (Gokhale et al., 2003), and, cancerostatic effects (Giovagnini et al., 2008). Copper-containing complexes with *N*, *N'*-donor ligands appear to be suitable for the preparation of potential therapeutic agents. These have shown notable cytotoxicity, with mechanisms

of action related to varied DNA interactions and redox metabolism alterations (Bravo-Gomez et al., 2009; Marzano et al., 2009; Palanimuthu et al., 2013). The 1,10-phenanthroline-based ligands (known as Casiopeinas®) were the first reported copper(II) complexes that inhibited tumour growth *in vivo* (Dwyer et al., 1965). Since then, many copper(II) complexes containing 1,10-phenanthroline (phen) and other bidentate *N*-donor heterocyclic ligands (N-N) have been synthesized and studied to determine if they have anticancer properties. Therefore, we studied Casiopeina®-like complexes having the general composition [Cu(N-N)(*qui*)]X.*y*H_2O. Specifically, four copper(II) mixed-ligand complexes with the general composition [Cu(*qui1*)(L)]Y·*x*H_2O (**1**-**3**) and [Cu(*qui2*)(phen)]Y (**4**), where *Hqui1* = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone (Figure 2A), *Hqui2* = 2-(4-amino-3,5-dichlorophenyl)- *N*-propyl-3-hydroxy-4(1*H*)-quinolinone-7-carboxamide (Figure 2B), Y = NO_3 and L = 1,10-phenanthroline (phen) (**1**), 5-methyl-1,10-phenanthroline (mphen) (**2**), and bathophenanthroline (bphen) (**3**) (Figure 3; **1**-**4**) were evaluated to investigate their ability to influence the expression of major drug-metabolizing enzymes and transcriptional activities of steroid receptors, nuclear receptors, and xenoreceptors. Copper(II) – quinolinone complexes were designed and prepared at the Department of Inorganic Chemistry, Palacký University, Olomouc (Buchtik et al., 2012; Buchtik et al., 2011; Krikavova et al., 2016; Travnicek et al., 2014).

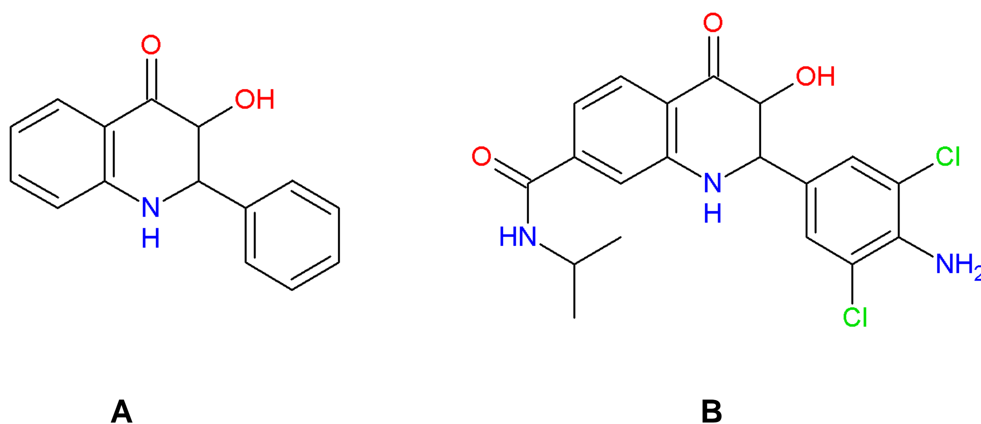


Figure 2:

The structural formulas of N-donor heterocyclic ligands of 2-phenyl-3-hydroxy-4(1H)-quinolinone (Hqui1) (A) and 2-(4-amino-3,5-dichlorophenyl)-N-propyl-3-hydroxy-4(1H)-quinolinone-7-carboxamide (Hqui2) (B) (ACD/ChemSketch).

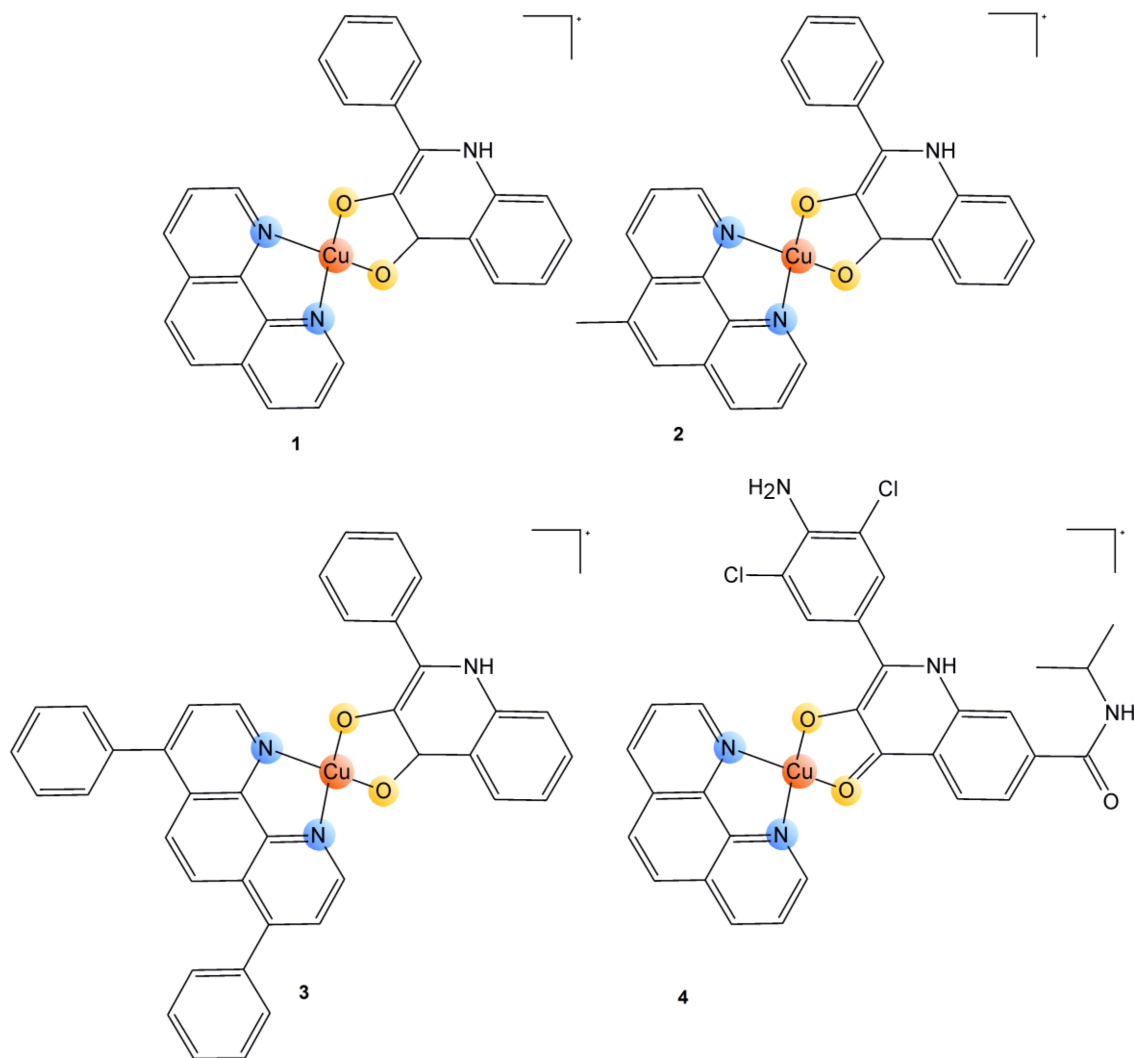


Figure 3:

The structural formulas of copper(II) mixed ligand complexes (ACD/ChemSketch):

[Cu(qui1)(phen)]$NO_3.H_2O$ (1)

[Cu(qui1)(mphen)]$NO_3.H_2O$ (2)

[Cu(qui1)(bphen)]$NO_3.H_2O$ (3)

[Cu(qui2)(phen)]NO_3 (4)

3.1.3 Gold

Gold is a transition metal located in the 6th period and 11th group of the periodic table. Gold compounds occur in oxidation states ranging from -I to +V. The elemental forms of gold are extremely stable under a wide range of conditions, such as those in aqueous systems. Generally, they do not react with oxygen and non-oxidizing acids, and do not react directly with sulphur, though they react very well with halogens. However, in the presence of stabilizing ligands, they can be auto-oxidized to the +I or +III state, which are the most stable oxidation states; they exhibit extensive chemistries in media, such as aqueous media (Greenwood and Earnshaw, 1997). Gold compounds play important roles in biological systems, and could be used for the treatment of several diseases via current medicinal practices (Russell et al., 1997).

Since ancient times, gold has been known as an element that has medicinal effects. Talismans or medallions were used for protection against illnesses and evil spirits. In many cases, potions containing gold powders were administered to ill patients. After alchemists learned to use aqua regia to dissolve gold, compounds as well as elemental gold were used in medicinal treatments (Higby, 1982). Interest in gold compounds increased after the discovery of the use of gold cyanide $K[Au(CN)_2]$ as an anti-tuberculosis drug by Robert Koch in 1890 (Koch, 1890). However, gold cyanide is too toxic as a drug; therefore, scientists focused on gold thiolate salts against pulmonary tuberculosis (Benedek, 2004). Though treatment with gold complexes was ineffective as therapy, led to significant reduction in joint pain. This was the impetus required by the French physician Jacques Forestier, who began his research about the treatment of rheumatoid arthritis with gold compounds in 1935. Nowadays, gold(I) thiolate salts myochrysin (sodium aurothiomalate; sodium [(2-carboxy-1-carboxylatoethyl)thiolato] gold(I) (Figure 4A), solganol (aurothioglucose; [(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)-oxane-2-thiolato] gold(I) (Figure 4B) and ridaura (gold(I) phosphine compound Auranofin; triethylphosphine-(2,3,4,6-tetra-O-acetyl-β-D-thiopyranosato) gold(I) (Figure 4C) are the most important gold compounds that are clinically used for the treatment of rheumatoid arthritis (Eisler, 2003). These disease-modifying anti-rheumatic agents impede and sometimes cause remissions of the disease. These drugs majorly show an anti-inflammatory activity; however, they are expected to have additional mechanisms of action (Madeira et al., 2012).

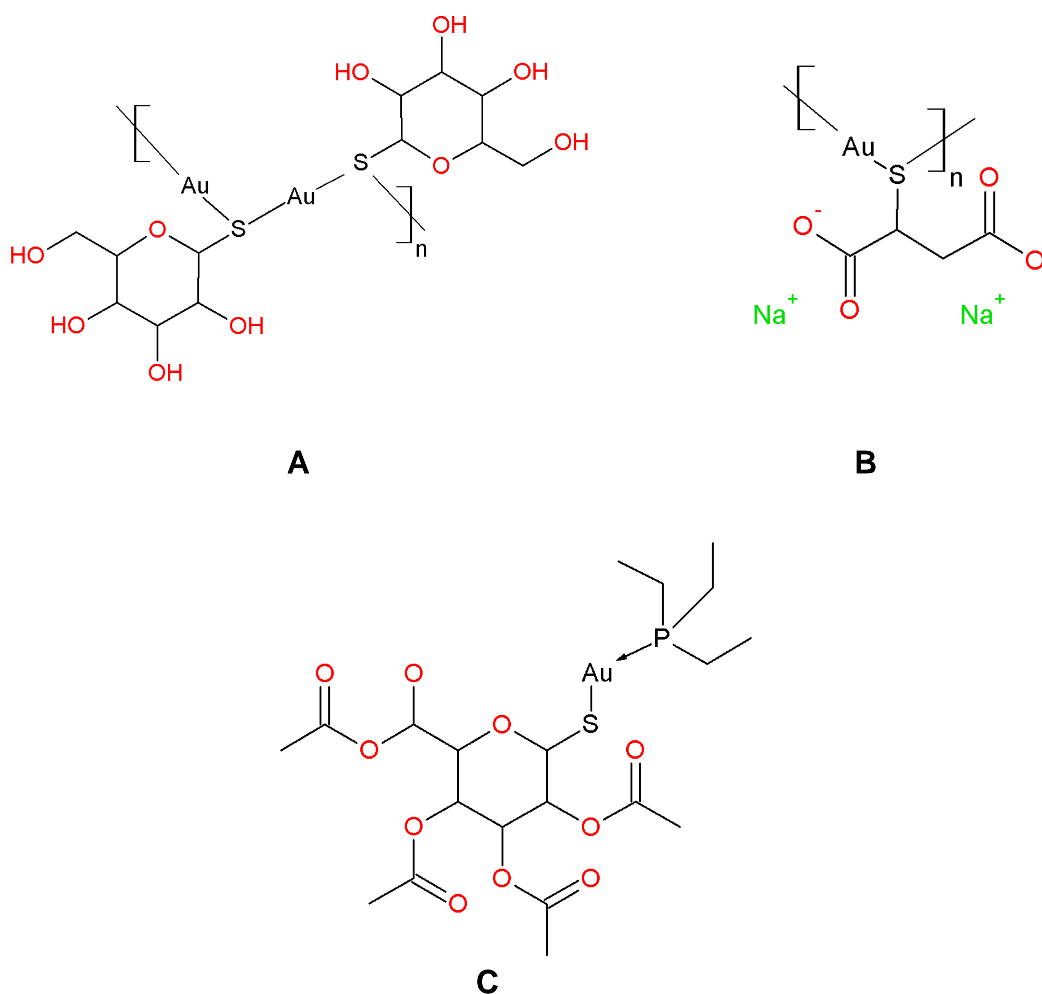


Figure 4:

The structural formulas of gold-containing anti-inflammatory drugs: Solganol (A), Myochrysin (B), Auranofin (C) (ACD/ChemSketch).

3.1.4 Gold(I) - 9-deazahypoxanthine complexes

The 9-deazahypoxanthine derivatives (6-oxo-9-deazapurines) (Figure 5B) are inhibitors of purine nucleoside phosphorylases (PNPs), and are grouped as selective immunosuppressive agents (Bzowska et al., 2000), which are beneficial in the treatment of T-cell proliferative and autoimmune diseases, such as T-cell leukaemia and lymphomas (Bantia et al., 2001). Moreover, *C9*-substituted 9-deazahypoxanthines are efficient PNP inhibitors with two members, ImmucillinH and DADMe-ImmucillinH, for which clinical trials are being conducted, for determining their effectiveness to treat T-cell and B-cell cancers (Dummer et al., 2014).

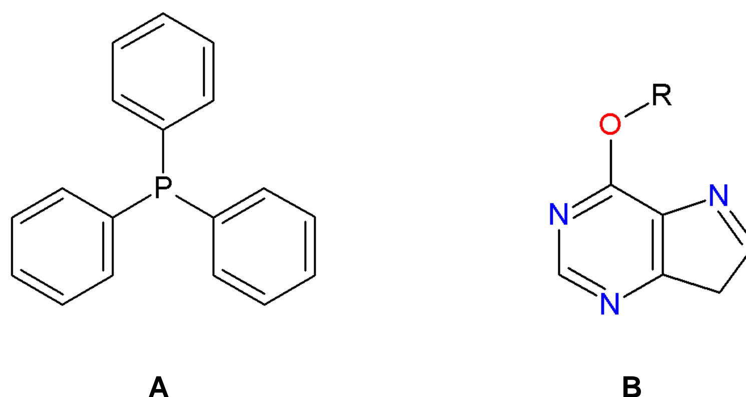


Figure 5:

The structural formulas of triphenylphosphine (A) and the deprotonated form of O-substituted derivatives of 9-deazahypoxanthine (B) (ACD/ChemSketch).

The complexes of gold with *O*-substituted 9-deazahypoxanthine were found to be highly biologically active (Galikova and Travnicek, 2015; Vanco et al., 2017). It is generally known that the substitution of organic ligands, such as the aforementioned N6-benzyladenines, which constitute the structures of transition metal complexes, modulate the biological activity of such complexes (Lemoine et al., 2004; Sorenson, 1976). This fact motivated us to focus on gold(I) triphenylphosphine complexes during the present study.

Gold(I) complexes containing organic phosphine and heterocyclic *N*-donor ligands have received attention because of their biological activity, predominantly because of their cytotoxic (Lima and Rodriguez, 2011), biocidal (Navarro, 2009) and anti-inflammatory activities (Travnicek et al., 2012). Gold(I) coordination compounds are known to play several biochemical roles (Gielen et al., 2005). It has been shown that the gold(I) complexes that exhibit the cytotoxic and antitumor activities do not primarily target DNA; their main targets are the components of the proteasome. In addition, it was shown that gold(I) compounds interact with the active site of cytosolic and mitochondrial thioredoxin reductases (TrxR); acting as potent TrxR inhibitors (Buac et al., 2012).

The gold(I) complexes having the composition $[Au(L_n)(PPh_3)]$ exhibited selective activity against some human cancer cell lines. Moreover, these complexes also showed anti-inflammatory activities that were similar to that of a commercially used anti-arthritis drug, Auranofin (Vanco et al., 2014). Therefore, we studied gold(I) phosphine mixed-ligand complexes having the general formula $[Au(L_n)(PPh_3)]$ (**1-3**),

where HL_n stands for various *O*-substituted derivatives of 9-deazahypoxanthine such as 6-isopropyloxy-9-deazahypoxanthine (HL_1) (**1**), 6-phenethyloxy-9-deazahypoxanthine (HL_2) (**2**), 6-benzyloxy-9-deazahypoxanthine (HL_3) (**3**) (Figure 6; **1**-**3**), and PPh_3 which represents triphenylphosphine (Figure 5A). These compounds were evaluated for their capability to influence the expression of major drug-metabolizing enzymes and transcriptional activities of steroid receptors, nuclear receptors and xenoreceptors. Gold(I) – 9 -deazahypoxanthine complexes were designed and prepared at the Department of Inorganic Chemistry, Palacký University, Olomouc (Krikavova et al., 2014; Vanco et al., 2014).

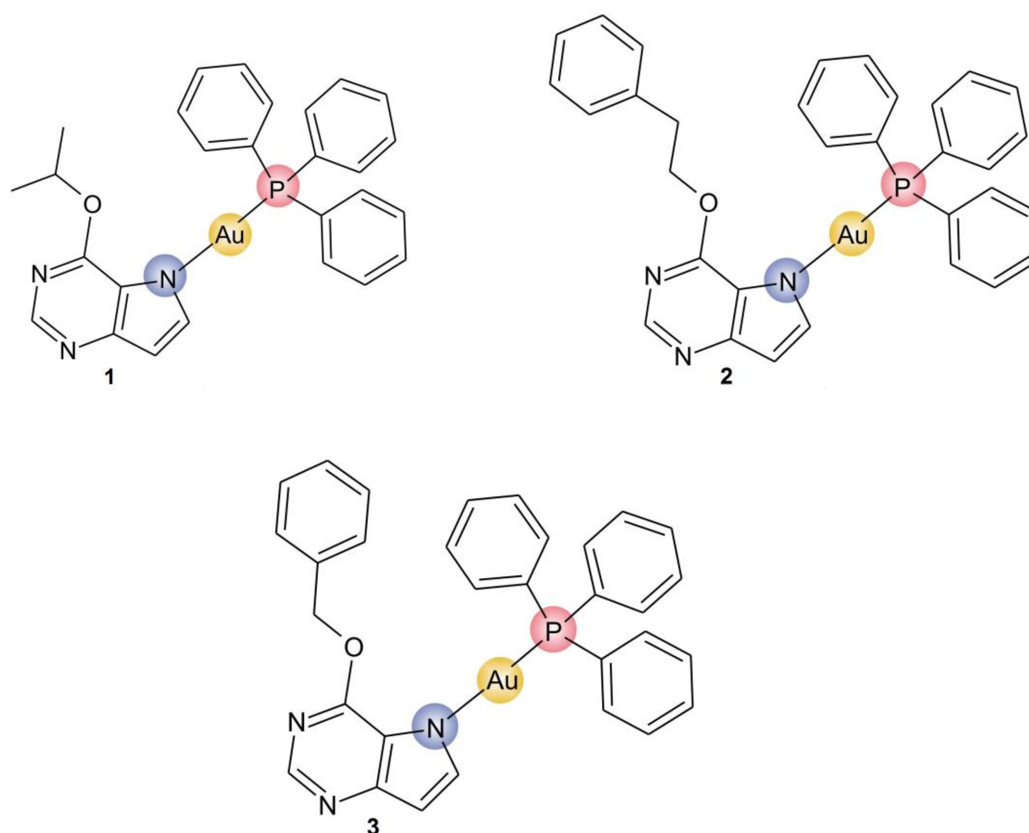


Figure 6:

The structural formulas of gold(I) mixed-ligand complexes (ACD/ChemSketch):

$[Au(HL_1)(PPh_3)]$ (1)

$[Au(HL_2)(PPh_3)]$ (2)

$[Au(HL_3)(PPh_3)]$ (3)

3.2 METABOLISM OF XENOBIOTICS

Xenobiotics are substances that are foreign to the living organism, and enter the organism in different ways. These exogenous substances include industrially manufactured chemical compounds such as pharmaceuticals, preservatives, and food stabilizers, industrial chemicals, cosmetic ingredients, fertilizers, fungicides, herbicides, insecticides, and detergents. Most of the xenobiotics are more or less subjected to extensive transformations in the body by highly effective mechanisms. The pharmacokinetics of xenobiotics include the overall fate of xenobiotics, which includes their absorption, distribution, metabolism and elimination (Casarett et al., 2008).

3.2.1 Absorption & Distribution

Xenobiotics can enter the body via different routes, including oral, nasal, intravenous, intramuscular, intraperitoneal, or subcutaneous application. Xenobiotics enter the body through the air, drinks, foods, etc.; their physicochemical properties (volatility, reactivity, hydrophobicity) influence their entry into the organism. Xenobiotics could interfere with the proper functioning of the organ/body barriers, which might make absorption easier (Hanninen et al., 1979). Within the digestive tract, most substances are absorbed in the intestines or the stomach (Hanninen et al., 1987). They are carried either through the portal vein to the liver (polar xenobiotics) or through the lymphatic system (non-polar substances), after which they enter into the systemic circulation. Xenobiotics interact directly with the epithelial cells (lung, skin, stomach) in the gastrointestinal tract (GIT). In general, polar (hydrophilic) xenobiotics poorly penetrate cell membranes, and are transported into the cell by different channels and transporters. They occur in the free form in the blood; they do not need blood transporters, and they are easily excreted in urine. Conversely, non-polar (hydrophobic) xenobiotics freely diffuse across the cell membrane, but this requires the transport proteins to be present in the blood; otherwise, their elimination from the body is delayed. Therefore, it is necessary to modify the molecule into a form, that could be easily removed from the organism (Nassar, 2010).

3.2.2 Biotransformation and elimination

The conversion of xenobiotics into inactive substances and causing an increase in their polarity to facilitate excretion, i.e., detoxification, is the goal of biotransformation. Biotransformation is affected by many factors, including age,

gender, genetic predisposition, diseases, etc. (Shimada and Guengerich, 2006). The process of biotransformation occurs mainly in the liver, and also occurs in the skin, lungs, nasal mucosa, eyes, intestines, kidneys, pancreas, heart, brain, ovaries, erythrocytes, etc. (Krishna and Klotz, 1994). Additionally, intestinal microflora plays an important role in the biotransformation of certain xenobiotics (Hanninen et al., 1987). Xenobiotic biotransformation enzymes (XMEs) are located primarily in the smooth endoplasmic reticulum (microsomes) or soluble fraction of the cytoplasm (cytosol), and in lesser amounts in the mitochondria, nuclei, and lysosomes. XMEs are also often involved in the metabolism of endogenous substances. The biotransformation enzymes play the key role of converting hydrophobic xenobiotics into hydrophilic xenobiotics (Lang and Pelkonen, 1999). Biotransformation occurs in phases I and II (see Table 2) (Hodgson, 2004). Some substances undergo both phases, and some enter directly the second phase, while others might be excreted without undergoing phase II.

In phase I of biotransformation, (i) a polar moiety is introduced into the structure of a xenobiotic molecule, and/or (ii) a less polar moiety is converted into a more polar moiety, and/or (iii) a polar moiety is released after hydrolytic reactions. Lipophilic xenobiotics are often first metabolized by phase I enzymes, to make xenobiotics more polar and provide sites for conjugation reactions. Modifications occur mainly on the smooth endoplasmic reticulum by oxidation (aromatic and aliphatic hydroxylation, epoxidation, dealkylation, nitrogen oxidation, and oxidative deamination), reduction (reductive dehalogenation, nitroreduction, azoreduction), and hydrolytic reactions (Vrzal et al., 2004). Biotransformation phase I reactions are catalysed by a large number of enzymes (see Table 2). A prominent representative of phase I enzymes is the enzyme system of cytochrome P450 (CYP), which is responsible for the oxidation of approximately 75% of all drugs (Zuber et al., 2002). Other phase I enzymes include flavin monooxygenases, alcohol dehydrogenases, aldehyde dehydrogenases, carbonyl reductases, peroxidases, xanthinoxidases, carboxylesterases, etc. (Hodgson, 2004).

The phase II of biotransformation is represented by conjugation reactions; the product of phase I of biotransformation reacts with a small hydrophilic endogenous molecule (glucuronic acid, glutathione, sulphate, cysteine or acetate) to form a more polar metabolite (conjugate), which allows renal or biliary excretion (Klaassen and Lu, 2008). Enzymes from the class of transferases, including glutathione transferases, UDP-glucuronosyl-transferases, sulfotransferases, methyltransferases and N-acetyltransferases, etc. (see Table 2) catalyse most conjugation reactions. The conjugation reactions usually occur in the cytosol. For a majority of xenobiotics

undergoing biotransformation, the conjugates are pharmacologically inactive. However, conjugation reactions might generate metabolites with properties that are the same as or are similar to the parental substances; in some cases, biotransformation might result in the formation of a toxic side product (ROS) and biologically active metabolites, which could readily and irreversibly interact with biomacromolecules (e.g. DNA, RNA and proteins). Some of these metabolically derived reactive metabolites are epoxides, radicals, carbonium ions, and nitrenium ions (Ioannides, 2002). In addition, some drugs are biologically ineffective precursors, and their activation occurs through biotransformation in the body (Lang and Pelkonen, 1999).

Transporter proteins, acting as pumps, are also involved in the metabolism of xenobiotics; therefore, the transport of xenobiotics in/out of the cells is sometimes referred to as phase III. The transporters are expressed in many tissues such as the liver, intestine, kidney, and brain. Xenobiotic transporters carry molecules in and/or out of the cell, thereby influencing the intracellular concentration of the drug. The most important transporter that pumps xenobiotics out of cells is P-glycoprotein (P-gp), the first known ATP-binding cassette transporter. The P-gp mediates the trans-epithelial efflux of hydrophobic, neutral, or positively charged molecules (Lin and Yamazaki, 2003). The inhibition and induction of P-gp causes DDIs to occur (Lin, 2003). The most important membrane transporters that carry xenobiotics from the extracellular space into the cells, include organic anion transporting polypeptides (OATPs) and organic cation transporters (OCTs). A gene superfamily of OATPs mediates the transport of amphipathic-organic solutes such as conjugates with glucuronic acid, sulphate, or glutathione. Most OATPs are expressed in multiple organs, including the blood–brain barrier, choroid plexus, lung, heart, intestine, kidneys, placenta, and testis. Some OATPs are selectively expressed in the liver (Hagenbuch and Meier, 2003). OCTs, which are mainly expressed in the liver (Faber et al., 2003), transport small organic cations, such as tetramethylammonium, tetraethylammonium, thiamine, choline, dopamine, serotonin, histamine, adrenalin, and noradrenalin.

***Table 2**:*

Classification and subcellular localization of XMEs (Hodgson, 2004).

REACTION	ENZYME	LOCALIZATION
	Phase I	
Oxidation	Cytochrome P450	Microsomes
	Flavin-monooxygenases	Microsomes
	Monoamine oxidase	Mitochondria
	Prostaglandin H synthase	Microsomes
	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
Reduction	NADPH:P450 oxidoreductase	Microsomes
	Aldehyde reductase	Cytosol
	Nitro reductase	Cytosol, microsomes, intestinal bacteria
	Azo reductase	Microsomes, intestinal bacteria
	Epoxide hydrolase	Microsomes, cytosol
Hydrolysis	Carbonylesterase/Amidase	Microsomes, mitochondria, cytosol
	Epoxide hydrolase	Microsomes, cytosol
	Phase II	
Glucuronide Conjugation	UDP-glucuronosyltrasferase	Microsomes
Sulfate Conjugation	Sulfotransferase	Cytosol
Glutathione Conjugation	Glutathion-S-transferase	Cytosol, microsomes
Acetylation	N-acetyltransferase	Cytosol
Methylation	S-methyltransferase	Cytosol, microsomes, blood
Amino acid Conjugation	Acyltransferase	Mitochondria, microsomes

3.3 CYTOCHROME P450

The evolutionary development of cytochromes P450 (henceforth CYPs) dates back to 1.5 billion years. Their presence was first reported in 1962 (Omura and Sato, 1964). CYPs are biotransformation enzymes responsible for the metabolism of xenobiotics. The gene superfamily of CYPs contains the haeme structure as a cofactor in its molecules. At present, more than 2,000 cytochromes are known, out of which 59 genes occur in humans. These are divided into 18 families and 43 subfamilies of CYP genes that differ in structure, catalytic activity and substrate specificity (Pavek and Dvorak, 2008). CYPs are constitutively expressed and their expression could be induced by xenobiotics.

3.3.1 Nomenclature

The term “cytochrome” has a historical significance due to the use of the enzymes involved in electron transport (e.g. the respiratory chain). The second part of the name, i.e., "P450", was derived from the discovery of a rat liver pigment that absorbed light at a wavelength of 450 nm after haem iron was reduced by carbon monoxide (Klingenberg, 1958). A systematic nomenclature was established for the large number of enzymes in the “CYP superfamily“ in 1996, to ensure that there is clarity in the terminology employed. The nomenclature for this superfamily is based on the similarity of its members to the amino acid apoprotein (Nelson et al., 1996). The enzyme superfamily is referred to by the abbreviation CYP (cytochrome P450). The abbreviation CYP is followed by the Arabic numerals 1-118 that indicate the gene family (e.g. CYP1A2) that the enzyme is derived from; thus, the similarity in the protein sequence of two cytochrome P450 molecules is higher than 40%. The numeral is followed by a capital letter from A-Q that specifies the subfamily (CYP1A2); the similarity in the protein sequence of two cytochrome P450 molecules is higher than 55%. Finally, an Arabic numeral indicates a specific CYP isoform (CYP1A2). The name of the gene (*CYP1A2*) that the enzyme is derived from needs to be italicised.

3.3.2 Localization and structure

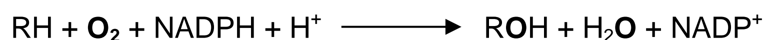
The CYP enzymes are firmly bound to the membranes of the endoplasmic reticulum and mitochondria in eukaryotes, while they are dissolved in the cytosol of bacteria. In the human body, CYPs are located primarily in the liver, and also located in the nasal mucosa, lungs, gastrointestinal tract, and kidneys. The CYP levels in

individual human tissues cannot be considered absolute because they depend on many factors such as nutrition, age, sex, smoking, alcohol consumption, external environment, drugs, etc. (Pavek and Dvorak, 2008).

CYP is a type b haemoprotein that consist of a polypeptide chain and porphyrin ring (i.e., protoporphyrin IX), as well as substituents attached to the protoporphyrin core. The iron(III) ligand is bound to the porphyrin ring by four ligands. The cysteine-derived thiolate anion is the fifth ligand and is bound to its own enzyme. The sixth ligand is an oxygen group belonging to the haeme molecule, and is added into the enzyme structure during the reaction.

3.3.3 The mechanism of activation of the oxygen atom

CYPs, also called hepatic microsomal monooxygenases, are enzymes that bind molecular oxygen; however, there is a distinct mechanism by which each oxygen atom becomes bound. One oxygen atom is incorporated into a substrate molecule (RH), while the other is reduced to water with reducing equivalents derived from nicotinamide adenine dinucleotide phosphate (NADPH) (Anzenbacher and Anzenbacherova, 2001). The CYP-catalysed monooxygenase reaction is summarized by the following equation:



$$RH + \mathbf{O_2} + NADPH + H^+ \longrightarrow R\mathbf{O}H + H_2\mathbf{O} + NADP^+$$

The catalytic cycle of CYP is quite complex (Figure 7). The xenobiotic substrate (RH) is bound to an oxidized form of CYP (complex $RH\text{-}Fe^{3+}$), leading to a change in the conformation of CYP. (These changes facilitate the single-electron transport from NADPH and the reduction of Fe^{3+} to Fe^{2+}). The $RH\text{-}Fe^{3+}$ complex is converted to $RH\text{-}Fe^{2+}$ by NADPH, through a single-electron reduction reaction, which is brought about by cytochrome P450 oxidoreductase (CYPOR). The oxygen atom is bound to the complex and a ternary complex ($RH\text{-}Fe^{2+}\text{-}O_2$) is formed. In this step, the second reduction reaction occurs. The complex receives a second electron from CYP or cytochrome b5 to form a peroxide complex. The complex reacts with two protons to form a water molecule and a $RH\text{-}FeO^{3+}$ complex. There is a breakdown of the O-O bond. The iron bound oxygen atom is attached to the RH molecule. The oxidized substrate (product) is released, after which the enzyme could return to its initial state (Ortiz de Montellano, 1995). If the catalytic cycle is interrupted after the introduction of the first electron, oxygen is released in the form of a superoxide anion. If the cycle is interrupted after the introduction of the second electron, oxygen is released in the form

of hydrogen peroxide. For this reason, certain CYP reactions could be supported by hydroperoxides in the absence of CYPOR and NADPH (Hollenberg et al., 2008).

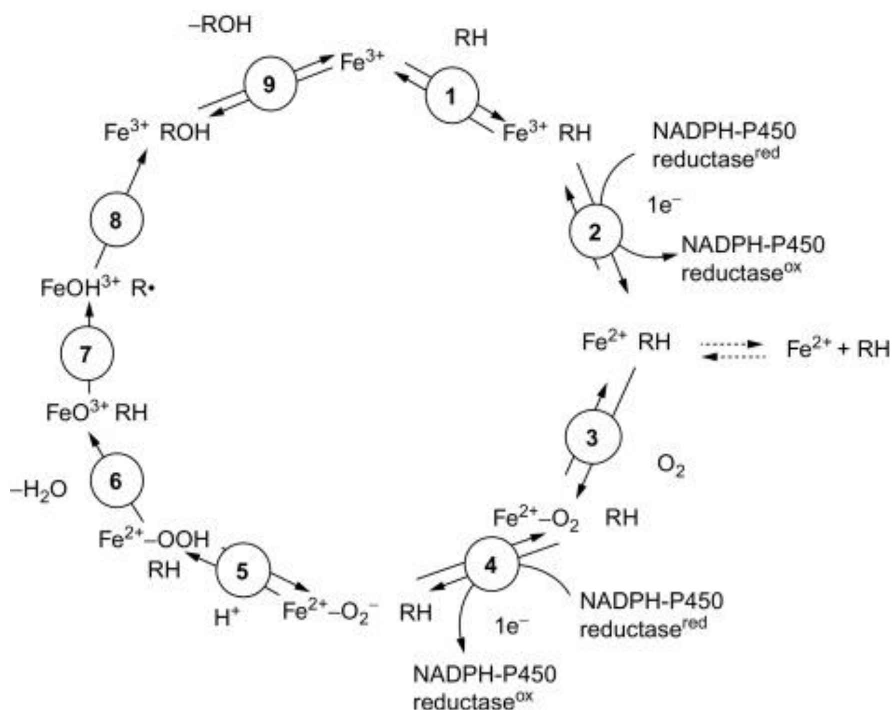


Figure 7:
Catalytic cycle of cytochrome P450 (Cederbaum, 2015).

3.3.4 Drug-metabolizing CYP isoenzymes

The sequencing of the human genome revealed the presence of 107 human CYP genes, comprising 59 active and 48 pseudogenes (Nelson et al., 1996). The function of the enzymes corresponding to these genes is known only to some extent. The main CYP forms involved in drug metabolism are CYP2C9, CYP2C19, CYP2D6, and CYP3A4, whereas the CYP1A1, CYP1A2, CYP1B1, CYP2E1, and CYP3A4 isoforms are responsible for the metabolic activation of pre-carcinogens (Ingelman-Sundberg, 2004). Broad and overlapping substrate specificity is a typical feature of these CYPs. The most abundant isoform is CYP3A4, which metabolizes a majority of drugs. In contrast, CYP2D6 accounts for less than 5% of the total CYPs, but metabolizes about a quarter of all known drugs (Figure 8). CYPs are also involved in the metabolism of endogenous substrates (Ortiz de Montellano, 1995). In addition, CYP isoenzymes are strongly inducible, and their levels might increase dramatically under the influence of xenobiotics. The high inter-individual variability in the content of

CYP isoforms is attributable to environmental factors (Honkakoski and Negishi, 2000), age (Sotaniemi et al., 1997) and pathological conditions (Villeneuve and Pichette, 2004).

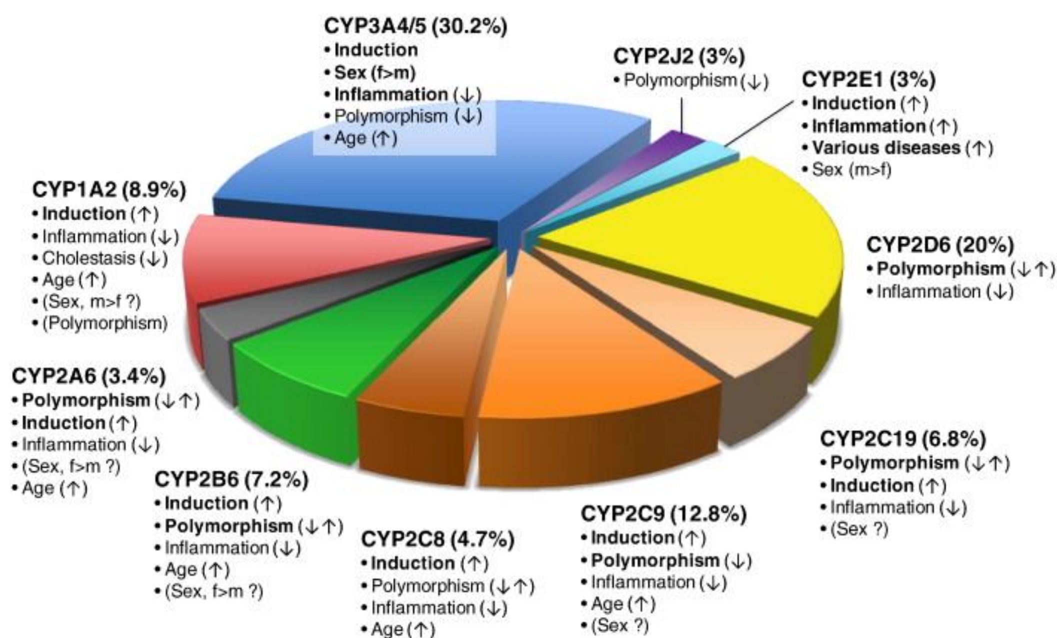


Figure 8:

Factors influencing the expression of CYP isoforms (Zanger and Schwab, 2013).

Genetic polymorphisms significantly influence the physiological activity of CYP enzymes. They are inherited DNA changes that lead to inadequate production, insufficient inducibility, or protein synthesis of CYPs with altered catalytic activity (Martignoni et al., 2006). Consequently, individuals metabolize the same xenobiotic at different rates, based on which patients could be grouped as poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultra-rapid metabolizers (UMs). The PM phenotype is characterized by total or partial metabolic disability, and affects the metabolism of up to 20% of all clinically used drugs. IMs carry one or more defective alleles in their genotype and are characterized by a lowered metabolic activity. In EMs, both alleles of the gene are functional (e.g. they encode a fully active protein or a protein with a relatively low activity); therefore, drugs are metabolized normally. The EMs form a majority of the population. UMs have multiple genetic copies of the enzymes, because of which these enzymes are highly expressed. The different, capabilities of patients to metabolize certain drugs necessitates the proper adjustment of the drug dose (Zanger et al., 2004).

The factor that is particularly responsible for the occurrence of DDIs is the interaction between a xenobiotic and an enzyme, which results in the inhibition of catalytic activity via several mechanisms. In addition, the expression of many XMEs is induced by xenobiotics, i.e. their amount in a cell increases upon the incubation with the drug (see Table 3). The induction is mediated through nuclear receptors, steroid receptors, and xenoreceptors. The inhibition and induction of CYP could lead to an alteration in the pharmacokinetics of the drug, thereby influencing the toxicity, efficacy, and adverse effects of the drug.

3.3.4.1 The CYP1 family

The CYP1 family comprises three functional genes, i.e., CYP1A1, CYP1A2, and CYP1B1. The enzymes of the CYP1 family are found in different tissues, and their expression is mainly regulated by the aryl-hydrocarbon receptor (AhR) (Nguyen and Bradfield, 2008).

3.3.4.1.1 CYP1A1

CYP1A1 is an extrahepatic enzyme, and is detectable only after its induction (Anzenbacher and Anzenbacherova, 2001). Typical inducers of CYP1A1 include polyaromatic hydrocarbons (PAHs) and halogenated hydrocarbons (HAHs). CYP1A1 is an important detoxifying enzyme with a wide substrate specificity, and it is distributed throughout the body (Pavek and Dvorak, 2008). However, it can generate highly carcinogenic metabolites; for example, CYP1A1 catalyses the hydroxylation and epoxidation of benzo[a]pyrene, which can lead to the formation of ROS.

3.3.4.1.2 CYP1A2

CYP1A2 is expressed mainly in the liver, but it also occurs in the lungs (Wei et al., 2002). CYP1A2 levels could be increased by AHR activators, such as PAHs, aromatic amines, indoles (e.g. indole-3-carbinol present in cruciferous vegetables), flavonoids, or drugs (e.g. rifampicin, barbiturates and omeprazole) (Lewis, 2004). This enzyme metabolizes caffeine, theophylline, tizanidine, tacrine, zolmitriptan, R-warfarin, aromatic amines, aflatoxin B1 etc. (Pavek and Dvorak, 2008; Pelkonen et al., 2008). The typical inhibitors of CYP1A2 include disulfiram, furafylline, ciprofloxacin, estradiolvalerate, and levonorgestrel (Laine et al., 1999).

Table 3:

General overview of human CYPs (CYP1-CYP3) (Ingelman-Sundberg, 2004).

CYP	Main tissue localization	Substrate	Inductor
1A1	lungs, liver, brain, GIT, lymphocytes, heart, skin	polycyclic aromatic hydrocarbon (PAH)	PAH, dioxins, omeprazole
1A2	liver, lungs	aromatic amines, PAH, caffeine, theophylline	PAH, β-naphthoflavone, phenobarbital
1B1	steroid tissues, skin, brain, heart, lungs, spleen, liver, kidneys, GIT	PAH	dioxin
2A6	liver, nasal mucosa, lungs	coumarin, nicotine	barbiturates, dexamethasone
2B6	liver, heart, brain, kidneys, placenta, lungs, nasal mucosa	cyclophosphamide, tramadol	barbiturates
2C8	liver, kidneys	arachidonic acid, retinoic acid	barbiturates, imatinib
2C9/10	liver	warfarin, ibuprofen, rosuvastatin	rifampicin, barbiturates, prednisone
2C18	liver, skin	warfarin	rifampicin
2C19	liver, heart	diazepam	barbiturates, rifampicin
2D6	liver, brain, heart, duodenum	codeine, tamoxifen, tramadol	----
2E1	liver, lungs, brain, heart, bone marrow, nasal mucosa, kidneys, testicles, GIT, ovaries	ethanol, paracetamol, salicylic acid	acetone, ethanol, starvation
2F1	lungs, nasal mucosa, liver	coumarins	---
3A4/5	liver, GIT, kidneys, lungs, brain, endothelium, placenta, lymphocytes	cyclosporine A, diazepam, paracetamol, atorvastatin	steroids, imatinib, gingko biloba
3A7	foetal liver, uterine endometrium	like 3A4	steroids, barbiturates
3A43	liver, prostate	probably similar to 3A4	probably similar to 3A4

3.3.4.1.3 CYP1B1

CYP1B1 is expressed in the uterus, breast, ovary, testis, prostate, adrenal gland, kidney, thymus, spleen, brain, heart, lung, colon and intestine (Shimada et al., 1996). It is an important enzyme because it metabolizes oestradiol (Hayes et al., 1996) and diverse pro-carcinogens, such as benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (Crespi et al., 1997). CYP1B1 is over-expressed in human cancer tissues, such as cancer tissues of the skin, brain, testis (Murray et al., 2001), and breast (McKay et al., 1995). Therefore, the induction of CYP1B1 is a significant factor for determining the risk of developing tumours, especially in the tissues that are sensitive to hormonal changes.

3.3.4.2 The CYP2 Family

The drug-metabolizing mammalian CYP2 family involves the subfamilies 2A, 2B, 2C, 2D, and 2E. The enzymes belonging to the CYP2 family are abundantly expressed in the endothelium, myocardium, and kidney in humans (Chen and Wang, 2015).

3.3.4.2.1 CYP2A6

CYP2A6 is expressed mainly in the liver, where its levels represent approximately 4% of the hepatic CYP pool (Shimada et al., 1994). The transcriptional regulation of human CYP2A6 involves the signalling pathways of the pregnane X receptor (PXR) (Itoh et al., 2006), constitutive androstane receptor (CAR), glucocorticoid receptor (GR) (Onica et al., 2008), and oestrogen receptor (ER) (Higashi et al., 2007). It is a polymorphic enzyme that metabolizes low-molecular-weight substances containing a keto- or nitro group (e.g. nicotine, cotinine, and coumarin) (Raunio and Rahnasto-Rilla, 2012). It is the only drug-metabolizing enzyme that catalyses coumarin 7-hydroxylation (Pelkonen et al., 2000). Furthermore, CYP2A6 metabolises approximately 3% of all the therapeutic drugs (disulfiram, pilocarpine, tamoxifen, ifosfamide, and valproic acid) and procarcinogens (nitrosamines and aflatoxin B1) (Di et al., 2009). It also participates in the biotransformation of several endogenous compounds (steroids, retinoic acids) (Abu-Bakar et al., 2012).

3.3.4.2.2 CYP2B6

Human CYP2B6 is mainly expressed in the brain, and its levels have been reported to be higher in smokers and alcoholics (Miksys et al., 2003). Low levels of CYP2B6 were found in the kidneys (Aleksa et al., 2005), heart (Michaud et al., 2010), placenta (Wang et al., 2010), lungs and nasal mucosa (Ding and Kaminsky, 2003). In contrast, no expression of CYP2B6 was observed in the skin, keratinocytes (Du et al., 2006), and intestine (Paine et al., 2006). The induction of CYP2B6 is controlled mainly by CAR (Sueyoshi et al., 1999) and PXR (Wang et al., 2003). CYP2B6 inducers are typically PXR/CAR ligands, such as rifampicin and barbiturates (Faucette et al., 2004). CYP2B6 is involved in the metabolism of cyclophosphamide, efavirenz, bupropion, artemisinin, ketamine, and methadone (Turpeinen and Zanger, 2012; Zanger et al., 2007).

3.3.4.2.3 CYP2C9

CYP2C9 is expressed mainly in the liver and is involved in the metabolism of warfarin, phenytoin, valproic acid, candesartan, losartan, tolbutamide, diclofenac, and ibuprofen (Lee et al., 2002). The CYP2C9 enzyme also metabolizes endogenous substances, particularly arachidonic acid and some steroids. The transcriptional regulation of CYP2C9 involves PXR, CAR, GR, vitamin D receptor (VDR) and oestrogen receptor alpha (ERα) (Chen and Goldstein, 2009). CYP2C9 expression is induced by genetic polymorphisms in CYP2C9, together with the induction or inhibition of CYP2C9 by xenobiotics; these are also pivotal determinants of warfarin metabolism, and of other CYP2C9-metabolized drugs (Lu et al., 2008; Niemi et al., 2003).

3.3.4.2.4 CYP2C19

CYP2C19 is expressed mainly in the liver, and also in the duodenum. The transcriptional regulation of CYP2C19 requires factors that are similar to those required for CYP2C9; however, it is not a typical inducible enzyme. Genetic polymorphism of CYP2C19 results in the occurrence of inter-individual differences in the metabolism of drugs such as S-mephenytoin (Kupfer and Preisig, 1984). The CYP2C19 enzyme is involved in the metabolism of antidepressants (Brosen, 2004), antiepileptics, proton pump inhibitors (omeprazole, lansoprazole) and anticoagulants (clopidogrel) (Hulot et al., 2006; Kazui et al., 2010). Endogenous substrates of CYP2C19 include progesterone and melatonin.

3.3.4.2.5 CYP2D6

CYP2D6 is expressed in the liver, enterocytes, various regions of the human brain, right ventricle, duodenum, and jejunum, but is not expressed in the ileum and large intestine. It is a constitutively expressed enzyme and is regulated via the hepatocyte nuclear factor 4 alpha (HNF4α) signalling pathway (Hara and Adachi, 2002). A large number of therapeutically used drugs are metabolized by CYP2D6, though relatively low amounts of it are expresses in the liver. CYP2D6 metabolizes approximately 25% of all clinically used drugs, including the anti-arrhythmic (mexiletine), anti-depressant (amitriptyline), anti-psychotic (risperidone), β-blocker (bufuralol), anti-cancer and opioid analgesic (tramadol, codeine) drugs (Zanger and Hofmann, 2008). Genetic polymorphisms of CYP2D6 were first demonstrated in population and family pharmacokinetic studies conducted in the 1970s, which showed that patients treated with spartein (Mahgoub et al., 1977) and debrisoquinone (Eichelbaum et al., 1979; Ingelman-Sundberg, 2005) exhibited different therapeutic responses. Therapeutic differences have also been observed in different sexes, suggesting that the activity of this enzyme was lower in females (Franconi et al., 2007).

3.3.4.3 The CYP3 family: CYP3A4

Only one subfamily of the CYP3 family (CYP3A) occurs in humans, and it is comprised of four enzymes: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 are the most abundant CYPs in the human liver and GIT. They are involved in the metabolism of steroids and approximately 52% of clinically used drugs (Guengerich, 2003; Nebert and Russell, 2002). CYP3A4 is the most important enzyme of the CYP3A subfamily (Kolars et al., 1992). The CYP3A4 protein has a relatively bulky and flexible active site, which allows it to be able to bind to variable substrates (Anzenbacher et al., 2008; Ekroos and Sjogren, 2006). CYP3A4 exhibits an inter-individual variability that might vary up to 10-fold in individuals (Floyd et al., 2003). This variability is attributable to genetic polymorphisms, permanent induction or inhibition by endogenous compounds, and food-borne xenobiotics. Some examples of food-born inhibitors and inducers of CYP3A4 are furanocoumarin (grapefruit juice) and hyperforin (St. John Wort), respectively. The transcriptional regulation of CYP3A4 involves multiple factors and pathways, including HNF1α, HNF4α, PXR, CAR, GR, VDR or peroxisome proliferator-activated receptor alpha - PPARα.

3.4 NUCLEAR RECEPTORS

Nuclear receptors (NRs) are members of a large superfamily of evolutionarily related DNA-binding transcription factors (Germain et al., 2006). These ligand-activated transcription factors regulate the expression of CYPs and other genes that transform extracellular/intracellular signals into cellular responses by activating ligands (e.g. xenobiotics, eubiotics, or oxidation products). NRs play crucial and non-redundant roles, notably in the regulation of many biological processes such as cell growth and differentiation, development, homeostasis, or metabolism (Gronemeyer et al., 2004). The NR superfamily includes receptors for steroid ligands (nuclear hormone receptors) and non-steroid ligands (e.g. retinoid acid, prostaglandins, fatty acid, etc.). The orphan receptors are those for which no physiologically relevant activators or ligands are known (Enmark and Gustafsson, 1996).

A typical NR consists of six domains (designated A–F), ranging from the N-terminus to C-terminus of the protein (Figure 9A). The N-terminal Activation Function domain (AF-1) is highly variable, depending on the receptor (A/B region). The central DNA binding domain (DBD) is the most conserved and is responsible for direct interactions with DNA and the specifity of the binding of DNA with the P-box (C region). Additional sequences in the DBD are involved in the binding of sequence-specific hormone response elements (HREs) on target genes, either as homodimers or heterodimers. The C-terminal ligand-binding domain (LBD) carries Activation Function domain (AF-2), and usually interact with small hydrophobic molecules (E region). The DBD and LBD are linked by a flexible hinge (D region), which contains the nuclear localization signals (NLSs). Some receptors might also contain a COOH-terminal domain (F region) with unknown functions (Berg, 1989). Regions C and E are evolutionarily conserved, unlike the A/B, D, and F regions, which are divergent (Giguere et al., 1986).

Over 48 distinct NRs have been identified till date (Germain et al., 2006). A nomenclature system has been proposed, based on the sequence alignment of the two well-conserved domains (DBD and LBD) and phylogenetic tree construction, resulting in six evolutionary subfamilies of unequal sizes (Auwerx et al., 1999). There is a correlation between a DNA-binding and dimerization abilities of each NR and its phylogenetic position. Subfamily 1 is comprised of NRs that form heterodimers with retinoid X receptors (RXRs) such as TR, VDR, PPAR, CAR, and PXR receptors, as well as the liver X receptor (LXR) and retinoic acid receptor (RAR). Subfamily 2 includes RXRs, COUP-TF and HNF-4. Subfamily 3 includes steroid hormone receptors

such as OeR, GR, AR, progesterone receptor (PR), and mineralocorticoid receptor (MR). Subfamilies 4, 5, and 6 are small groups that contain orphan receptors such as NGFI-B, FTZ-1/SF-1, and GCNF.

Despite a highly conserved evolutionary structural organization, the function and the mode of action of NRs are very diverse. Some NRs are localized in the cell nucleus regardless of the presence of a ligand, and bind to the DNA response elements (REs) of their target genes along with corepressors (type II NR; RXR heterodimers), while others are predominantly bound to chaperone proteins (HSP; heat-shock protein) in the cytoplasm in the absence of a ligand (type I NR), and are translocated to the nucleus only after ligand binding (Figure 9B) (Guiochon-Mantel et al., 1996). Cell signalling in NRs is initiated by the binding of a small lipophilic molecule (ligand) to a receptor, which is followed by a conformational change in the receptor. Ligand-binding results in the dissociation of chaperone proteins, nuclear translocation of the receptor, specific binding of DNA to REs, recruitment of coactivators, and activation of target genes for type I NRs. Ligand-binding results in the dissociation of corepressors, recruitment of coactivators, and activation of target genes for type I NRs (Grimaldi et al., 2015).

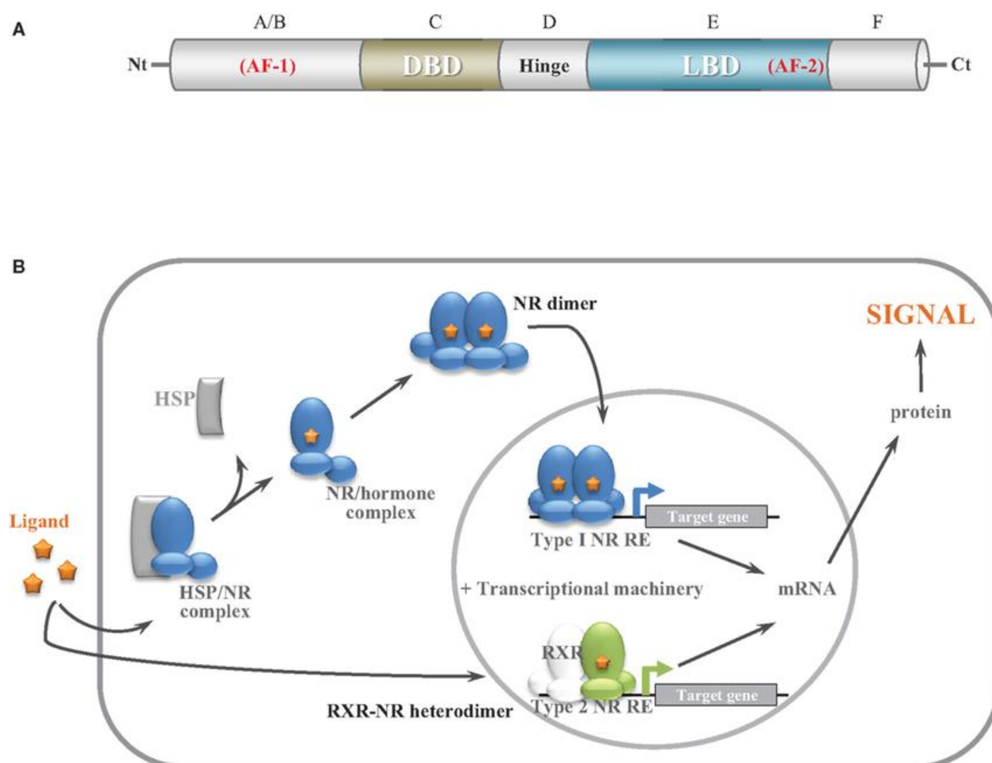


Figure 9.

General NR structure and function (Grimaldi et al., 2015). *Simplified scheme of the NR basic domain (A). Schematic model of the NR function (B).*

Drug-metabolizing enzymes are transcriptionally regulated by several NRs such as xenoreceptors (AhR, PXR, CAR) (Harper et al., 2006; Honkakoski et al., 1998; Pascussi et al., 2008), and also by steroid receptors (ER, GR) (Dvorak and Pavek, 2010; Safe and Wormke, 2003) and nuclear receptors (TR, RAR, PPAR, VDR) (Drocourt et al., 2002; Fallone et al., 2005; Monostory et al., 2009; Murphy et al., 2007), which are a platform for cross-talk between xenoreceptors and signalling pathways of intermediary metabolism to occur. Hence, the possible modulation of transcription factors, such as AhR, CAR, and PXR, which are involved in regulation of the expression of CYP isoforms has been focused upon, in order to understand the molecular mechanism of action of synthetic drugs (Waxman, 1999).

3.4.1 Aryl hydrocarbon receptor (AhR)

AHR is a ligand-activated transcriptional factor, also known as dioxin receptor, which mediates the toxic, carcinogenic and teratogenic effects of environmental pollutants such as persistent planar hydrophobic halogenated aromatic hydrocarbons (HAHs) (dioxins, dibenzofurans, biphenyls) and polycyclic aromatic hydrocarbons (PAHs) (benzo[*a*]pyrene, benzanthracene, 3-methylcholantrene) (Denison et al., 2002). It is well-known that the human population is exposed to AhR ligands via cigarette smoke, combustible products, and contaminated food (dioxins, PAH) daily, and that the toxic responses of these environmental pollutants are a direct consequence of AhR activation. TCDD is an especially widespread environmental contaminant that causes a wide range of alterations in the immune system, hormonal and endocrine imbalances, and changes in cell proliferation and differentiation (Pohjanvirta and Tuomisto, 1994). Tryptophan derivatives, arachidonic acid metabolites, equilenin, haeme metabolites, and indigoids have been characterized as endogenous AhR ligands (Nguyen and Bradfield, 2008). It is has been also reported that AhR plays a role in tumour-progression (Moennikes et al., 2004). Beside its function in tumour-initiation and tumour-promotion, reports have suggested that AhR also plays a role in tumour-progression (Chang et al., 2007; Villano et al., 2006). Interestingly, naturally occurring compounds such as indoles, flavonoids, and drugs (omeprazole, lansoprazole, TSU-16) might also act as AhR activators (Curi-Pedrosa et al., 1994; Matsuoka-Kawano et al., 2010).

AhR belongs to the basic helix-loop-helix/Per/ARNT/Sim (bHLH/PAS) superfamily (Hankinson, 1995) that is responsible for dimerization of proteins. Cytosolic AhR forms a complex with two molecules of Hsp90 (heat-shock protein 90 kDa) when it is in the

resting state, including at least one immunophilin homologous protein XAP2 (hepatitis B virus X-associated protein 2) and the co-chaperone Hsp90-interacting protein p23 (Figure 10) (Carver and Bradfield, 1997). These proteins are involved in the process of folding and stabilization of AhR (Gu et al., 2000). After joining the ligand (dioxin or PAH), a conformational change occurs, the receptor is released from the complex of chaperone proteins and is translocated to the nucleus, in which it forms a heterodimer with ARNT (AhR nuclear translocator) (Pollenz et al., 1994). The AhR/ARNT heterodimer interacts with dioxin-responsive element (DRE; 5′-GCGTG-3′ DNA sequence) and leads to the transcription of CYP genes, including CYP1A1, CYP1A2, CYP1B1, UGTs (uridin-5´-diphosphate-glucuronosyltransferases; UGTA1), GSTs (glutathione-S-transferase; GSTA1), NADPH reductase, etc. (Figure 10) (Nebert et al., 2004).

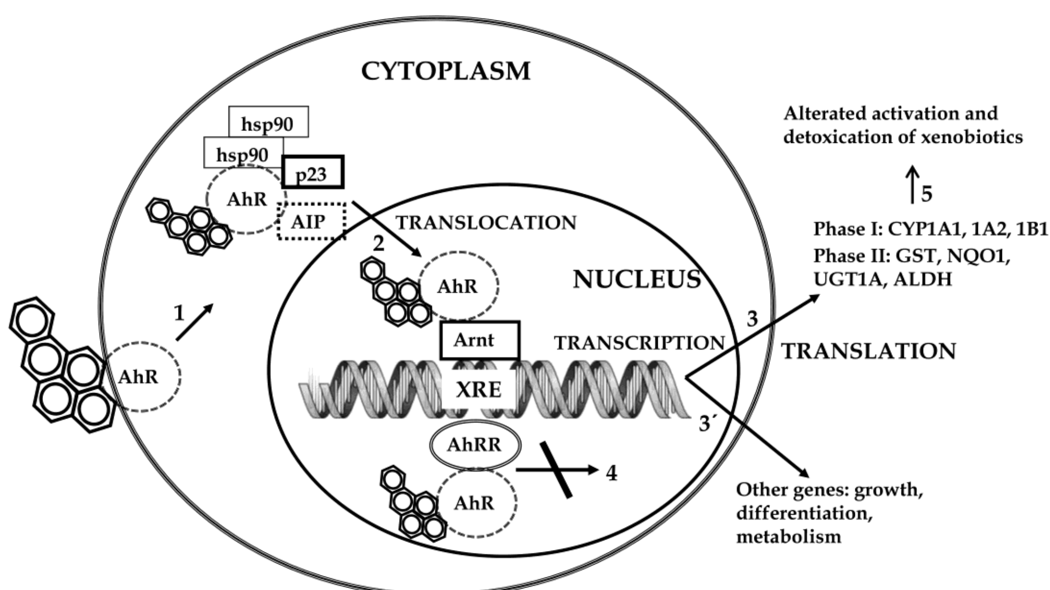


Figure 10.
Schematic representation of the AhR signalling pathway (Pocar et al., 2005).

The transcriptional activity of AhR could be modulated by different co-regulatory proteins. Co-activators such as the p160 proteins (NcoA1/SRC-1, NcoA2/TIF2/GRIP1 and NcoA3/pCIP) (Beischlag et al., 2002), CREB binding protein/p300 (CBP/p300) (Kobayashi et al., 1997), receptor-interacting protein 140 (RIP140) (Kumar et al., 1999), oestrogen receptor associated protein 140 (ERAP140) (Nguyen et al., 1999), and nuclear receptor coactivator 4 (NcoA4) (Kollara and Brown, 2006) have been shown to enhance the transcriptional activity of AhR. On the other hand, co-repressors such as SMRT (silencing mediator of retinoid and thyroid receptors) inhibit AhR activity.

Furthermore, the activity of the AHR-ARNT complex is also attenuated by a second mechanism, i.e. the upregulation of a transcriptional repressor known as the aryl hydrocarbon receptor repressor (AHRR). The AHRR is another bHLH/PAS protein that exhibits a high sequence similarity with the AHR. The AHRR represses AHR transcriptional activity by binding ARNT to DREs (Mimura et al., 1999). This attenuation of AHR activity by means of a negative feedback loop and receptor degradation might serve to protect the organism from the consequences of transcriptional hyperstimulation by potent agonists, and to provide precise temporal control of this pathway.

Protein kinase C and tyrosine kinase are also involved in modulation of transcriptional activity. The binding of DNA to the AHR/ARNT heterodimer requires the phosphorylation of both these proteins (Perdew and Hollenback, 1990). The phosphorylation of AhR tyrosine 9 does not appear to be essential for ARNT dimerization, but is a critical residue required for the binding of DNA to the full-length protein (Berghard et al., 1993). However, the phosphorylation of serine/threonine residues belonging to either the AHR or ARNT regulates events that occur following DNA binding and transcriptional activation (Li and Dougherty, 1997).

The AHR-dependent pathway appears to be well-conserved across species and occurs widely expressed not only in the liver, but also in several extrahepatic tissues and cell types, such as the skin, spleen and lymphoid tissues, lung, ovary, placenta, thymus, kidney, small intestine, heart, and pancreas (Dolwick et al., 1993; Harper et al., 2006; Mason and Okey, 1982).

3.4.2 Pregnane X receptor (PXR)

The pregnane X receptor (PXR) (NR1I2) belongs to the NR superfamily of ligand-regulated transcriptional factors that protect the body from toxic chemicals (Kliewer et al., 1998). The human PXR (hPXR) ortholog was subsequently reported as the steroid and xenobiotic receptor (SXR) and pregnane activated receptor (PAR) (Bertilsson et al., 1998; Blumberg et al., 1998). PXR is highly expressed in the liver and intestine, where detoxification reaction s occur (Lehmann et al., 1998), but low levels of expression have also been found in the peripheral blood monocytes, blood-brain barrier, uterus, ovary, placenta, breast, osteoclasts, heart, adrenal glands, bone marrow, and specific brain regions of various species (Lamba et al., 2005; Miki et al., 2005). The hPXR responds to structurally distinct ligands that induce the expression of the CYP3A genes and other genes (e.g. CYP2B6, CYP2C8, CYP2C9, and CYP2C19)

involved in the detoxification and elimination of these potentially harmful chemicals (Drocourt et al., 2001; Jones et al., 2000; Tolson and Wang, 2010). These compounds include phenobarbital, rifampicin, dexamethasone, nifedipine, taxol, and hyperforin, the active agent of the herbal remedy named St. John's wort (Lehmann et al., 1998). PXR is also activated by many clinical drugs and endogenous ligands, including pregnanes, bile acids, hormones, and dietary vitamins. PXR also controls the expression of phase II (UDP-glucuronosyltransferases, glutathione-S-transferases, and sulfotransferases) and phase III (multidrug resistance 1 and multidrug resistance protein 2) detoxifying enzymes (Orans et al., 2005; Xu et al., 2005). The biological and physiological implications of PXR activation are broad, ranging from drug metabolism and drug-drug interactions (Fuhr, 2000) to the homeostasis of numerous eubiotics such as glucose (Kodama et al., 2007), lipids (Zhou et al., 2006), steroids (Zhai et al., 2007; Zhang et al., 2010), bile acids (Xie et al., 2001), bilirubin (Staudinger et al., 2001), retinoic acid (Wang et al., 2008), and bone minerals (Pascussi et al., 2005).

In the resting state, the cytosolic PXR forms a complex with the co-chaperones cytoplasmic CAR retention protein (CCRP) and Hsp90. After binding to a ligand, the PXR dissociates from co-regulators, and the activated receptor translocates from the cytoplasm to the nucleus (Squires et al., 2004). PXR then binds to a variety of DNA response elements (direct repeats DR-3, DR-4, and DR-5, and everted repeats ER-6 and ER-8) in the promoter regions of target genes as a heterodimer of the retinoid X receptor α (RXRα). PXR is also capable of recruiting of co-regulators of the p160 family such as steroid receptor coactivators 1 (SRC-1), TIF/GRIP (SRC-2), and peroxisome proliferator-activated receptor gamma coactivator 1a (PGC-1a) (Mangelsdorf and Evans, 1995). It activates the transcription of the target genes (Waxman, 1999).

Moreover, PXR and RXRα are induced by GR, which inderictly participates in the regulation of CYP3A. The activation of GR by glucocorticoids (e.g. dexamethasone) leads to the induction of PXR/RXR and CAR, and GR is deactivated by the IL-6 inflammatory cytokines (Pascussi et al., 2000).

In addition, PXR regulates the genetic expression of the OATP2 and MRP2 transporters involved in bile acid homeostasis (Fischer et al., 1996).

3.4.3 Constitutive androstane receptor (CAR)

The nuclear orphan receptor CAR also belongs to the NR superfamily (NR1I2) of transcription factors that are also involved in the process of CYP transcriptional regulation. CAR is predominantly expressed in the liver and kidneys, though a low basal level of

expression has been detected in the brain, heart, and intestinal tissues (Timsit and Negishi, 2007). The CAR responds to signals and constitutes a structurally diverse panel of ligands, because different amino acid residues could interact with these ligands (Jyrkkarinne et al., 2005). The ligands modulate CAR activity, and thus mediate the induction of CYP2B, CYP3A, CYP2C, and phase II enzymes of drug metabolism (GSTs, SULTs and UGTs) (Sueyoshi et al., 1999). These deactivators and agonists include androstane steroidal compounds, retinoic acids, clotrimazole, chlorpromazine, and hydrocarbons such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) and 2,3,3',4',5',6-hexachlorobiphenyl, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3] thiazole-5-carbaldehyde-*O*-(3,4dichlorobenzyl)oxime (CITCO) (Maglich et al., 2003; Tzameli et al., 2000). However, only TCPOBOP and CITCO are shown to specifically bind to mouse CAR (mCAR) and human CAR (hCAR), respectively. CAR has been implicated in energy metabolism, including lipid metabolism and glucose homeostasis (Kodama et al., 2004; Miao et al., 2006; Ueda et al., 2002). In addition, CAR regulates the detoxification and excretion of toxic endogenous metabolites, such as bilirubin (Sugatani et al., 2002) and bile acids (Eloranta and Kullak-Ublick, 2005).

In the absence of ligands, CAR exists in the cytoplasm in complexes with its co-chaperone partner, i.e. cytoplasmic CAR retention protein (CCRP), the membrane associated subunit of protein phosphatase 1β (PPP1R16A) and Hsp90 chaperone proteins. Under the influence of ligand, CAR dissociates from the cytoplasmic complex and translocates into the nucleus, where it forms a heterodimeric complex with RXR. The CAR/RXR heterodimer binds to a conservative sequence called PBREM (phenobarbital-responsive enhancer module) in the 5' region of *CYP2B* and to the ER6 and DR3 regions of *CYP3A4*. Moreover, PXR could bind to the DR4 sequence in PBREM and regulate the expression of CYP2B (Honkakoski et al., 1998; Maglich et al., 2003; Sueyoshi et al., 1999). CAR does not immediately interact with the PBREM, and phosphorylation/ dephosphorylation processes play an important role. The interaction of the CAR/RXR heterodimer with PBREMs is promoted by coactivators such as the glucocorticoid receptor interacting protein-1 (GRIP-1), proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), and steroid receptor coactivator-1 (SRC-1). A corepressor of this interaction known as the small heterodimer partner interacting leucine zipper protein (SMILE) has also been reported (Xie et al., 2009).

4 METHODOLOGICAL PART

4.1 BIOLOGICAL MATERIAL

4.1.1 Human hepatocytes

Primary human hepatocyte cultures were obtained from two sources: (i) *long-term human hepatocytes in monolayer "*Batch HEP220879" (female; 65 years) were isolated after liver biopsy at Biopredic International (France); (ii) liver segments were resected from the multiorgan donors HH61 (male; 64 years), HH64 (male; 73 years), HH65 (male; 34 years), and HH66 (male; 65 years) at Faculty Hospital, Olomouc. The tissue acquisition protocol and the use of liver cells was approved by the "Ethical committee at Faculty Hospital, Olomouc", and the protocol was in accordance with the Transplantation Act #285/2002 Coll. Cells were seeded in collagen-coated plates in a hormonally and chemically defined medium consisting of a mixture of William's E and Ham's F-12 [1:1 (v/v)] (Isom et al., 1985; Pichard-Garcia et al., 2002) and were stabilized for 24 h before treatment. Cultures were maintained in a humidified incubator (37 ºC; 5% CO_2). Hepatocytes were treated with the studied complexes, inducers, and/or the vehicle (DMF; 0.1%, v/v) for 24 h (RNA) and 48 h (protein).

4.1.2 Human cancer cell lines

Human Negroid cervix epithelioid carcinoma cells or HeLa cells (ECACC No. 93021013), human Caucasian hepatocellular carcinoma cells or HepG2 cells (ECACC No. 85011430), human Caucasian colon adenocarcinoma cells or LS180 cells (ECACC No. 87021202), human Caucasian breast adenocarcinoma cells or MCF7 cells (ECACC No. 86012803) and human prostate carcinoma epithelial cells or 22Rv1 cells (ECACC No. 05092802) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). Cell lines HeLa, HepG2, LS180, and MCF7 were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 µg/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids, 4mM L-glutamine, and 1 mM sodium pyruvate. The cell line 22Rv1 was cultured in RPMI 1640 Medium (RPMI) enriched with the same components as DMEM, except for the non-essential amino acids. Cell cultures were maintained at 37 ºC and 5% CO_2 in a humidified incubator. AZ-GR (Novotna et al., 2012), AZ-AHR (Novotna et al., 2011) PZ-TR (Illes et al., 2015) and AIZ-AR cells (Bartonkova et al., 2015) were incubated under the same conditions that were provided while culturing as parental cells.

4.2 COMPOUNDS AND REAGENTS

N,N´-dimethylformamide (DMF), dimethylsulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hygromycin B, dexamethasone (DEX), rifampicin (RIF), dihydrotestosterone (DHT), 3,3´,5-triiodo-*L*-thyronine (T3), resveratrol (RVT), culture medium (DMEM, RPMI), non-essential amino acids, L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Ultra Scientific (USA). 25-hydroxyvitamin D3 (VD3) was purchased from Santa Cruz Biotechnology Inc. (Germany). TRI Reagent® was acquired from the Molecular Research Center (USA). M-MuLV Reverse Transcriptase and random hexamers were purchased from New England Biolabs (USA). The oligonucleotide primers used in qRT-PCR reactions were synthesized by Generi Biotech (Czech Republic). Light-Cycler® 480 Probes Master and protease/phosphatase inhibitor cocktails were purchased from Roche Diagnostic Corporation (Czech Republic). Luciferase lysis buffer and FuGENE® HD Transfection Reagent were obtained from Promega (USA). Reagents used for Simple Western Blotting by Sally Sue™, antibody diluent, a goat anti-rabbit IgG antibody, and a goat anti-mouse IgG antibody were purchased from ProteinSimple (California). Primary antibodies against CYP1A1 (goat polyclonal; sc-9828, G-18), CYP1A2 (mouse monoclonal; sc-53614, 3B8C1), CYP2A6 (mouse monoclonal; sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal; sc-67224, H-110), CYP3A4 (mouse monoclonal; sc-53850, HL3), a rabbit anti-goat IgG secondary antibody (sc-2768, H0712), secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology Inc. (Germany). A β-actin antibody (mouse monoclonal; 3700S, 8H10D10) was obtained from Cell Signaling Technology (USA). A Light Shift Chemiluminescent EMSA Kit was purchased from Thermo Scientific (USA). All other chemicals purchased were of the highest quality commercially available.

Gold(I) triphenylphosphine complexes with the general compositions of $[Au(L_n)(PPh_3)]$, **1** (L_1 = 6-isopropyloxy-9-deazahypoxanthine); **2** (L_2 = 6-phenethyloxy-9-deazahypoxanthine); **3** (L_3 = 6-benzyloxy-9-deazahypoxanthine) were synthesized and characterized at the Department of Inorganic Chemistry, Palacký University, Olomouc as described previously (Vanco et al., 2014). The copper(II) quinolinone complexes with general formulas $[Cu(qui1)(L_n)]NO_3 \cdot H_2O$ and $[Cu(qui2)(phen)]NO_3$, where *qui1* = 2-phenyl-3-hydroxy-4(1H)-quinolinone, *qui2* = 2-(4-amino-3,5-dichlorophenyl)-N-propyl-3-hydroxy-4(1H)-quinolinone-7-carboxamide, L_1 = 1,10-phenanthroline (phen), L_2 =

5-methyl-1,10-phenanthroline (mphen), L_3 = bathophenanthroline (bphen) were synthesized and characterized at the Department of Inorganic Chemistry, Palacký University, Olomouc as described previously (Buchtik et al., 2011; Krivavova et al., 2016; Travnicek et al., 2014).

4.3 METHODS

4.3.1 Cell viability assay

The human cancer cell lines 22Rv1, HeLa, HepG2, and LS180 were seeded in the 96-well culture plates (3×10^5 cells per well). After 16 h of pre-incubation, the cells were treated with copper(II) and gold(I) complexes, in concentrations ranging from 1 nM to 50 µM (unless the solubility was lower) for 24 h. In parallel, the cells were treated with the vehicle (DMF; 0.1%, v/v; negative control) and Triton X-100 (TX100; 1%, v/v; positive control) to assess the minimal and maximal cell damage, respectively. Thereafter, the medium was removed, the cells were washed with phosphate buffered saline 1× (PBS) and incubated with MTT (100 µL; 0.3 mg/ml) for 1 h at 37°C. The reaction was stopped by removing MTT solution and adding 40-100 µl of DMSO. Absorbance was measured spectrophotometrically at 570 nm, using Infinite M200 (Schoeller Instruments, Czech Republic). The data were expressed as a percentage of cell viability, where 100% and 0% represented negative and positive controls, respectively. The data were acquired from three independent experiments (cell passages). The concentrations that caused a decline in viability that was no greater than 20% were considered to be non-toxic, and were used for further experiments.

4.3.2 Plasmids

A chimeric p3A4-luc reporter construct containing the basal promoter (-362/-53), in which the proximal PXR response element (ER6) and distal xenobiotic-responsive enhancer module (-7836/-7208) of the CYP3A4 gene 5´-flanking region was inserted to the pGL3-Basic reporter vector (Promega, UK) has been described (Goodwin et al., 1999). The expression plasmid for human PXR (pSG5-hPXR) that encoded human PXR was provided by Dr. S. Kliewer (University of Texas, Dallas, USA). The expression plasmid pSG5-hVDR that encoded human VDR was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). The pCYP24-luc plasmid containing the 5´-flanking region (−1200 to −22) of the human CYP24 gene subcloned

into the pGL3 plasmid upstream of the firefly luciferase reporter gene was provided by Dr. J. M. Pascussi (INSERM U632, Montpellier, France). The luciferase reporter construct pSG5-luc and pGL3 plasmid were purchased from Promega.

4.3.3 Luciferase inhibition assay

The highest concentration of tested compounds used during the previous experiments were added to cell lysates containing firefly luciferase, and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.3.4 Transfection and luciferase gene reporter assay

Stably transfected human luciferase gene reporter cell lines AZ-AHR, AZ-GR, AIZ-AR, and PZ-TR were used to examine the transcriptional activities of the aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), androgen receptor (AR), and thyroid hormone receptor (TR), respectively. Transiently transfected LS180 cells were used for the analysis of the transcriptional activity of vitamin D receptor (VDR) and pregnane X receptor (PXR) (Vrzal et al., 2011).

Reporter cell lines were seeded into to the 96-well plates (density 3×10^5 cells per well). After 16 h of incubation, the cells were treated with the tested compounds, TCDD (5 nM), DEX (100 nM), DHT (100 nM), T3 (10 nM) and/or vehicle (DMF; 0.1%; v/v). LS180 cells were transiently transfected by lipofection (FuGENE®HD Transfection Reagent) using 300 ng per well of the reporter gene (*pCYP24-luc* or *p3A4-luc*) and 100 ng per well of expression vectors (hPXR or hVDR) in 24-well plates. Prior to the treatments, cultures were stabilized for 16 h and then incubated for 24 h with the tested compounds, RIF (10 µM), VD3 (50 nM), and/or vehicle (DMF; 0.1%, v/v). After the treatments, cells were lysed (Reporter Lysis Buffer) and the luciferase activity was measured using the Infinite M200 plate luminometer (Schoeller Instruments, Czech Republic).

4.3.5 RNA isolation, reverse transcription and qRT-PCR

LS180 cells and primary human hepatocytes were seeded in 6-well plates (density: $1\text{x}10^6$ cells per well). After 16 h of incubation, the cells were treated with the tested compounds, TCDD (5 nM), RIF (10 µM), and/or vehicle (DMF; 0.1%, v/v). The total RNA was isolated using TRI Reagent®. The cDNA was synthesized from 1000 ng of total RNA, according to a common protocol that use M-MuLV Reverse Transcriptase

and random hexamers at 42 ºC for 60 min. The real-time quantitative reverse transcription polymerase chain reaction (real time qRT-PCR) was carried out using Light Cycler® 480 Probes Masteron a Light Cycler 480 II instrument (Roche Diagnostic Corporation, Czech Republic). The mRNA levels were determined using primers and the Universal Probes Library (UPL; Roche Diagnostic Corporation, Czech Republic) technology described previously (Vrzal et al., 2015). The sequences of primers are shown below (see Table 4).

Table 4:

Primer sequences with appropriate UPL numbers.

Gene symbol	Forward primer sequence	Reverse primer sequence	UPL number
CYP1A1	CCAGGCTCCAAGAGTCCA	GATCTTGGAGGTGGCTGCT	33
CYP1A2	ACAACCCTGCCAATCTCAAG	GGGAACAGACTGGGACAATG	34
CYP2A6	CCACGGGACTTCATTGACTC	CCCAATGAAGAGGTTCAACG	63
CYP2B6	TTCACTCATCAGCTCTGTATTCG	GCCCCAGGAAAGTATTTCAA	106
CYP2C9	GAAGCACCCAGAGGTCACAG	CTCGTGCACCACAGCATC	92
CYP3A4	TGTGTTGGTGAGAAATCTGAGG	CTGTAGGCCCCAAAGACG	38
TAT	GCACCCCTAGAAGCTAAGGAC	CAGGTCTTGGAACCAGGATG	37
SPOT14	CATGCACCTCACCGAGAA	TGTCTTCTATCATGTGAAGGGATCT	79
GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC	60

The steps used were as follows: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s; annealing with elongation at 60 °C for 30 s). The measurements were performed in triplicates. The gene expression levels were normalized to those of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Data were processed by using the delta-delta method (Pfaffl, 2001). Results are expressed in terms of the x-fold induction over DMF-treated cells.

4.3.6 Simple western blotting by SallySue^{TM}

The total protein extracts were obtained as follows: the cells were washed twice with ice-cold PBS, scraped into 1 mL of PBS, and a suspension was centrifuged (4500 rpm/5 min/4°C). The pellet was re-suspended in 150 μL of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 0.1% (w/v) SDS, anti-protease cocktail, 1% (v/v) Triton X-100, anti-phosphatase cocktail, 1% (v/v) sodium deoxycholate, 5 mM EDTA). After homogenization, the mixture was vortexed and incubated for 10 min on ice and centrifuged (13500 rpm/15 min/4°C). The supernatant was collected, and the protein content was determined using the Bradford assay. The samples were stored at – 80°C.

The Sally Sue Simple Western System was used for the detection of CYP proteins using to the ProteinSimple manual (Sally Sue, USA). The following diluted primary antibodies were used: CYP1A1 (dilution 1:25), CYP1A2 (dilution 1:250), CYP3A4 (dilution 1:10000), and β-actin (dilution 1:100). Target proteins were identified using primary antibodies and immune-probed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was analysed and quantified by Compass Software, version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the signals were normalized to those of β-actin, which was used as a loading control.

4.3.7 Electrophoretic mobility shift assay (EMSA)

MCF-7 cells (density: $2x10^6$ cells per well) were seeded in 6-well plates. After 16 h of pre-incubation, the cells were treated for 2 h with 1 μM of the copper(II) complexes (**1**–**4**), TCDD (5 nM), and vehicle, used as a negative control (DMF; 0.1%, v/v). Nuclear and cytoplasmic fractions were isolated using the Nuclear Extract Kit according to the protocol provided by the manufacturer (Active Motif). The following double-stranded
5´-biotinylated oligonucleotides containing DNA-binding sequences specific for AhR, which corresponded to the 27-bp protein binding site of DRE 3 were used (Denison et al., 1988):

sense 5′ − GATCCGGCTCTTCTCACGCAACTCCGAGCTCA − 3′;
antisense 5′ − GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG − 3′

A gel mobility shift assay was performed using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA). The nuclear protein extract (15 μg) was pre-incubated in binding buffer (final concentrations: 10 mMTris, 50 mM KCl, 1 mM DTT; pH 7.5), along with 2.5% glycerol, 0.05% NP-40, ddH_2O and non-specific competitor Poly (dI.dC); then, the biotin-labelled probe was added (10 fmol/μL). An un-labelled probe (2 pmol/μL) was added to the reaction mixture for the competitive assay. The complete reaction mixture was incubated at room temperature for 20 min. Finally, 5 μL of 5X loading buffer was added before loading the mixture in the wells of a non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein-DNA complexes were electro-blotted to a positively charged nylon membrane. Thereafter, transferred DNA was cross-linked using a UV-light cross-linker instrument. Biotin-labelled DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in a Light-Shift Chemiluminescent EMSA Kit (Thermo Scientific, USA).

4.3.8 Statistical analysis

Student's *t*-test and GraphPad Prism 6 software (GraphPad Software, USA) were used to calculate the half-maximal inhibitory concentrations (IC_{50}) and half maximal effective concentrations (EC_{50}).

5 RESULTS

5.1 COPPER(II) MIXED-LIGAND COMPLEXES

5.1.1 The effects of copper(II) complexes on the transcriptional activity of steroid receptors, nuclear receptors, and xenoreceptors

In the first series experiments, the cytotoxicity of copper(II) complexes (**1**-**4**) in AIZ-AR, AZ-AHR, AZ-GR, PZ-TR and LS180 cells was determined using the MTT assay. Based on the cytotoxicity data, gene reporter assays were performed using concentrations of up to 50 μM (unless the solubility of substances was lower). The cells were incubated with the complexes (**1**-**4**) and vehicle in the absence (*agonist mode*) and/or presence (*antagonist mode*) of the model agonist for each receptor. The luciferase activity was measured and half-maximal inhibitory concentrations (IC_{50}) were calculated.

Androgen receptor: The cytotoxicity and transcriptional activity of AR were evaluated in the transgenic reporter cell line AIZ-AR. Complexes **1, 2,** and **4** were not cytotoxic until their concentration was up to 10^{-6} M, while complex **3** displayed cytotoxicity at a concentration of 10^{-6} M (Figure 11; upper left panel). Dihydrotestosterone (DHT; 100 nM), the model agonist for AR increased luciferase activity by an average of 11-fold, as compared to vehicle-treated cells (Figure 11; middle right panel). Copper(II) complexes (**1**–**4**) did not influence either the basal or DHT-inducible luciferase activity of AR (Figure 11). A decline in the luciferase activity of cells treated with complexes **2, 3,** and **4** at concentrations higher than 10^{-6} M was because of their cytotoxic effects.

Glucocorticoid receptor: The cytotoxicity of tested compounds was assessed in the gene reporter cell line AZ-GR. Complexes **1** and **3** were non-toxic when their concentration was up to 1 μM, whereas 1 μM of the complexes **2** and **4** decreased the viability down to approximately 75% (Figure 11; upper right panel). Dexamethasone (DEX; 100 nM), the model agonist of GR, increased the luciferase activity by an average of 43-fold, as compared to vehicle-treated cells. The basal activity of GR was dose-dependently decreased by complex **1**, suggesting that is exhibits inverse agonist behaviour (Figure 11; middle right panel). Dexamethasone-inducible luciferase activity was decreased by all the tested compounds, and this decline was attributable to their intrinsic cytotoxicity (Figure 11; lower right panel).

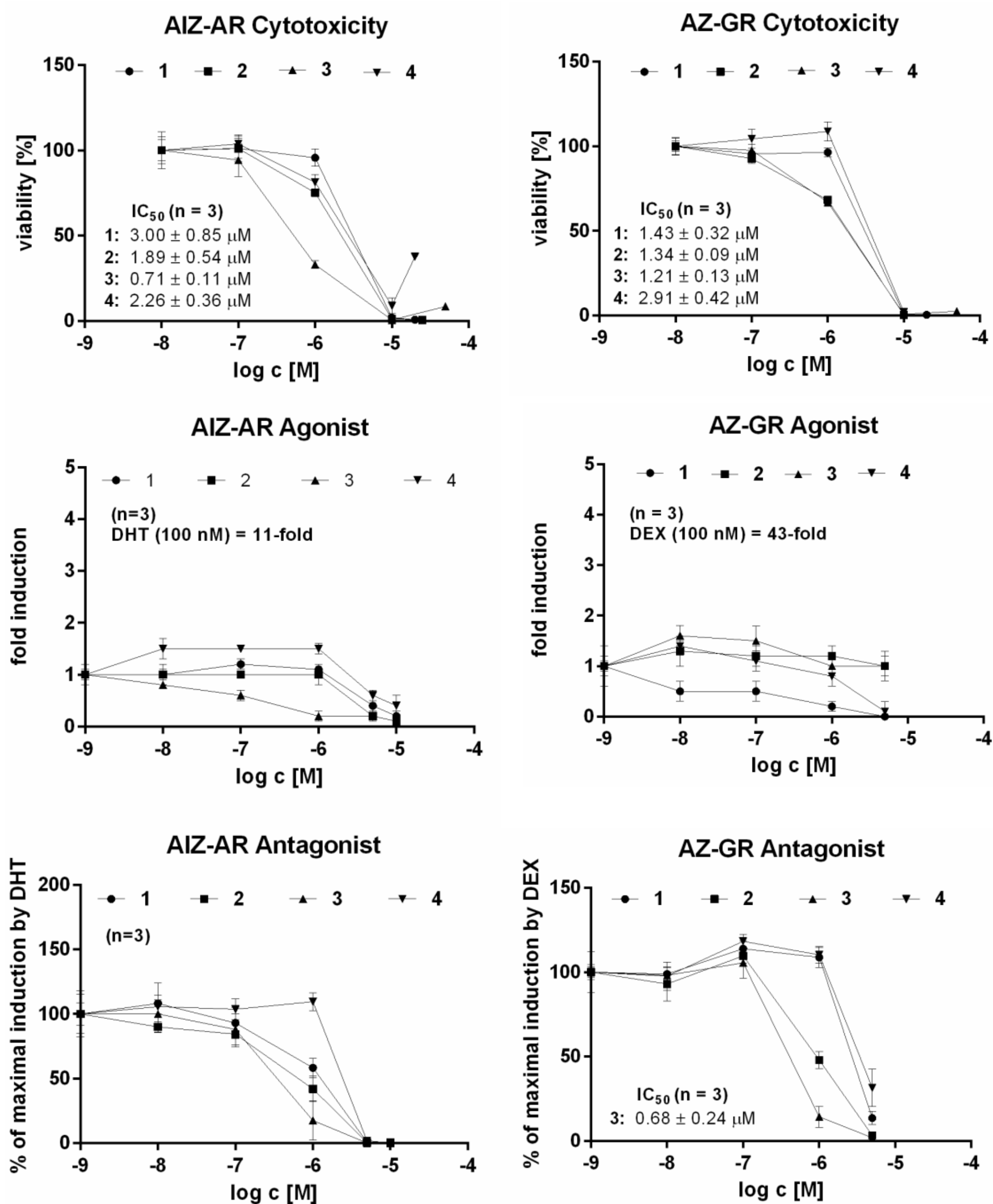


Figure 11: The effects of copper(II) complexes on the transcriptional activities of steroid receptors. *The cells were stabilized (16 h) and then incubated (24 h) with the copper(II) complexes (**1-4**) at concentrations ranging from 1 nM to 50 µM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

Aryl hydrocarbon receptor: The effects of copper(II) complexes and the transcriptional activity of AhR were assessed in the transgenic reporter cell line AZ-AHR. All complexes were cytotoxic if present in concentrations higher than 1 μM (Figure 12; upper left panel). An AhR agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM), increased luciferase activity by an average value of 669-fold, as compared to vehicle-treated cells. Complexes **1** and **2** caused the dose-dependent induction of luciferase activity, which peaked at 10^{-6} M, while at higher concentrations, the luciferase activity was decreased, probably due to the cytotoxicity of the compounds (Figure 12; middle left panel). The calculated EC_{50} values were 0.14 ± 0.02 μM and 0.15 ± 0.02 μM for complex **1** and **2,** respectively. The efficacies of complexes **1** and **2** were about 6.5% of that obtained by TCDD. Only a negligible induction of luciferase activity was observed for complexes **3** and **4**. All complexes dose-dependently decreased the TCDD-inducible AhR-dependent luciferase activity (in non-cytotoxic concentrations), revealing the partial agonist behaviour of those compounds. The effects were much more pronounced for complexes **3** (IC_{50} 0.87± 0.2 μM) and **4** (IC_{50} 0.54 ± 0.3 μM), as compared to those for complexes **1** and **2** (Figure 12; lower left panel).

Pregnane X receptor: The transcriptional activity of PXR was assessed in LS180 cells transiently transfected with the *p3A4-luc* reporter construct. With the exception of complex 3, which moderately decreased the cell viability, the remaining complexes were non-cytotoxic at concentrations of up to 1 μM (Figure 12; upper right panel). PXR agonist rifampicin (RIF; 10 μM) increased the luciferase activity by an average value of 3-fold, as compared to vehicle-treated cells. None of the tested compounds affected either the basal or RIF-inducible luciferase activity of PXR (Figure 12). The decrease in the luciferase activities by higher concentrations of the complexes was attributable to their cytotoxicity.

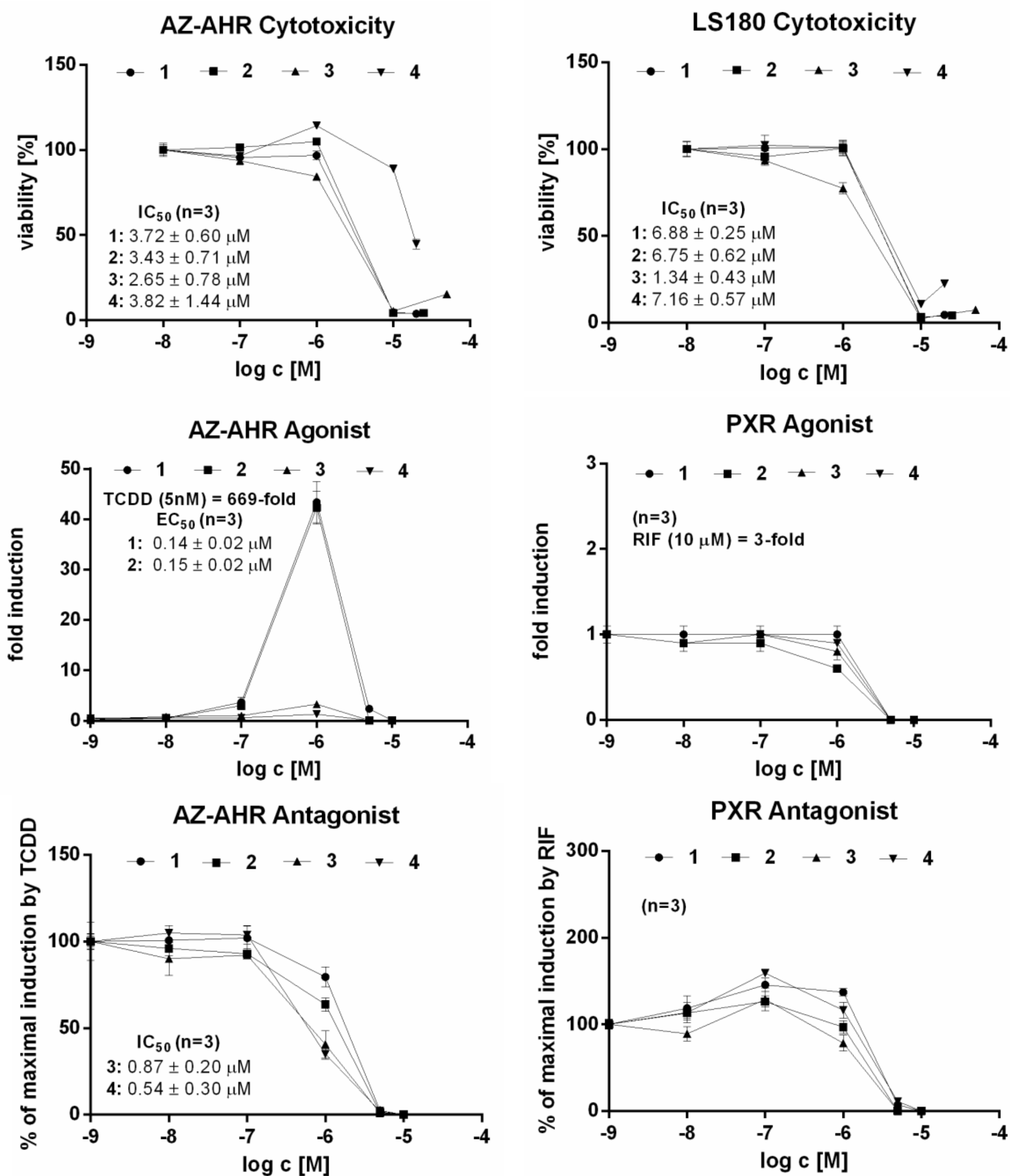


Figure 12. The effects of copper(II) complexes on the transcriptional activities of xenoreceptors. *The cells were stabilized (16 h) and then incubated (24 h) with the copper(II) complexes (**1-4**) at concentrations ranging from 1 nM to 50 µM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

Thyroid hormone receptor: The transcriptional activity of the nuclear receptor TR was evaluated in the transgenic reporter cell line PZ-TR. A cell viability assay revealed that tested complexes are not cytotoxic at concentrations of up to 1 μM (Figure 13). A model agonist for TR named 3,3′,5-triiodo-L-thyronine (T3; 10 nM) induced luciferase activity by an average value of 3-fold, as compared to vehicle-treated cells. All complexes dose-dependently decreased the basal transcriptional activity of TR (Figure 13), which was probably due to their cytotoxicity. Interestingly, complexes **1** and **4** slightly potentiated the T3-inducible luciferase activity concentrations of 5 μM (Figure 13).

Vitamin D receptor: The transcriptional activity of VDR was assessed in LS180 cells transiently transfected with the *pCYP24-luc* reporter construct. As observed with other cell lines besides complex **3**, the remaining complexes were not cytotoxic up to a concentration of 1 μM concentration (Figure 13). A model agonist of VDR, 1,25-dihydroxyvitamin D3 (VD3; 50 nM), induced luciferase activity by an average value of 47-fold as compared to vehicle-treated cells. In the agonist mode, the tested complexes were inactive, while in antagonist mode, the luciferase activity were decreased by higher concentrations of the complexes (Figure 13).

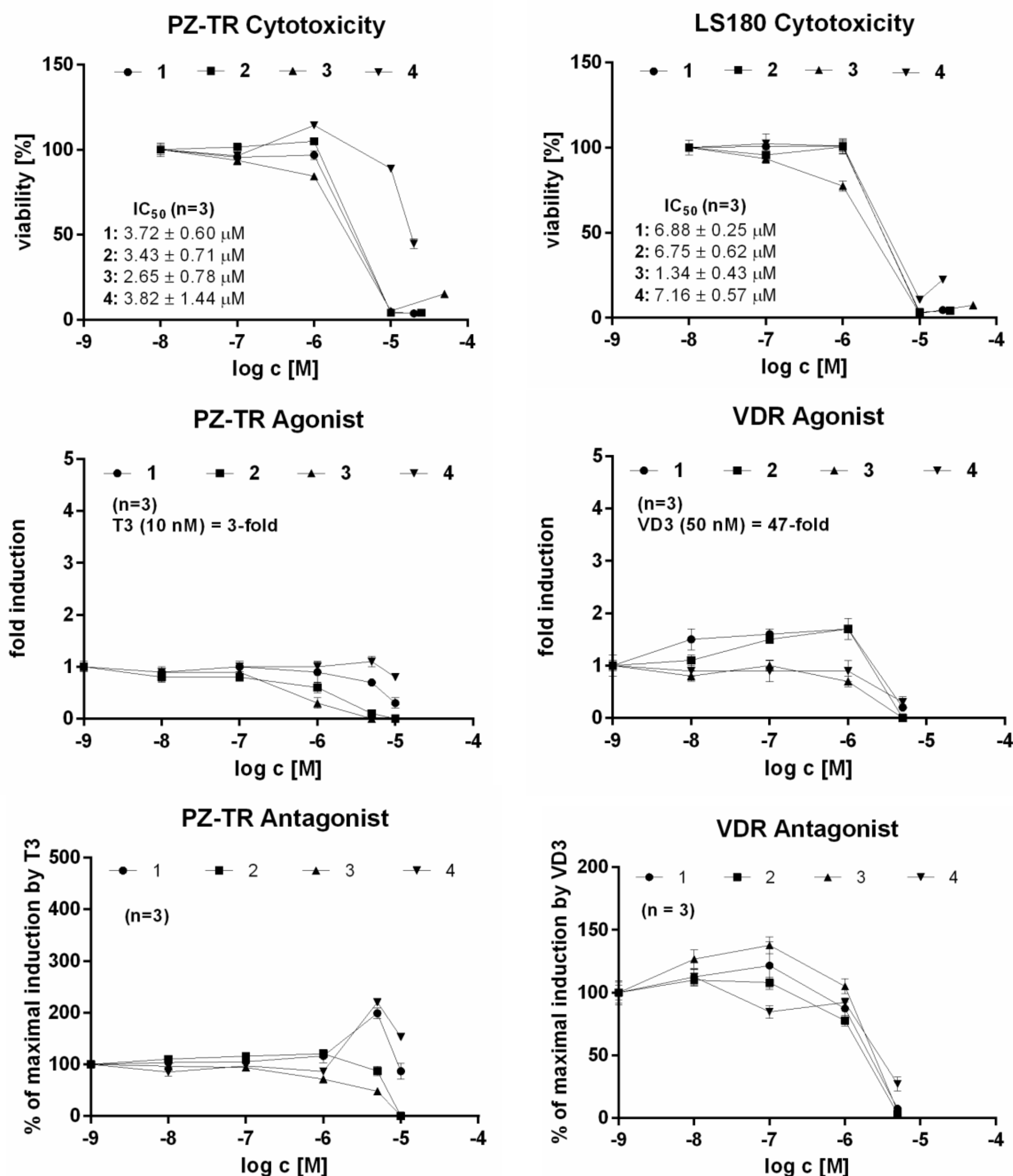


Figure 13. The effects of copper(II) complexes on the transcriptional activities of nuclear receptors. *The cells were stabilized (16 h) and then incubated (24 h) with the copper(II) complexes (**1-4**) at concentrations ranging from 1 nM to 50 μM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

5.1.2 The effects of copper(II) complexes on the expression of *CYP1A1* mRNA in LS180 cells and AhR transcriptional activity in AZ-AHR cells

In the next series of experiments, we examined whether the induction of luciferase activity in AZ-AHR cells by Cu(II) complexes involved the use of AhR. For this reason, we treated AZ-AHR with copper(II) complexes, vehicle, and TCDD in the presence or absence of resveratrol (RVT; 100 µM), a known AhR antagonist (Casper et al., 1999). The positive control TCDD significantly activated AhR in three consecutive passages of AZ-AHR cells, and an activation of 1305-fold, 387-fold and 487-fold was observed, as compared to DMF-treated cells. Complexes **1**, **2,** and **3** induced 48-fold, 20-fold and 3-fold luciferase activities respectively, on an average. An induction of luciferase activity by the complexes and TCDD was significantly ($p < 0.05$) abolished by RVT (Figure 14), implying the involvement of the AhR in the process.

We also tested whether copper(II) complexes caused the AhR-dependent induction of CYP1A1 mRNA. Dioxin strongly induced *CYP1A1* mRNA expression in three consecutive passages of LS180 cells, and 787-fold, 528-fold and 314-fold activations were observed. Complexes **1**, **2,** and **3** induced *CYP1A1* mRNA (20-fold to 40-fold) expression in three independent experiments. The levels of inductions were drastically diminished with RVT, affirming the AhR-dependent mechanism of induction (Figure 14).

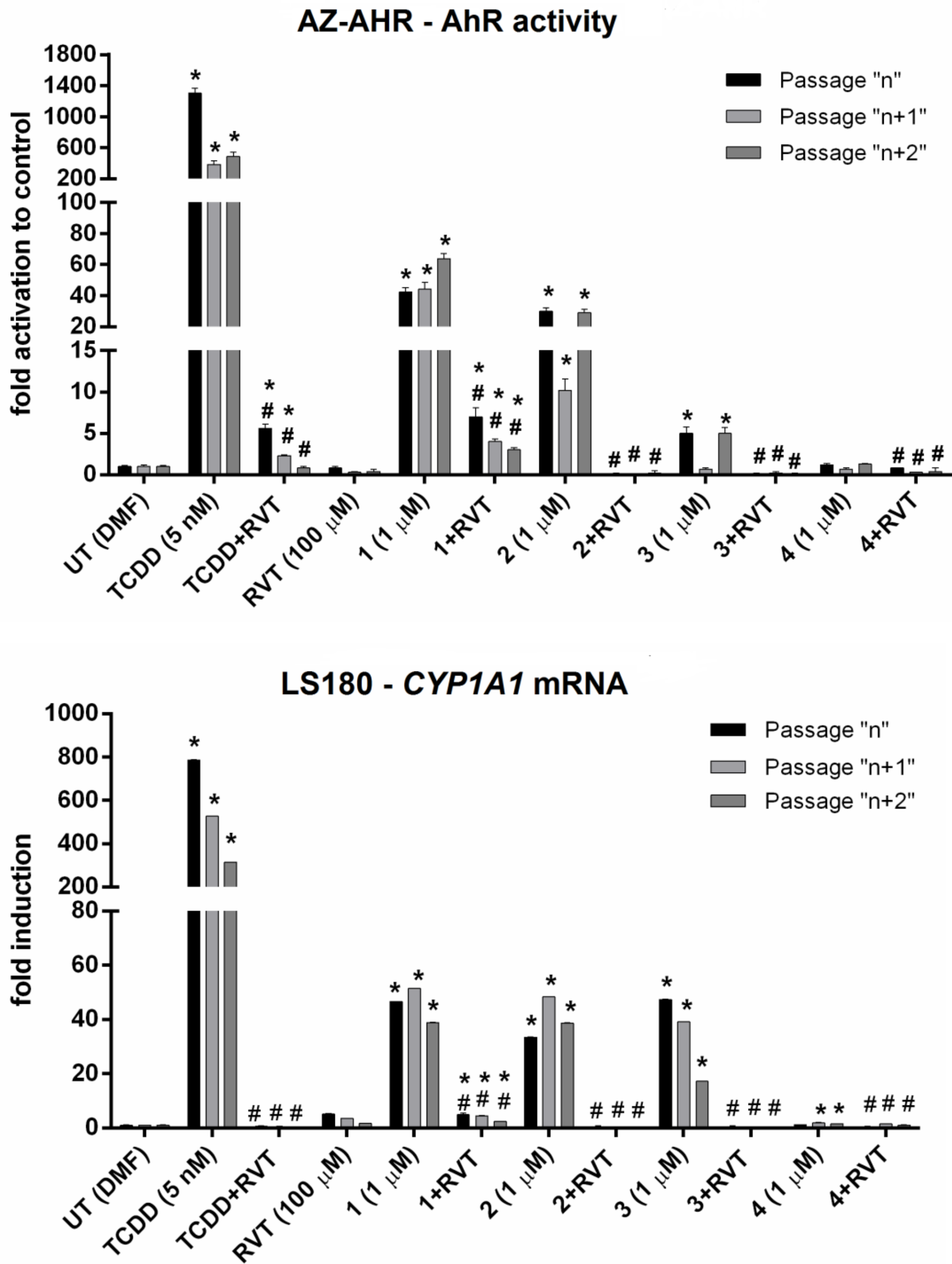


Figure 14. The effects of Cu(II) complexes and resveratrol on the expression of CYP1A1 mRNA in LS180 and AhR activity in AZ-AHR cells. *The cells were stabilized (16 h) and then treated (24 h) with copper(II) complexes (**1–4**; 1 μM), vehicle (DMF; 0.1%, v/v), and dioxin (TCDD; 5 nM) in the absence or presence of resveratrol (RVT; 100 μM). The data are presented as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. * = values that are significantly different from those of DMF-treated cells (p < 0.05). # = significant difference between cells incubated in the presence or absence of RVT (p < 0.05). Graphs are shown from three consecutive cell passages. Upper panel: Gene reporter assays in AZ-AHR cells. Lower panel: qRT-PCR analyses of CYP1A1 mRNAs in LS180 cells. The data were normalized to GAPDH mRNA levels.*

5.1.3 The effects of copper(II) complexes on binding of AhR to DNA

We examined whether tested compounds could elicit the binding of the AhR receptor to specific DNA response elements. We treated MCF7 cells with complexes **1**-**4** (1 μM), TCDD, and vehicle. TCDD and complex **2** strongly increased the formation of the AhR-ARNT DNA binding complex as compared to that of the vehicle. Complex **1** merely had a moderate effect, and complexes **3** and **4** were inactive (Figure 15).

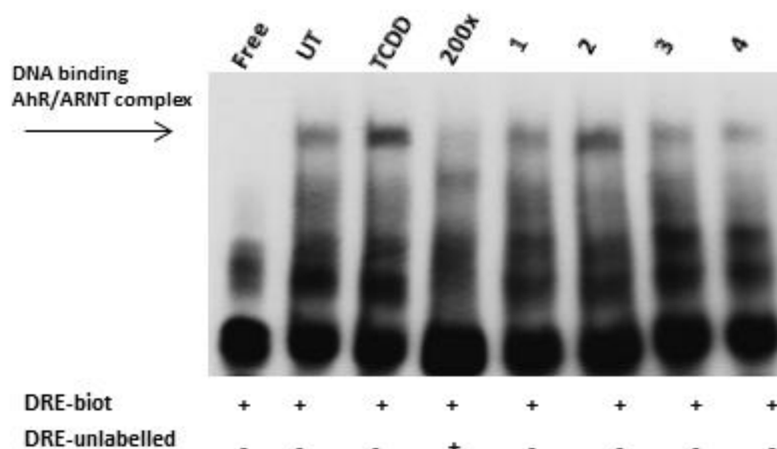


Figure 15. The effects of copper(II) complexes on binding of AhR to DNA. *MCF-7 cells at 100% confluence were treated with TCDD (5 nM), copper(II) complexes (**1**–**4**; 1 μM), and/or vehicle as a negative control (DMF; 0.1%, v/v) for 2 h. Nuclear extracts were incubated with a biotin-labelled probe containing the AhR binding site and separated on a 5% polyacrylamide gel. A representative gel is showned. Experiments were performed in three independent cell passages.*

5.1.4 The effects of copper(II) complexes on the expression of drug-metabolizing CYPs in primary human hepatocytes

In the final series of experiments, we investigated the effects of tested complexes on the expression of selected drug-metabolizing CYPs in primary cultures of human hepatocytes. We examined the ability of the copper(II) complexes to induce the AhR-mediated expression of *CYP1A1* and *CYP1A2* mRNAs and proteins. We also measured the expression of GR-dependent gene *tyrosine aminotransferase (TAT)* and other xenobiotic-inducible drug-metabolizing CYPs, including *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4*. We incubated primary cultures of human hepatocytes from three donors (HH61, HH64, HH65) for 24 h (mRNA) and 48 h (protein) with copper(II) complexes (**1**-**4**; 1 µM; 10 µM) and model inducers, including dioxin (TCDD; 5 nM) and rifampicin (RIF; 10 µM). *CYP1A1* and *CYP1A2* mRNAs and proteins were strongly induced by TCDD in all human hepatocyte cultures. The complexes **1** and **2** slightly increased the expression of *CYP1A1/1A2* genes (Figure 16), which was consistent with data from gene reporter assays and mRNA analyses in LS180 cells. On the other hand, the expression of CYP1A1 and CYP1A2 proteins by compounds **1** and **2** was very weak (Figure 17). Rifampicin, a model activator of PXR, induced the expression of *CYP2A6*, *CYP2B6,* and *CYP3A4* mRNAs in each culture of human hepatocytes (Figure 16). The induction of CYP2C9 mRNA by RIF was observed only in one culture (HH61), probably due to the high constitutive levels of this gene. Tested complexes slightly altered the expression of *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4* mRNAs, but these effects were inconsistent, and reflected the inter-individual variability between liver donors (Figure 16). Moreover, we observed that the studied complexes exhibited weak anti-glucocorticoid effects towards GR in gene reporter assays (Figure 11). Hence, the *tyrosine aminotransferase (TAT)* mRNA level was also measured, as a typical GR-dependent gene. We observed that the level of *TAT* mRNA was reduced by tested complexes; however, the effects did not correlate with data obtained from the gene reporter assay used to examine AZ-GR cells, reflecting the cytotoxicity of complexes against AZ-GR cells (Figure 16).

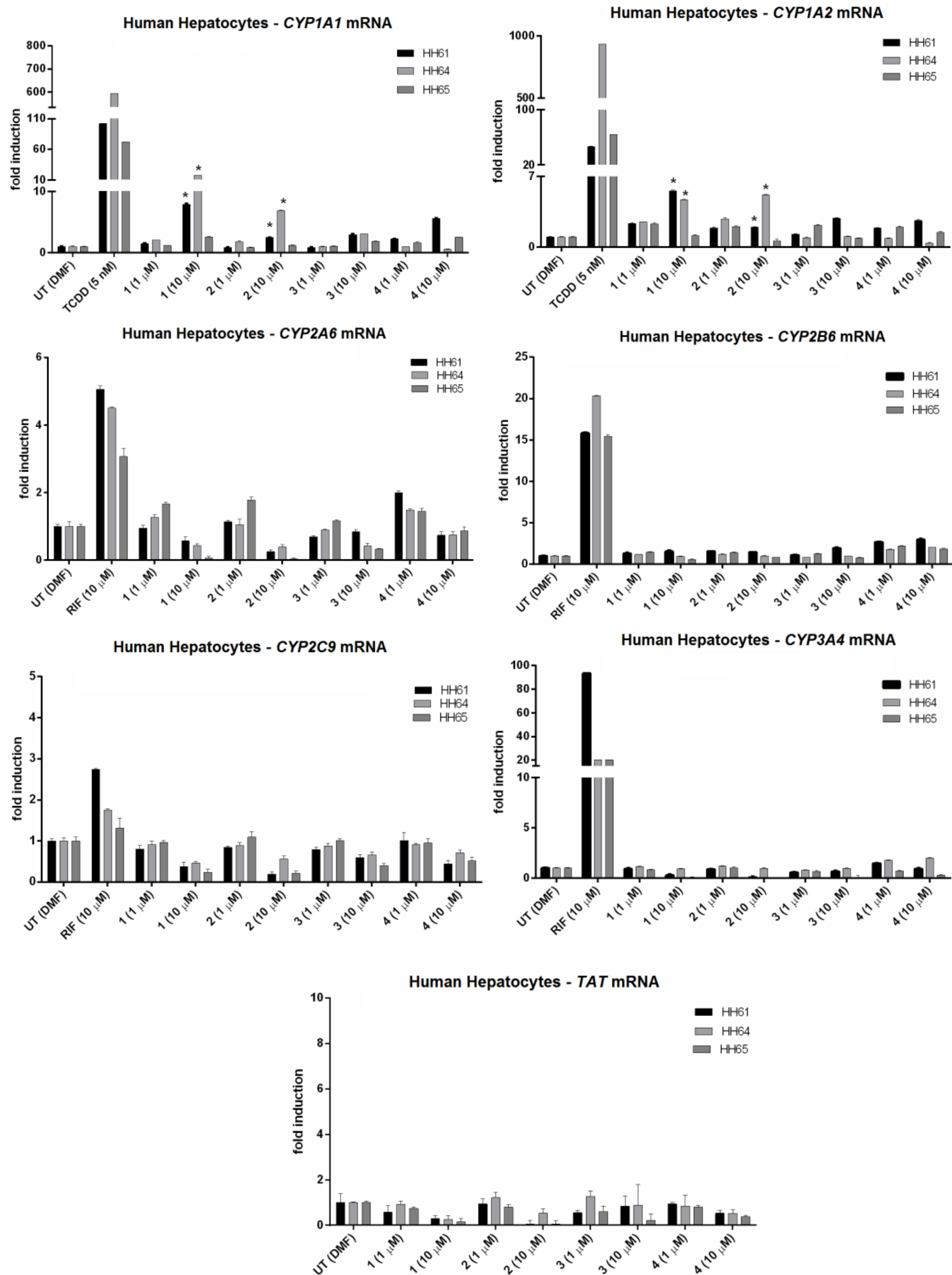
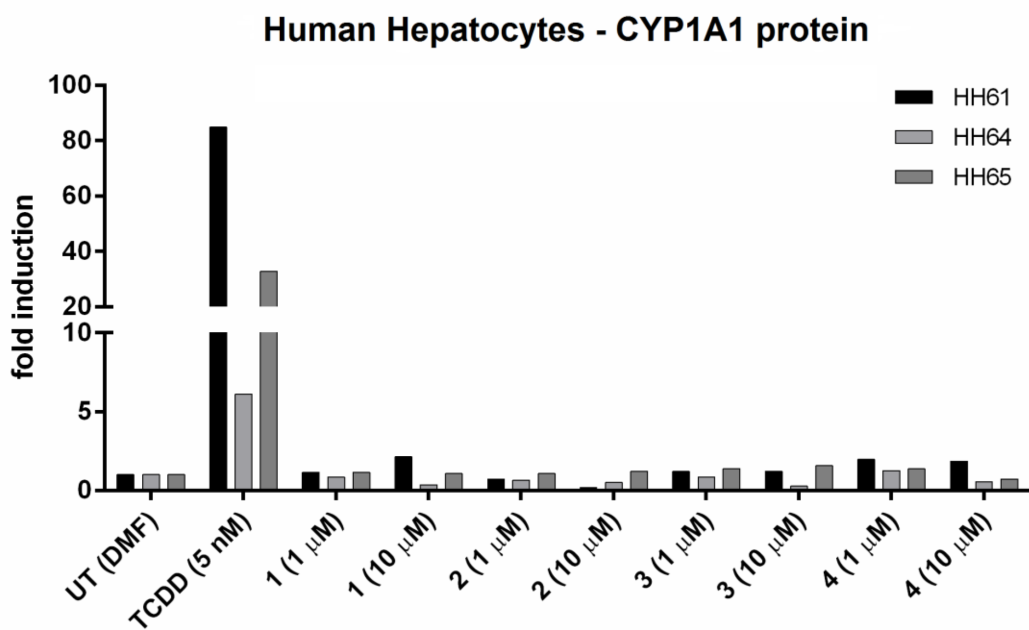


Figure 16. The effects of copper(II) complexes on the mRNA expression of drug-metabolizing CYPs and tyrosine aminotransferase (TAT) in primary human hepatocytes. *Human hepatocytes from three different donors (HH61, HH64, HH65) were treated (24 h) with copper(II) complexes (**1**–**4**; 1 μM and 10 μM), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and the vehicle (DMF; 0.1%, v/v). Bar graphs show qRT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, and TAT mRNAs. The data are presented as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. The data were normalized to GAPDH mRNA levels.*



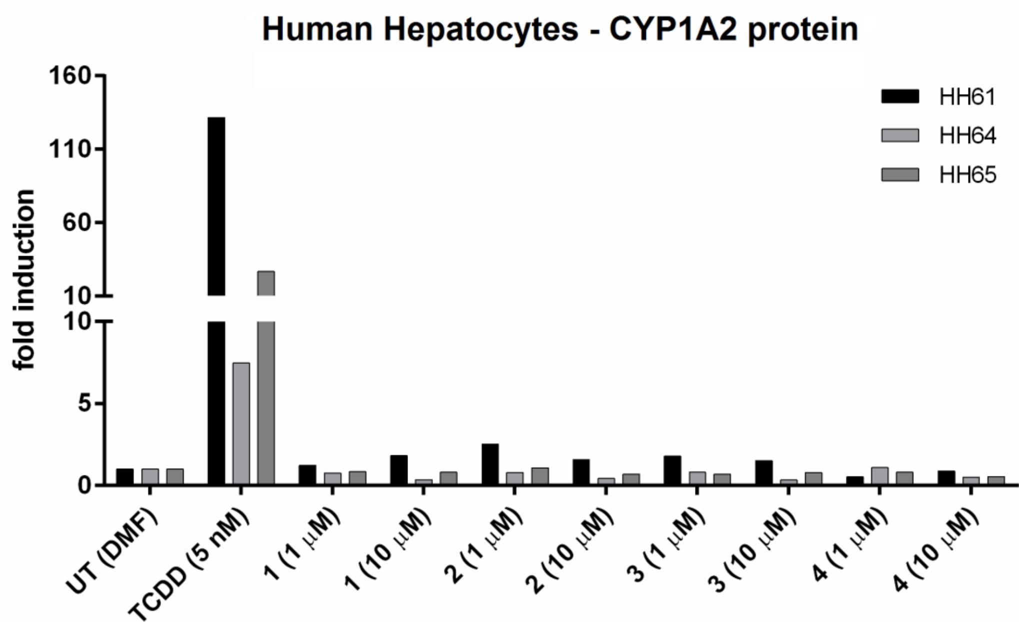


Figure 17. The effects of copper(II) complexes on the protein expression of drug-metabolizing CYPs in primary human hepatocytes. *Human hepatocytes from three different donors (HH61, HH64, HH65) were treated (48 h) with copper(II) complexes (**1–4**; 1 µM and 10 µM), dioxin (TCDD; 5 nM), and the vehicle (DMF; 0.1%, v/v).Bar graphs show the analyses of CYP1A1 and CYP1A2 proteins by Simple Western SallySue. The data are presented as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. The data were normalized to β-actin levels.*

5.2 GOLD(I) MIXED-LIGAND COMPLEXES

5.2.1 The effects of gold(I) complexes on the transcriptional activity of steroid receptors, nuclear receptors, and xenoreceptors

Firstly, the cytotoxicity of the studied compounds was examined and the concentration range for ongoing experiments was defined (Figure 7). Based on the data obtained from cytotoxicity assays, gene reporter assays were carried out using concentrations ranging from 1 nM to 50 μM (unless the solubility of substances was lower) (Figures 18, 19, 20). We performed detailed dose-response experiments, using stably (AIZ-AR, AZ-AHR, AZ-GR, PZ-TR) or transiently (LS180) transfected human cell lines. The transcriptional activities of AR, GR, PXR, AhR, VDR and TR were evaluated in the absence (*agonist mode*) or presence (*antagonist mode*) of the model agonist for each receptor. The half-maximal effective concentrations (EC_{50}) and half-maximal inhibitory concentrations (IC_{50}) were calculated.

Androgen receptor: Tested complexes **2** and **3** were not cytotoxic if their concentraiotns were up to 10 μM, while complex **1** (10 μM) decreased cell viability down to 40% of that of the control. Model AR agonist DHT activated AR, and the average induction of luciferase activity was 11-fold as compared to that of DMF-treated cells. All tested complexes dose-dependently antagonized both basal and DHT-inducible AR-dependent luciferase activity, thus revealing their inverse agonist behaviour. The IC_{50} values were 1.8 ± 0.3 μM and 4.0 ± 0.1 μM for complexes **2** and **3**, respectively. The inhibitory effects of complex **1** on DHT-inducible luciferase activity were rather due to the intrinsic cytotoxicity of the compound than due to the receptor antagonism (Figure 18).

Glucocorticoid receptor: Tested complexes were not cytotoxic if their concentrations were up to 10 μM in the AZ-GR cell line, as revealed by the MTT test (Figure 19). The positive control DEX activated GR with an average induction of 43-fold, as compared to DMF-treated cells. The gold(I) complexes did not have any effect on the basal activity of GR, while GR-driven luciferase activity was dose-dependently decreased by complexes **1, 2** and **3** with IC_{50} values 2.9 ± 1.9 μM, 5.0 ± 0.1 μM, and 2.5 ± 0.1 μM, respectively (Figure 18).

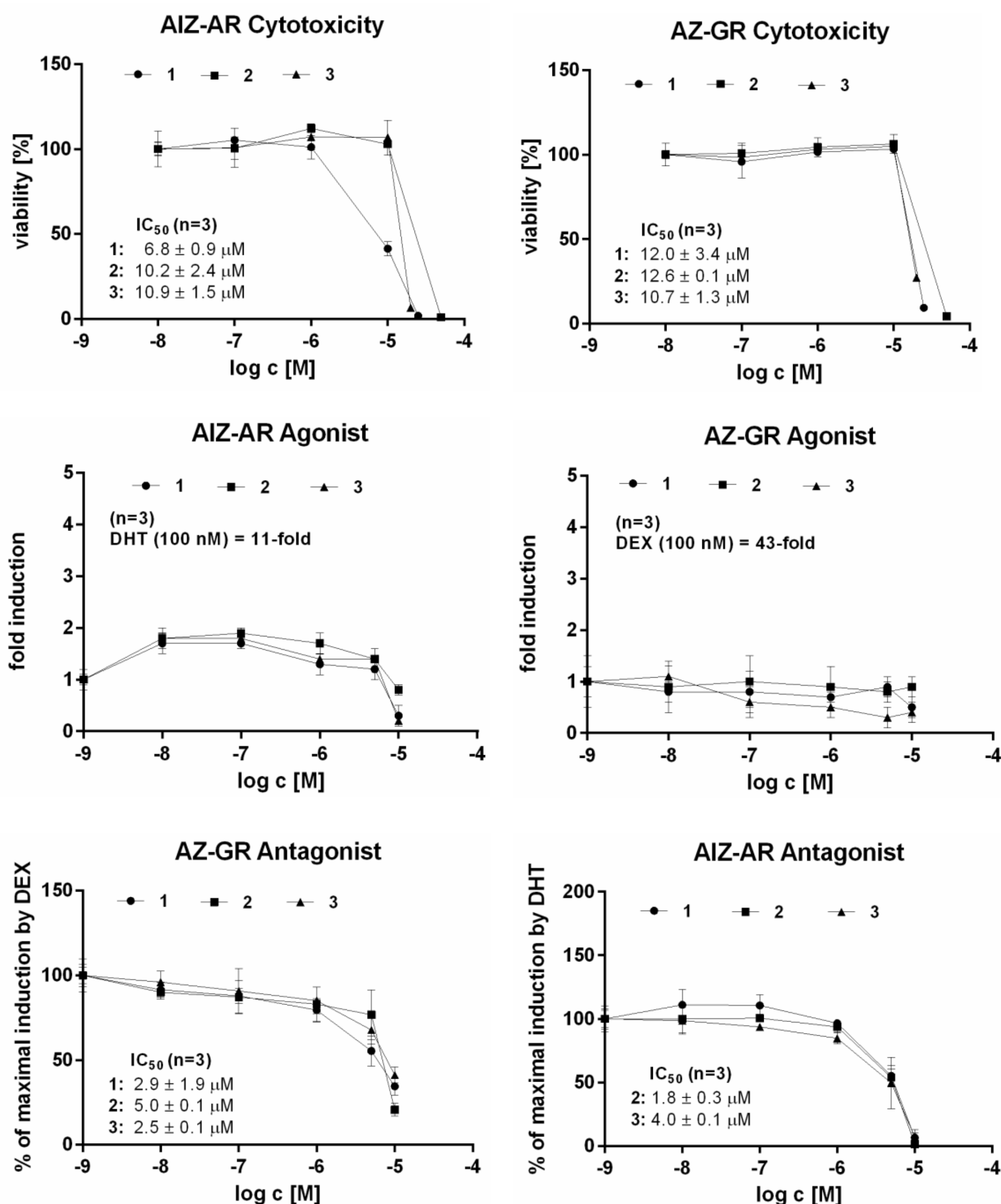


Figure 18. The effects of gold(I) complexes on the transcriptional activities of steroid receptors. *The cells were stabilized (16 h) and then incubated (24 h) with the gold(I) complexes (**1-3**) at concentrations ranging from 1 nM to 50 µM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

Aryl hydrocarbon receptor: The tested complexes did not influence the viability of AZ-AHR cells when their concentration was up to 10 μM (Figure 19). The positive control TCDD activated AhR 669-fold, as compared to DMF-treated cells. Complexes **1, 2,** and **3** dose-dependently increased the luciferase activity, and their EC_{50} values were 5.2 ± 0.1 μM, 5.0 ± 0.1 μM, and 3.8 ± 1.7 μM, respectively (Figure 19). The complexes exhibited weak efficacies ranging from 0.5% to 1.5% of that exhibited by TCDD, in the order **2**~**3** > **1**. None of the tested complexes showed antagonistic effects towards AhR (Figure 19).

Pregnane X receptor: The tested complexes were not cytotoxic if their concentrations were up to 5 μM in LS180 cells, as revealed by the MTT test (Figure 19). The positive control RIF activated PXR 3-fold as compared to DMF-treated cells. Complexes **1, 2,** and **3** dose-dependently increased the luciferase activity, and EC_{50} values ranged from 0.4 μM to 1.0 μM (Figure 19). The efficacy of complexes was similar to that of rifampicin in the order **2** > **1** >**3**. The PXR-driven luciferase activity was dose-dependently inhibited by the complexes **1** and **3**, but was slightly augmented by complex **2** (Figure 19).

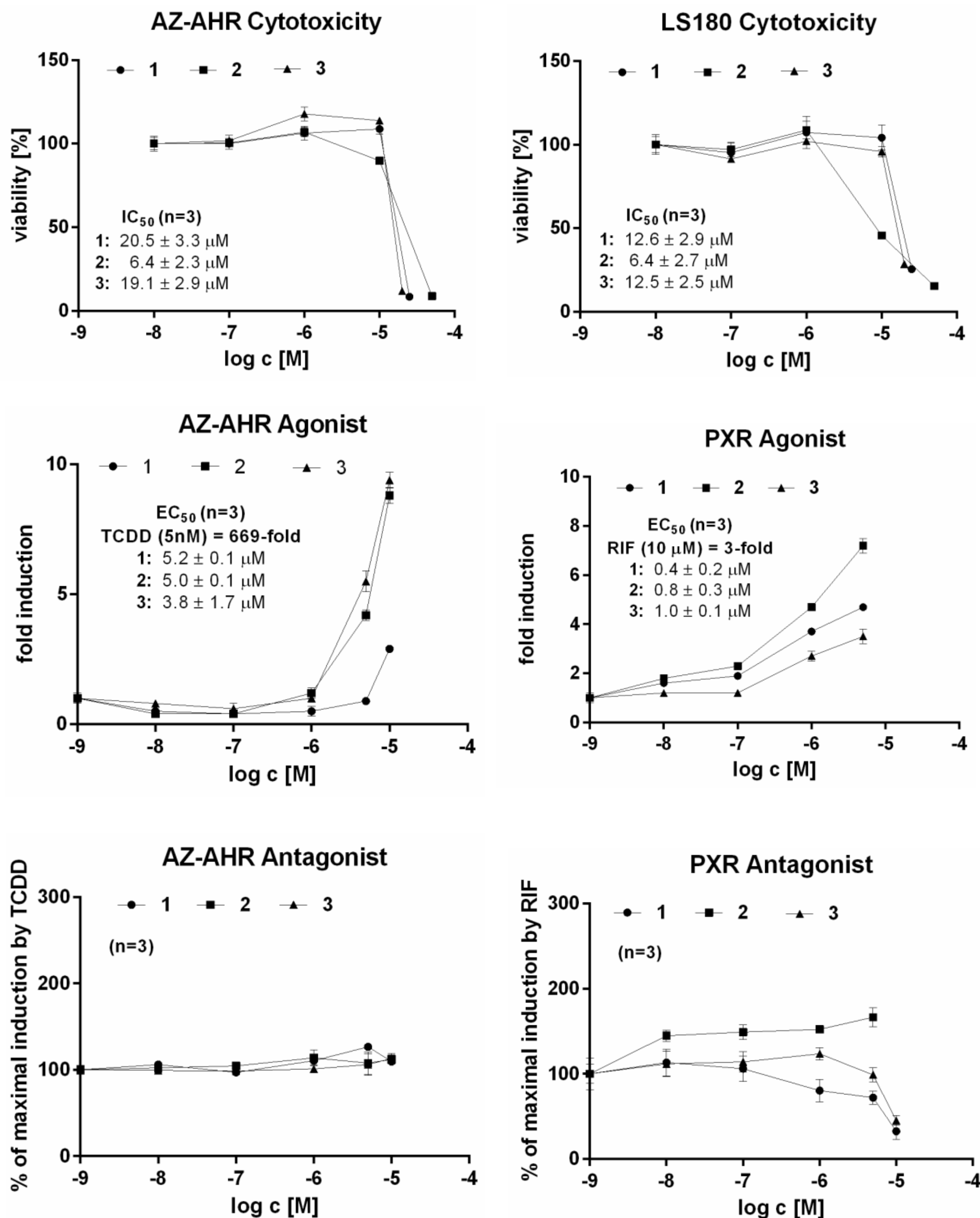


Figure 19. The effects of gold(I) complexes on the transcriptional activities of xenoreceptors. *The cells were stabilized (16 h) and then incubated (24 h) with the gold(I) complexes (**1-3**) at concentrations ranging from 1 nM to 50 µM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

Thyroid hormone receptor: Tested complexes were not cytotoxic if their concentraions were up to 10 μM in PZ-TR cells (Figure 20). The positive control T3 activated TR 3-fold as compared to DMF-treated cells. All complexes dose-dependently induced TR-mediated luciferase activity in the PZ-TR cell line (Figure 20). The calculated EC_{50} values were 3.4 ± 1.7 μM, 3.9 ± 1.7 μM, and 4.6 ± 0.8 μM for complexes **1, 2,** and **3**, respectively. The efficacies of all complexes were about 70-100% of that of 10 nM of T3 (Figure 20). Surprisingly, the combined treatment of PZ-TR cells with T3 revealed strong and dose-dependent additive effects on T3-inducible luciferase activity.

Vitamin D receptor: Tested complexes were not cytotoxic if their concentrations were up to 5 μM concentration in LS180 cells, as revealed by the MTT test (Figure 20). The positive control VD3 activated VDR 47-fold as compared to DMF-treated cells. In the *agonist mode*, all tested complexes dose-dependently decreased the basal activity of VDR, revealing their inverse agonist behaviour (Figure 20). In *antagonist mode*, complexes **1** and **3** slightly decreased VD3-stimulated luciferase activity, which suggested that they exhibited antagonist behaviour (Figure 20). Calculated IC_{50} values were 2.5 ± 0.1 μM and 2.7 ± 0.9 μM for complexes **1** and **3**, respectively. The inhibitory effect of complex **2** reflects its cytotoxic effects in LS180 cells.

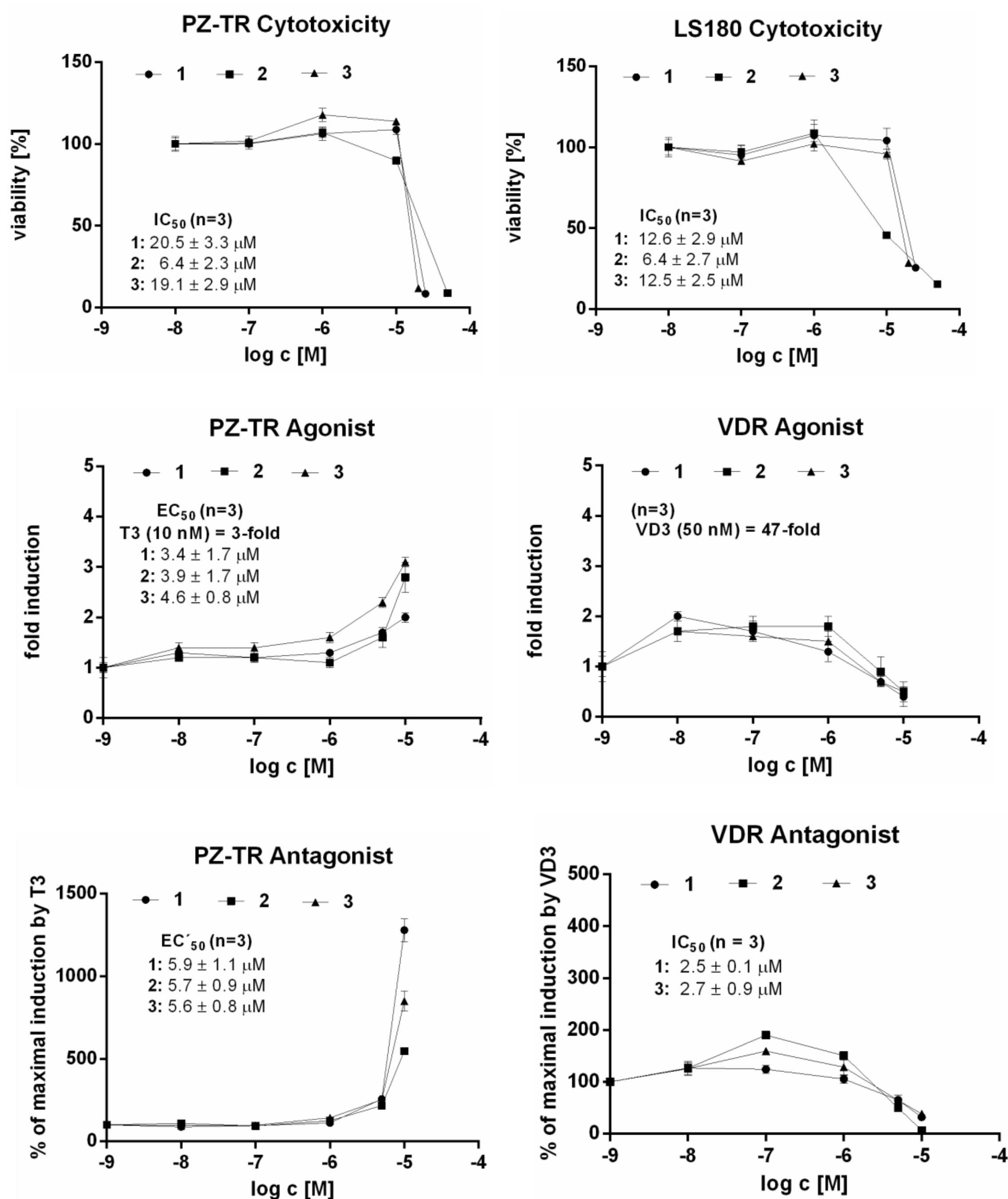


Figure 20. The effects of gold(I) complexes on the transcriptional activities of nuclear receptors. *The cells were stabilized (16 h) and then incubated (24 h) with the gold(I) complexes (**1-3**) at concentrations ranging from 1 nM to 50 μM, using 96-well culture plates. The vehicle used was DMF (0.1%, v/v). Upper panel: MTT assay - the data are expressed as the percentage of viability of control cells. Middle panel: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Lower panel: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of the maximal induction attained by model ligands. Representative graphs are shown. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. The IC_{50}/EC_{50} values were calculated where appropriate, and they are indicated in the plots.*

5.2.2 The effects of gold(I) complexes on the expression of *CYP1A1*, *CYP3A4,* and *SPOT14* mRNAs in LS180 and PZ-TR cell lines

Since tested compounds activated the expression of AhR, PXR, and TR, we evaluated the expression of their target genes, i.e., *CYP1A1*, *CYP3A4,* and *SPOT14*, respectively. TCDD strongly induced the expression of *CYP1A1* mRNA (1113-fold, 1300-fold and 1280 fold) in LS180 cells after 24 h of incubation. All tested Au(I) complexes significantly induced *CYP1A1* mRNA expression in three consecutive passages (Figure 21). Results are consistent with AhR activation by these compounds in reporter gene assays (Figure 19). RIF induced the expression of approximately 2.5-fold *CYP3A4* mRNA in LS180 cells after 24 h of incubation, but there was no *CYP3A4* mRNA induction by any tested complex (Figure 21), which is inconsistent with the data derived from reporter gene assays. T3 induced the expression of approximately 2.5-fold *SPOT14* mRNA in PZ-TR cells after 24 h of incubation. All tested complexes decreased the basal and T3-inducible *SPOT14* mRNA expression (Figure 21), which is opposite to the effects observed in gene reporter assays (Figure 19).

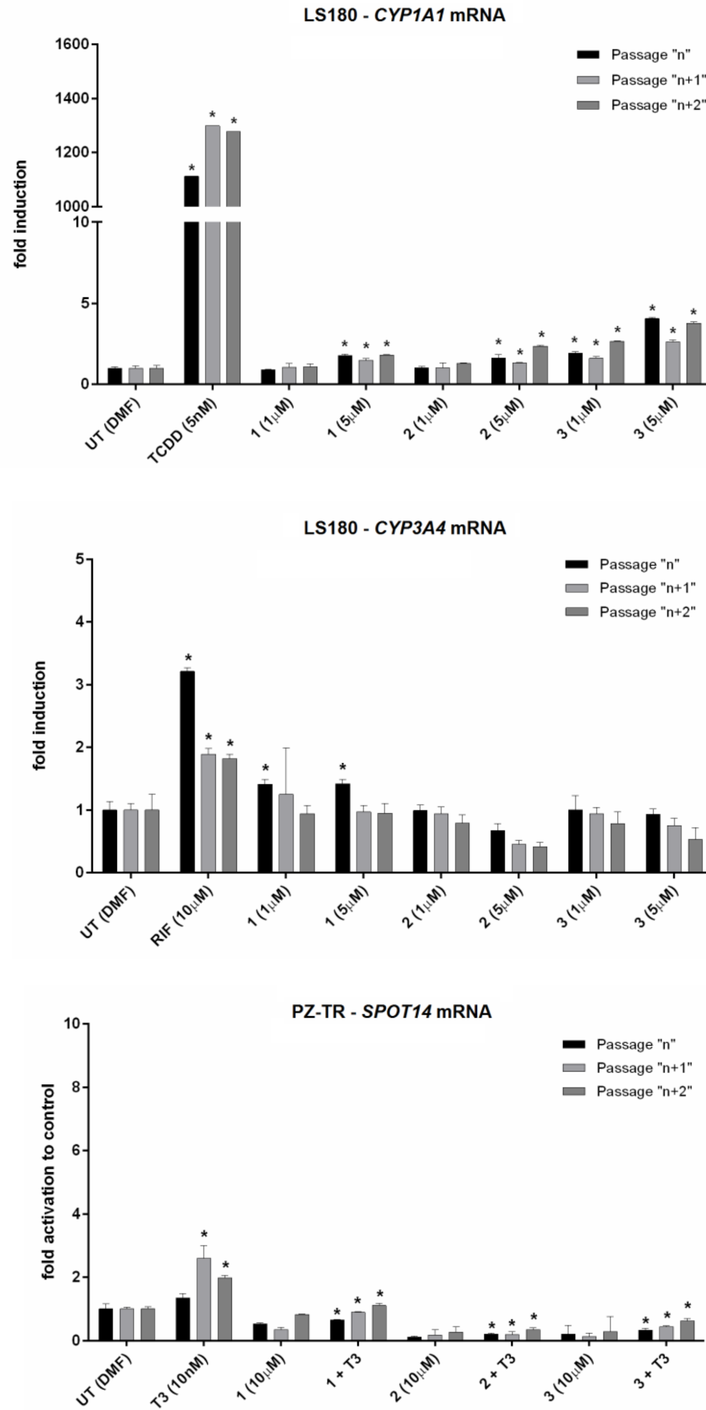


***Figure 21. The effects of gold(I) complexes on the expression of CYP1A1, CYP3A4, and SPOT14 mRNAs in LS180 and PZ-TR cells**. The cells were treated (24 h) with gold(I) complexes (**1**–**3**; 1 µM, 5µM, 10 µM), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 µM), 3,3´,5-triiodo-L-thyronine (T3; 10 nM), and the vehicle (DMF; 0.1%, v/v). Bar graphs show qRT-PCR analyses of CYP1A1, CYP3A4, and SPOT14 mRNAs. The data are presented as the mean ± SD from triplicate measurements, and are expressed as a fold induction over the DMF-treated cells. The data were normalized to GAPDH mRNA levels. * = values that are significantly different from those of DMF-treated cells ($p < 0.05$).*

5.2.3 The effects of gold(I) complexes on the expression of drug-metabolizing CYPs, *TAT,* and *SPOT14* in primary human hepatocytes

In the last series of experiments, we tested the effects of the gold(I) complexes on the expression of selected target genes for steroid receptors, xenoreceptors, and nuclear receptors in primary cultures of human hepatocytes. In the panel of CYP450 drug-metabolizing genes, we evaluated the expression of *CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4* mRNAs. We also measured the expression of GR and TR-target genes *TAT* and *SPOT*, respectively. We incubated primary cultures of human hepatocytes from five donors (HH61, HH64, HH65, HH66, Hep220879) for 24 h (mRNA analyses) and 48 h (protein analyses) with the gold(I) complexes (**1**-**3**; 1 µM; 10 µM), and model inducers, including dioxin (TCDD; 5 nM), rifampicin (RIF; 10 µM); 3,3′,5-triiodo-L-thyronine (T3; 2 nM or 10 nM), and vehicle (UT; 0.1% DMF v/v). TCDD strongly induced the expression of *CYP1A1* and *CYP1A2* mRNAs and proteins in five human hepatocytes cultures. Complexes **1, 2,** and **3** increased the levels of *CYP1A1* and *CYP1A2* mRNAs (Figure 22) and proteins (Figure 23), which is consistent with data regarding *CYP1A1* mRNA induction in LS180 cells and gene reporter assays in AZ-AHR cells. RIF (10 µM) induced the expression of *CYP2A6*, *CYP2B6*, *CYP2C9* and *CYP3A4* mRNAs in all human hepatocyte cultures. Tested compounds weakly altered *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4* mRNAs (Figure 22), and this is consistent with PXR activation in gene reporter assays. The effects of the gold(I) complexes on the expression of the target genes were varied between individual hepatocyte cultures, probably due to the inter-individual variability between the donors (Figure 22). No tested compound showed a significant influence on the expression of CYP3A4 protein (Figure 23).

Complexes **1**, **2,** and **3** dose-dependently decreased the activity of *TAT* mRNA (Figure 22), which is consistent with the downregulation of the transcriptional activity of GR in gene reporter assays (Figure 18). The expression of TR-regulated *SPOT14* mRNA was dose dependently decreased by all tested complexes, which further confirms the data derived from PZ-TR cells (Figure 22).

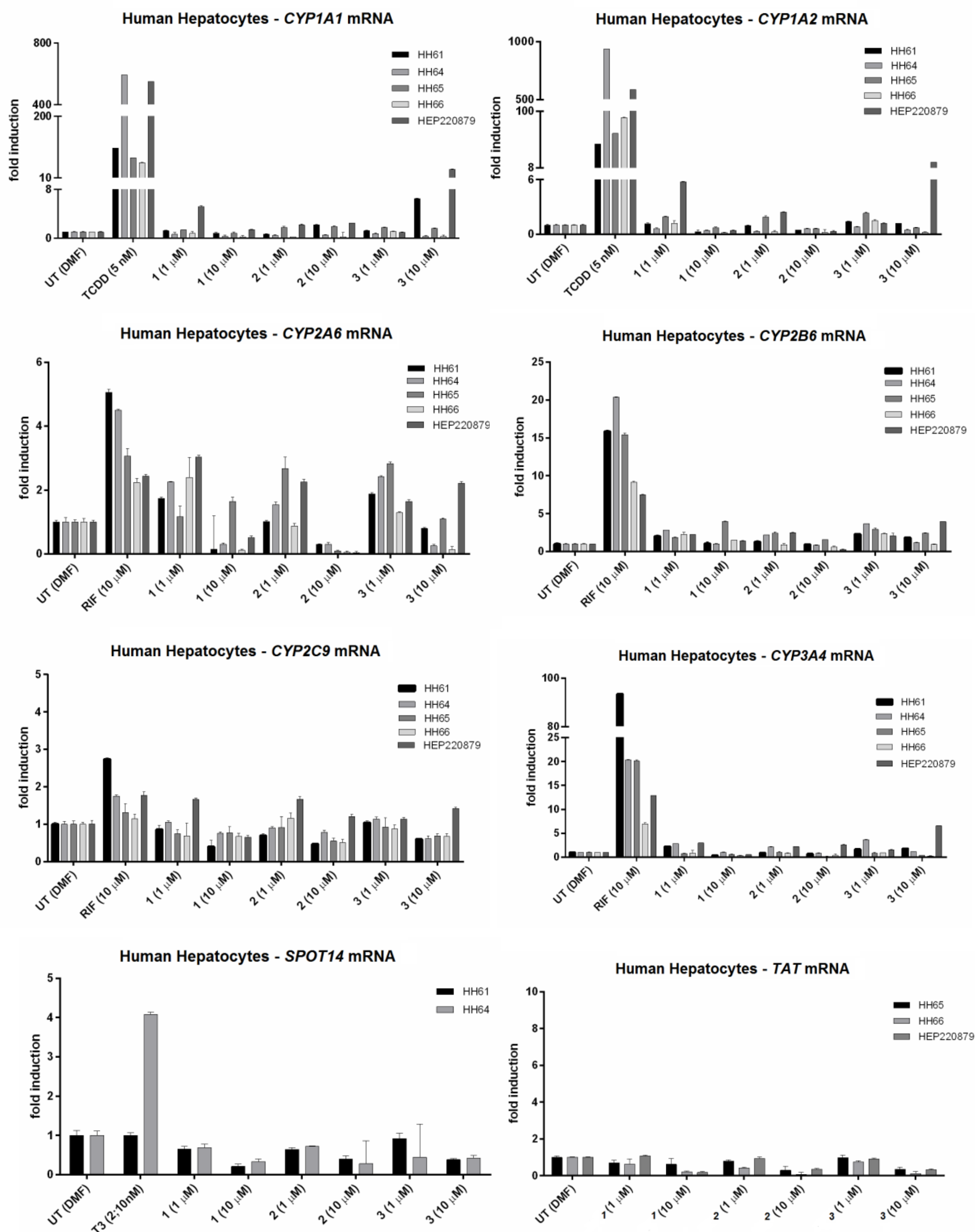
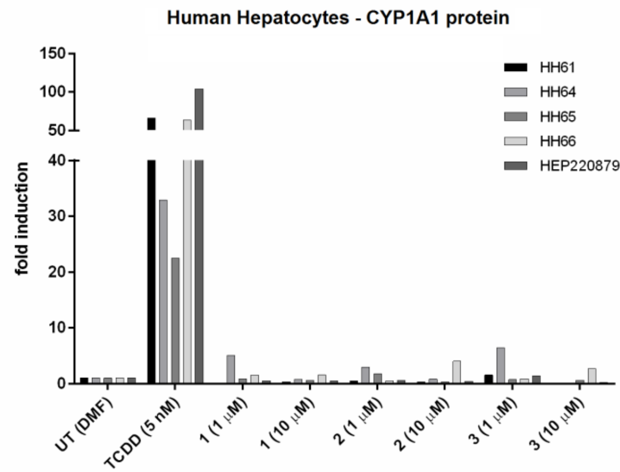
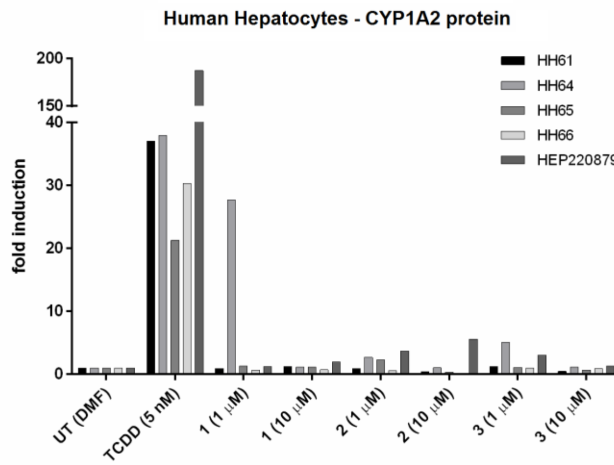


Figure 22. The effects of gold(I) complexes on the expression of drug-metabolizing CYPs, TAT, and SPOT14 mRNAs in primary human hepatocytes*. Human hepatocytes from five different donors (HH61, HH64, HH65, HH66, Hep220879) were treated (24 h) with gold(I) complexes (**1**–**3**; 1 μM and 10 μM), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), and the vehicle (DMF; 0.1%, v/v).Bar graphs show the results of the qRT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, TAT, and SPOT14 mRNAs. The data are presented as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. The data were normalized to GAPDH mRNA levels.*





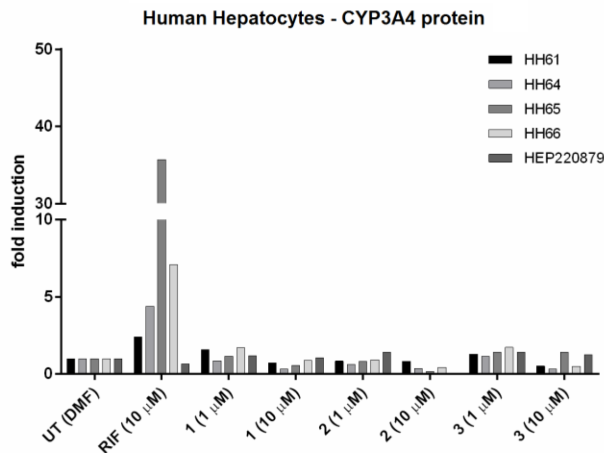


Figure 23. Effects of gold(I) complexes on the protein expression of drug-metabolizing CYPs in primary human hepatocytes. *Human hepatocytes from five different donors (HH61, HH64, HH65, HH66, Hep220879) were treated (48 h) with gold(I) complexes (1 µM, 10 µM), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 µM), and vehicle (DMF; 0.1%, v/v). Bar graphs show the results of CYP1A1 and CYP1A2 protein analyses by using Simple Western SallySue. The data are presented as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. The data were normalized to β-actin levels.*

6 DISCUSSION

In the present study, I investigated the effects of copper(II) and gold(I) mixed-ligand complexes on the activity of selected steroid receptors, xenoreceptors, and nuclear receptors, and the expression of their target genes. Two *in vitro* cellular models, i.e., primary human hepatocyte cultures and human cancer cell lines were used. For our biological studies, we have employed modern experimental methods such as the gene reporter assay, real-time quantitative reverse transcription polymerase chain reaction, western blotting, and electrophoretic mobility shift assay.

The aim of the first section of the study was to investigate whether quinoline-derived copper(II) complexes interacted with signalling pathways of the selected receptors, and whether these compounds had an impact on the expression of their target genes. I demonstrated that copper(II) complexes **1** and **2** significantly stimulated the AhR activity in the transgenic AZ-AHR cell line and accordingly induced the expression of the following AhR-dependent genes: *CYP1A1* mRNA in LS180 cells and *CYP1A1*/*CYP1A2* mRNAs in human hepatocytes (Kubesova et al., 2016). We found a slight inconsistency in case of complex **3**, which induced *CYP1A1* mRNA in LS180 cells, but not in human hepatocytes. Complex **4** did not stimulate AhR activity, and thus did not induce the expression of CYP1A1 and CYP1A2 in primary human hepatocytes and human cancer cell lines. This might be attributable to the fact that the human hepatocytes, unlike LS180 cells, are fully equipped with detoxification enzymes that might have caused the metabolic inactivation of complexes **3** and **4** (Li, 2007). The reversal of the induction of expression and AhR activation by resveratrol (AhR antagonist) revealed the involvement of AhR in the process. In addition, the complexes **1** and **2** induced the formation of the AhR-DNA complex, which is also a proof regarding the molecular activity of AhR. In general, the activation of AhR by foreign compounds is considered undesirable due to the involvement of AhR in a variety of cell functions, such as intermediary metabolism, cell differentiation, immune response, and/or chemically induced carcinogenesis (Go et al., 2015; Stejskalova and Pavek, 2011). After taking the values of EC_{50} for copper(II) complexes from gene reporter assays (~150 nM) into account, we might consider complexes **1** and **2** to have a medium to high level of potency, as compared to that of TCDD. On the other hand, the magnitude of AhR activation (efficacy) by complexes **1** and **2** was only about 6% of that by TCDD. The fold inductions of *CYP1A1* and *CYP1A2* mRNA expression by complexes **1** and **2** in human hepatocytes ranged from 2% to 7% of those by TCDD, implying that their low potential to cause AhR-based DDIs. Overall, transcriptional

activity of AhR in gene reporter assays, the expression of *CYP1A1* mRNA in LS180 cells, and the expression of CYP1A1/CYP1A2 in human hepatocytes were increased by copper(II) complexes in the following (descending) order: **1** > **2** > **3** > **4**. The difference between the effects of individual complexes on AhR-mediated activity and CYP1A induction might be explained by the different structures of copper(II) complexes, in particular, the type, size, and position of substituents (Buchtik et al., 2011; Sui et al., 1998). The active complexes **1** and **2** contained either unsubstituted or 5-methylated (small molecule) 1,10-phenanthroline, respectively, in their structure. The inactive complex **3** was 4,7-diphenyl substituted, and two large aromatic rings might account for the loss of AhR activation by the complex. While inactive complex **4** contained unsubstituted 1,10-phenanthroline in its molecule, it also contained large substituents at positions 2 and 7 in the quinoline-based part of the molecule. In the past, chelating 1,10-phenanthrolines were described as partial agonists or competitive antagonists of rat AhR (Mahon and Gasiewicz, 1992), which was consistent with our findings. Unlike for AhR, we found that tested complexes did not influence the basal and ligand-inducible transcriptional activities of AR, GR, TR, PXR, and VDR receptors. Moreover, the major drug-metabolizing CYPs in human hepatocytes, including CYP2B6, CYP2C9, and CYP3A4 were not induced. Finally, the quinoline-derived compounds are clinically used in the pharmacotherapy against a number of diseases (Andriole, 2005; Barlow, 1963; Sato et al., 1995), and they act as potent inhibitors of DNA-gyrases (Pommier et al., 2010). Therefore, the present study was highly relevant and timely. The data obtained favoured the potential use of the tested compounds in human pharmacotherapy, with regard to their (no) interactions with signalling pathways via steroid receptors, nuclear receptors, and xenoreceptors, and induction of xenobiotic-metabolizing enzymes.

In the second section of the present study, I have investigated the effects of gold(I) phosphine complexes on the transcriptional activities of selected nuclear receptors, steroid receptors and xenoreceptors. I found that the tested complexes had differential effects on the transcriptional activities of the receptors and the expression of their target genes (Kubesova et al., 2016). I demonstrated that the gold(I) complexes **1**, **2** and **3** slightly and dose-dependently activated PXR and AhR, and accordingly, they moderately induced the expression of the CYP3A4 and CYP1A1/CYP1A2 genes in human hepatocytes and LS180 cells, respectively. All tested gold(I) compounds weakly induced the expression of *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4* mRNAs in primary human hepatocytes. In contrast, there was no induction of *CYP3A4* mRNA

expression in LS180 cells by gold(I) complexes. This inconsistency might reveal the cell type-specific and tissue-specific regulation of CYP3A4 (Pavek et al., 2010). All tested gold(I) complexes displayed a dose-dependent and differential inhibitory activity against GR, AR, and VDR in gene reporter assays. The transcriptional activity of GR declined in the presence of GR ligand dexamethasone, which indicates its antagonistic behaviour. The decline of the basal transcriptional activities of AR and VDR is suggestive of their inverse agonist behaviour. The anti-glucocorticoid effects of the gold(I) complexes (**1**-**3**) were confirmed in human hepatocytes, where these compounds down-regulated the GR-target gene *TAT*. Because the compounds were not cytotoxic, it is assumed that they exhibited antagonist effects against GR. A significant activation of expression of TR was observed by all gold(I) complexes; however it had a very low potency, as compared to that of T3. The magnitude of TR activation (efficacy) by these complexes was about 70-100% of the induction attained by T3. All gold(I) complexes decreased both basal and T3-inducible *SPOT14* mRNA expression levels, which is in contrast to their effects on TR transcriptional activity in gene reporter assays. This might be attributable to the complex process of the transcriptional regulation of *SPOT14*, which could involve other regulators besides TR. Indeed, the hepatospecific regulation of *SPOT14* by CAR and PXR was demonstrated (Breuker et al., 2010). Since the use of drug inverse agonists (Khilnani and Khilnani, 2011) and antagonists is of therapeutic potential and value, the data obtained here might pave the way for targeted drug research in the future (Leurs et al., 2002).

Overall, our results conclusively prove that gold(I) and copper(II) complexes differentially and selectively influence the activity of selected human nuclear receptors, steroid receptors, and xenoreceptors, which might have consequences for their potential therapeutic use with regard to DDIs, endocrine disruption or perturbation of intermediary metabolism.

7 CONCLUSION

The aim of this study was to determine the effects of copper(II) and gold(I) mixed-ligand complexes on the expression of selected drug-metabolizing enzymes, and on the transcriptional activities of steroid receptors (AR, GR), xenoreceptors (AhR, PXR), and nuclear receptors (TR, VDR). It was demonstrated that:

(i) Copper(II) complexes (**1**, **2**) activate AhR and induce AhR-dependent genes in human hepatocytes and cancer cell lines.

(ii) Gold(I) complexes activate AhR and PXR, and induce their target genes.

(iii) Gold(I) complexes exhibit anti-androgen and anti-glucocorticoid activities.

The data presented in this thesis regarding the multiple roles of AhR and PXR in human physiology and pathophysiology might be clinically significant.

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9 CURRICULUM VITAE

Personal data

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Education

Present study (2013-2018):

Postgraduate studies in Molecular and Cell Biology in the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc.

Thesis topic: *In vitro biological activity of selected complexes of transient metals* (Supervisor: prof. RNDr. ZdeněkDvořák, DrSc. *et.* Ph.D.)

Completed study (2008-2010):

Master's program in Biochemistry, Palacký University, Olomouc.

Thesis topic: *The effect of clinically used benzodiazepines on transcriptional activity of xenoreceptors in human cancer cell lines*

Completed study (2005-2008):

Bachelor's program in Applied Chemistry, Palacký University, Olomouc.

Thesis topic: *Stability of Selected Structural Elements of Non-globular Protein p18(INK4c)*

Educational activities

Practical courses in cell biology II.

Practical courses in general genetics.

Research internships

May 2014

Presentation of results in English at the operational program education for competitiveness (CZ.1.07/2.3.00/30.0030) – Brno, Czech Republic - Nuclear receptors and PAS proteins in regulation of xenobiotic-metabolizing enzymes and cell functions (1 day)

February 2014

IT-LIVER Workshop: 3-Dimensional cell cultures and drug discovery - Vienna, Austria (3 days)

Research fellowship

The research fellowship was not undertaken, because of serious health complications. (3 months)

Participation in projects (as a researcher)

1. NPU I (LO1305), *Developing Centre of Advanced Technologies and Materials* (2014 – 2019)
2. CENTER OF EXCELLENCE, P303/12/G163, *Centre of Drug-Dietary Supplements Interactions and Nutrigenetics. Czech Scientific Foundation* (2012 – 2018)
3. EU ESF OP VK, CZ.1.07/2.2.00/28.0066, *Development and internationalization of chemical and biological study programs at Palacký University in Olomouc* (2012-2015)
4. FRUP 2014_2_063, *Completion of the laboratory of biological activity of coordination compounds* (2014)
5. EU ESF OP VK, CZ.1.07/2.3.00/20.0062, *Antabuse (disulfiram) as a pilot case of non-profit drug* (2011 - 2014)
6. EU EFPRR OP VaVpI, CZ.1.05/2.1.00/03.0058,*Regional Centre of Advanced Technologies and Materials* (2010 – 2014)
7. Student projects at Palacký University
    - PrF_2013_002, *Influence of artificial sweeteners on signalling AhR-CYP1A1 pathway in human cells* (2013)
    - PrF_2014_004, *Interactions between chiral drugs and cellular signalling paths* (2014)
    - PrF_2015_003, *Stereoselective effects of xenobiotics on metabolic drug signalling pathways* (2015)
    - PrF_2016_003, *Effect of enantiomers (itraconazole antimycotics) on the expression of cytochromes P450 metabolising xenobiotics* (2016)
    - PrF_2017_004, *mono-, di-, tri-methylated indole derivatives as selective and dual activators of human PXR and AhR* (2017)

Publications

Vrzal R. , **Kubesova K.**, Pavek P. , Dvorak Z. (2010). Benzodiazepines medazepam and midazolam are activators of pregnane X receptor and weak inducers of CYP3A4: Investigation in primary cultures of human hepatocytes and hepatocarcinoma cell lines. *Toxicol Lett 193*, 183 - 188**[IF_{2010} - 3.581]**

Kubesova K., Travnicek Z., Dvorak Z. (2016). Pleiotropic effects of gold(I) mixed-ligand complexes of 9-deazahypoxanthine on transcriptional activity of receptors for steroid hormones, nuclear receptors and xenoreceptors in human hepatocytes and cell lines. *Eur J Med Chem 121,* 530-540**[IF_{2016} - 4.519]**

Kubesova K., Vavrova (Doricakova) A., Travnicek Z., Dvorak Z. (2016). Mixed-ligand copper(II) complexes activate aryl hydrocarbon receptor AhR and induce CYP1A genes expression in human hepatocytes and human cell lines. *Toxicol Lett 255*, 24 - 35**[IF_{2016} - 3.858]**

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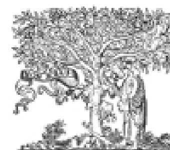
2. **Kubesova K.**, Travnicek Z., Dvorak Z.: In vitro cytotoxic activities of copper(II) and gold(I) complexes in human cancer cell lines. Interdisciplinary Toxicology Conference - TOXCON 2014, Stará Lesná, Slovakia, 23.9. – 26.9.2014. *Interdisciplinary Toxicology (*2014), **Vol.7 (Suppl. 1)** S56-S57.
3. **Kubesova K.**, Travnicek Z., Dvorak Z.: Effects of cytotoxic gold (I) complexes on transcriptional activity of various nuclear receptors. 13th European International Society for the Study of Xenobiotics - ISSX Meeting, Glasgow, Scotland, UK, 22.6. – 25.6.2015. (2015) **1 (Suppl 10)**:S110-S11, P143.
4. **Kubesova K.**, Travnicek Z., Dvorak Z.: Profiling of gold(I) complexes towards receptors for steroid hormones, nuclear receptors and xenoreceptors in human hepatocytes and cell lines. 13th European Biological Inorganic Chemistry Conference – EuroBIC 13, Budapest, Hungary, 28.8.2016 – 1.9.2016. *J Biol Inorg Chem* (2016), P187.



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APPENDIX I.

Kubesova K., Doricakova A., Travnicek Z., Dvorak Z. (2016). Mixed-ligand copper(II) complexes activate aryl hydrocarbon receptor AhR and induce CYP1A genes expression in human hepatocytes and human cell lines. *Toxicol Lett 255*, 24 – 35. **[IF_{2016} - 3.858]**

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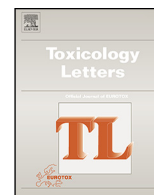

Mixed-ligand copper(II) complexes activate aryl hydrocarbon receptor AhR and induce CYP1A genes expression in human hepatocytes and human cell lines

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HIGHLIGHTS

- Mixed-ligand copper II complexes were studied.
- Transcriptional activity of TR, VDR, AR, GR, AhR and PXR receptors was measured.
- Target genes in primary human hepatocytes and cancer cell lines were determined.
- Two tested compounds activated AhR and induced CYP1A1 and CYP1A2.

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ABSTRACT

The effects of four copper(II) mixed-ligand complexes [Cu(qui1)(L)]$NO_3 \cdot H_2O$ (**1**–**3**) and [Cu(qui2)(phen)] NO_3 (**4**), where qui1 = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone, Hqui2 = 2-(4-amino-3,5-dichlorophenyl)-*N*-propyl-3-hydroxy-4(1*H*)-quinolinone-7-carboxamide, L = 1,10-phenanthroline (phen) (**1**), 5-methyl-1,10-phenanthroline (mphen) (**2**), bathophenanthroline (bphen) (**3**), on transcriptional activities of steroid receptors, nuclear receptors and xenoreceptors have been studied. The complexes (**1**–**4**) did not influence basal or ligand-inducible activities of glucocorticoid receptor, androgen receptor, thyroid receptor, pregnane X receptor and vitamin D receptor, as revealed by gene reporter assays. The complexes **1** and **2** dose-dependently induced luciferase activity in stable gene reporter AZ-AhR cell line, and this induction was reverted by resveratrol, indicating involvement of aryl hydrocarbon receptor (AhR) in the process. The complexes **1**, **2** and **3** induced CYP1A1 mRNA in LS180 cells and CYP1A1/CYP1A2 in human hepatocytes through AhR. Electrophoretic mobility shift assay EMSA showed that the complexes **1** and **2** transformed AhR in its DNA-binding form. Collectively, we demonstrate that the complexes **1** and **2** activate AhR and induce AhR-dependent genes in human hepatocytes and cancer cell lines. In conclusion, the data presented here might be of toxicological importance, regarding the multiple roles of AhR in human physiology and pathophysiology.

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1. Introduction

The use of metal-containing compounds for medicinal purposes dates back to ancient times. For instance, mercurous chloride (Hg_2Cl_2) and Arsphenamine (also known as Salvarsan or compound 606) were used for their diuretic, and antisyphilitic activities, respectively, and moreover, the latter was also used as the first modern compound in chemotherapy. An époque-making event was the introduction of the anti-cancer drug cisplatin to human chemotherapy, which was followed by other platinum-containing drugs such as oxaliplatin and carboplatin. The common

Abbreviations: AhR, aryl hydrocarbon receptor; AR, androgen receptor; CYP, cytochrome P450; DEX, dexamethasone; DHT, dihydrotestosterone; DMF, *N,N* dimethylformamide; GR, glucocorticoid receptor; PXR, pregnane X receptor; RIF, rifampicin; RVT, resveratrol; T3, 3,3′,5-triiodo-L-thyronine; RXRs, retinoid X receptors; TAT, tyrosine aminotransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TR, thyroid hormone receptor; VD3, 25-hydroxyvitamin D3; VDR, vitamin D receptor.

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drawbacks of these anticancer drugs are their adverse and undesirable effects such as nephrotoxicity, myelosuppression and intrinsic and acquired drug-resistance. For that reason, the search for alternative metallodrugs which could suppress the above-mentioned negative effects of currently used platinum-based chemotherapeutics represents one of the challenges for bioinorganic chemists at present. Currently, a plethora of metal-based drugs were approved for human medicinal and diagnostic purposes (Dabrowiak, 2009; Gaynor and Griffith, 2012). Among them, copper(II) mixed-ligand complexes involving the amino acids and 1,10-phenanthroline-based ligands, known as Casiopeinas®, received much more attention than the others due to their remarkable cytotoxicity (Becco et al., 2014; Serment-Guerrero et al., 2011; Valencia-Cruz et al., 2013). Therefore, we have previously focused on the study of Casiopeinas®-like complexes, containing *N*-donor heterocyclic ligands, such as 1,10-phenanthroline (phen) or 2,2′-bipyridine (bpy) or their derivatives abbreviated as N-N, and 2-phenyl-3-hydroxy-4(1*H*)-quinolinone and its derivatives (H*qui*) of the general composition [Cu(N-N)(*qui*)]X·*y*H_2O (where X = NO_3^- or BF_4^-) (Buchtik et al., 2012; Buchtik et al., 2011; Krikavova et al., 2016; Travnicek et al., 2012). The complexes were identified as promising cytotoxic agents on a broad spectrum of human cancer cell lines showing IC_{50} values in the micromolar and sub-micromolar levels.

Many cell functions, including proliferation, differentiation, immune response, energy production and storage, detoxification or apoptosis are transcriptionally regulated by nuclear receptors, receptors for steroid hormones and xenoreceptors. Nuclear and steroid receptors are key regulators of physiological and endocrine functions, while xenoreceptors control the expression of detoxification genes. Mutual and multiple cross-talks exist between xenoreceptors and receptors for steroid hormones and nuclear receptors (Pascussi et al., 2008). Indeed, xenoreceptors aryl hydrocarbon receptor (AhR) (Diani-Moore et al., 2013; Tanos et al., 2012) and pregnane X receptor (PXR) (Moreau et al., 2008) are involved in regulation of glucose and lipid homeostasis. On the other hand, nuclear receptors and steroid receptors including vitamin D receptor (VDR) (Drocourt et al., 2002), glucocorticoid receptor (GR) (Dvorak and Pavek, 2010), androgen receptor (AR), estrogen receptor (ER) (Monostory and Dvorak, 2011), thyroid receptor (TR) and retinoic X receptor (RXR) (Pascussi et al., 2003) are important transcriptional regulators of detoxification enzymes. The interactions between drugs and ligand-activated intracellular receptors may have several implications relevant for human health, including phenomenon of endocrine disruption (Heindel et al., 2015), drug–drug interactions (Chen et al., 2014) and food-drug interactions (Margina et al., 2015).

In the current paper, we examined the effects of the complexes **1**–**4** on the transcriptional activities of AhR, AR, GR, TR, PXR and VDR, employing gene reporter assays. We found that complexes **1** and **2** activate AhR but do not influence transcriptional activities of receptors AR, GR, TR, PXR and VDR. Additionally, complexes **1** and **2** transformed AhR in its DNA-binding form and they induced AhR-dependent genes CYP1A1 and CYP1A2 in LS180 cells and in human hepatocytes. We concluded that the data presented here might be of toxicological importance, regarding the multiple roles of AhR in human physiology and pathophysiology.

2. Materials and methods

2.1. Compounds and reagents

N,*N*-Dimethylformamide (DMF), hygromycin B, dexamethasone (DEX), rifampicin (RIF), dihydrotestosterone (DHT), 3,3′,5-triiodo-L-thyronine (T3) and resveratrol (RVT) were purchased from Sigma–Aldrich (Prague, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island, USA). 25-hydroxyvitamin D3 (VD3) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The copper(II) complexes (**1**–**4**) with general compositions [Cu(qui1)(L)]NO_3·H_2O (**1**–**3**) and [Cu(qui2)(phen)]NO_3 (**4**) were synthesized and characterized as previously described in the literature (Buchtik et al., 2012; Buchtik et al., 2011; Travnicek et al., 2012) (Fig. 1). Luciferase lysis buffer and FuGENE® HD Transfection Reagent were obtained from Promega (Madison, WI, CA). Oligonucleotide primers used in RT-PCR reactions were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). LightCycler 480 Probes Master and 480 SYBR Green I Master kit were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

2.2. Primary cultures of human hepatocytes

Human hepatocytes were isolated from human liver obtained from the multiorgan donors HH61 (M; 64 years), HH64 (M; 73 years) and HH65 (M; 34 years). Tissue acquisition protocol and the use of liver cells was approved by "Ethical committee at the Faculty Hospital Olomouc", and it was in accordance with Transplantation Act #285/2002 Coll. Cells were plated on collagen-coated dishes in hormonally and chemically defined medium (based on Ham's F12 medium and William's Medium E) as previously described (Pichard-Garcia et al., 2002) and stabilized for 24 h before the treatment. Hepatocytes were incubated in a serum-free medium for 24 h (for mRNA analyses) and 48 h (for protein analyses) with vehicle (UT; 0.1% DMF v/v), TCDD (5 nM), RIF (10 μM) and the tested complexes. Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. Human cancer cell lines

Human Caucasian colon adenocarcinoma cell line LS180 (ECACC No. 87021202) and human Caucasian breast adenocarcinoma cell line MCF7 (ECACC No. 86012803) were purchased from European Collection of Cell Cultures (ECACC). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Stably transfected gene reporter cell lines AZ-AHR (Novotna et al., 2011), AZ-GR (Novotna et al., 2012), AIZ-AR (Bartonkova et al., 2015) and PZ-TR (Illes et al., 2015) were described elsewhere. Cells were cultured at 37 °C and 5% CO_2 in a humidified incubator.

2.4. MTT cell viability assay

Cell lines AIZ-AR, AZ-AHR, AZ-GR, PZ-TR and LS180 were treated for 24 h with tested compounds (**1**–**4**) in concentrations ranging from 1 nM to 50 μM (unless the solubility was lower), using multi-well culture plates of 96 wells. In parallel, the cells were treated with vehicle (UT; 0.1% v/v DMF) and Triton X-100 (1%, v/v) to assess the minimal and maximal cell damage, respectively. MTT assay was performed and absorbance was measured spectrophotometrically at 570 nm on Infinite M200 (Schoeller Instruments, Prague, Czech Republic). The data were expressed as the percentage of cell viability, where 100% and 0% represent the treatments with negative control (DMF) and positive control (Triton X-100), respectively. Half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism 6 software (GraphPad Software, San Diego, USA). The tested concentrations causing the decline in viability no greater than 20% were considered as non-toxic for further experiments.

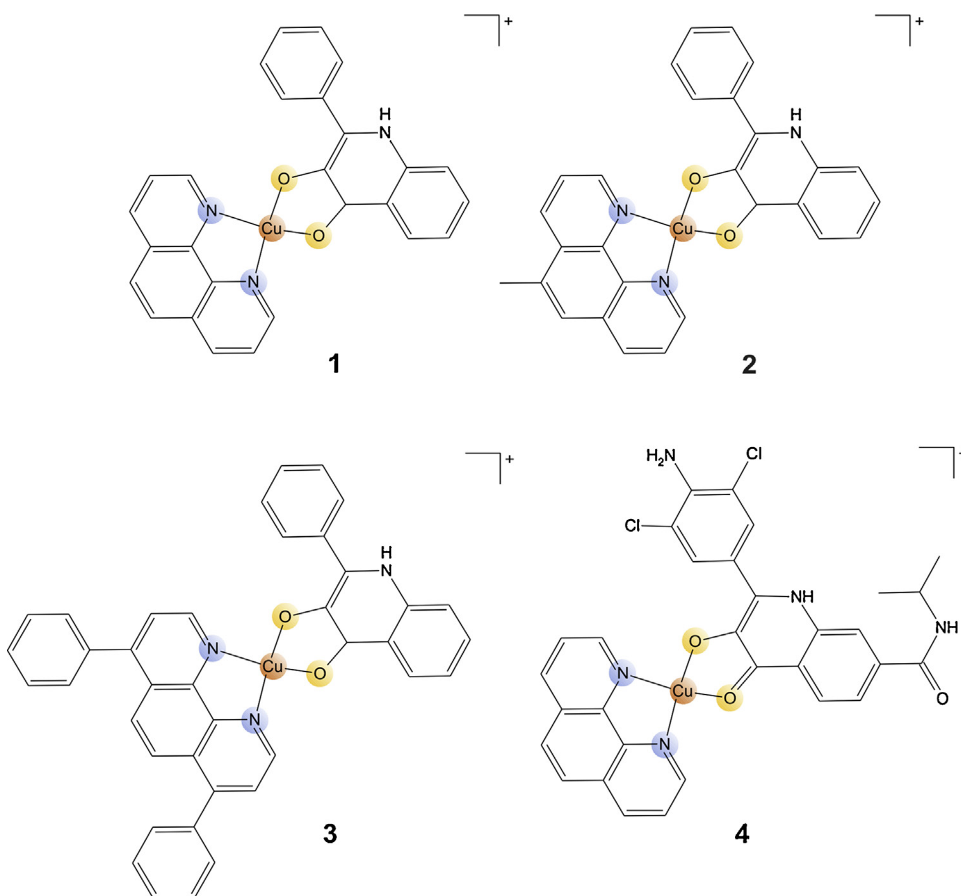


Fig. 1. Structural formulas of the studied copper(II) mixed-ligand complexes.

2.5. Gene reporter assays

Stably transfected gene reporter cell lines AZ-AHR (Novotna et al., 2011), AZ-GR (Novotna et al., 2012), AIZ-AR (Bartonkova et al., 2015) and PZ-TR (Illes et al., 2015) were used for examination of transcriptional activities of aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), androgen receptor (AR) and thyroid hormone receptor (TR), respectively. Transiently transfected LS180 cells were used for assessment of transcriptional activities of pregnane X receptor (PXR) and vitamin D receptor (VDR), as described elsewhere (Vrzal et al., 2011). The cells were stabilized for 16 h and then incubated for 24 h with tested compounds (**1**–**4**), TCDD (5 nM), DEX (100 nM), DHT (100 nM), T3 (10 nM), RIF (10 μM), VD3 (50 nM) and/or vehicle (UT; 0.1% DMF v/v). Thereafter, the cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI, USA) and the luciferase activity was measured spectrophotometrically on Tecan Infinite M200 plate luminometer (Schoeller Instruments, Prague, Czech Republic). Calculations of half-maximal effective concentrations EC_{50} and/or half-maximal inhibitory concentrations IC_{50} were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

2.6. Quantitative reverse transcriptase PCR (qRT-PCR)

The total RNA was isolated using TRI Reagent® (Molecular Research Center, Ohio, USA). cDNA was synthesized from 1000 ng of total RNA using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, Massachusetts, USA) and random hexamers (New England Biolabs). qRT-PCR was carried out on Light Cycler 480 II instrument (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, TAT and GAPDH mRNAs were determined using Universal Probes Library (UPL; Roche Diagnostic Corporation, Prague, Czech Republic) and probes described elsewhere (Vrzal et al., 2013). The measurements were performed in triplicates. The gene expression levels were normalized per *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene. Data were processed by delta–delta method.

2.7. Simple western blotting by Sally Sue™

The total protein extracts were obtained as described previously (Korhonova et al., 2015). Reagents used for simple western by *Sally Sue*™ were purchased from ProteinSimple (San Jose, California) and samples were prepared according to manufacturer's recommendations (www.proteinsimple.com). CYP1A1 (goat polyclonal, sc-9828, G-18) and CYP1A2 (mouse monoclonal, sc-53614, 3B8C1) primary antibodies, and rabbit anti-goat secondary antibody (sc-2768, H0712) were purchased from Santa Cruz Biotechnology Inc. β-actin (mouse monoclonal; 3700S, 8H10D10) primary antibody was from Cell Signalling Technology (Denvers, Massachusetts, USA). Target proteins were identified using primary antibodies and immune-probed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the signals were normalized to β-actin as a loading control.

2.8. Electrophoretic mobility shift assay (EMSA)

MCF-7 cells at 100% confluence were incubated for 2 h with vehicle as a negative control (UT; 0.1% DMF v/v), TCDD (5 nM) and studied complexes (**1**–**4**) at 1 μM. Nuclear fractions were isolated using Nuclear extract kit (Active Motif) according to manufacturer's protocol. The following double-stranded 5′-biotinylated oligonucleotides containing specific DNA-binding sequence for AhR corresponding to the 27-bp protein binding site of DRE 3 were used. Gel mobility shift assay was performed as follows: nuclear protein (15 μg) from each sample was pre-incubated in binding buffer (final: 10 mM Tris, 50 mM KCl, 1 mM DTT; pH 7.5) along with final concentrations of 2,5% glycerol, 0,05% NP-40, ddH_2O and nonspecific competitor Poly (dl.dC) before adding the biotin-labeled probe (10 fmol/μL). Unlabeled probe (2 pmol/μL) was added to reaction mixture for competitive experiment. The complete binding reaction was incubated at room temperature for 20 min. Finally 5 μL of 5 × loading buffer with bromophenol blue was added before loading in the non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein-DNA complexes were electro-blotted to positively charged nylon membrane. Thereafter, transferred DNA was cross-linked using a UV-light cross-linker instrument. Biotin-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in Light- Shift Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA). The sequences of DRE oligonucleotides were as follows:

sense 5′ – GATCCGGCTCTTCTCACGCAACTCCGAGCTCA – 3′;

antisense 5′ – GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG – 3′

2.9. Statistical analyses

The statistical significance was assessed by Student's *t*-test. Dose-response curve fittings and calculations of IC_{50} and EC_{50} values were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

3. Results

3.1. Effects of the copper(II) complexes on transcriptional activity of steroid receptors, nuclear receptors and xenoreceptors

Initially, we evaluated the effects of the copper(II) compounds on transcriptional activities of AR, GR, PXR, AhR, TR and VDR, using gene reporter assays. For this purpose, stably or transiently transfected human cancer cell lines were incubated for 24 h with tested compounds (**1**–**4**) and vehicle in the absence (*agonist mode*) or in the presence (*antagonist mode*) of model agonist for an individual receptor. Luciferase activity and MTT test were performed, and half-maximal effective concentrations (EC_{50}) and half-maximal inhibitory concentrations (IC_{50}) were calculated where appropriate.

3.1.1. Receptors for steroid hormones

Transcriptional activities of AR and GR were evaluated in transgenic reporter cell lines AIZ-AR and AZ-GR. Model ligands for AR (dihydrotestosterone DHT; 100 nM) and GR (dexamethasone DEX; 100 nM) increased luciferase activity in average by 11-fold and 43-fold, respectively. Tested complexes (**1**–**4**) did not influence basal or ligand-inducible transcriptional activity of AR and GR (Fig. 2A; middle and lower panel). Decrease in luciferase activities by 10^{-6} M and higher concentrations of the complexes was rather due to their cytotoxic effects in the cells (Fig. 2A; upper panel). The exception was complex (**1**), which dose-dependently decreased basal activity of GR in non-toxic concentration, suggesting inverse agonist behaviour.

3.1.2. Xenoreceptors

Dioxin (TCDD; 5 nM), a model agonist for AhR, increased luciferase activity in average by 669-fold in transgenic reporter cell line AZ-AhR. Complexes **1** and **2** dose-dependently increased luciferase activity up to 1 μM concentration, while in higher concentration luciferase activity dropped probably due to cytotoxicity of the compounds (Fig. 2B; upper panel). The potencies (EC_{50}) of **1** and **2** ranged between 140 nM and 150 nM (*cf.* 2 nM for TCDD). Only negligible induction of luciferase activity was observed for complexes **3** and **4**. The efficacies (magnitude of induction) of complexes **1** and **2** were about 6.50% of that by TCDD (Fig. 2B; middle panel). Ligand-inducible transcriptional activity of AhR was dose-dependently decreased by all complexes yet at non-cytotoxic concentrations, revealing partial agonist behaviour of those compounds. The effects were much more pronounced for complexes **3** (IC_{50} 0.87 μM) and **4** (IC_{50} 0.54 μM) as compared to complexes **1** and **2** (Fig. 2B; lower panel). Transcriptional activity of PXR was assessed in LS180 cells transiently transfected with *p3A4-luc* reporter construct. A model ligand and agonist for PXR rifampicin (RIF; 10 μM) increased luciferase activity in average by 3-fold as compared to vehicle-treated cells. Tested complexes did not influence basal or ligand-inducible transcriptional activity of PXR. Decrease in luciferase activities by high concentrations of the complexes was rather due to their cytotoxicity (Fig. 2B).

3.1.3. Nuclear receptors

Transcriptional activity of TR was evaluated in transgenic reporter cell line PZ-TR. 3,3′,5-triiodo-L-thyronine (T3; 10 nM), a model ligand and agonist for TR, induced luciferase activity in average by 3-fold, as compared to vehicle-treated cells. All complexes dose-dependently decreased basal transcriptional activity of TR, but these effects were rather due to cytotoxicity of complexes against PZ-TR cells. Interestingly, complexes **1** and **4** at 5 μM concentrations increased two-fold T3-inducible luciferase activity (Fig. 2C). Transcriptional activity of VDR was assessed in LS180 cells transiently transfected with *pCYP24-luc* reporter construct. A model ligand and agonist for VDR, 1,25-dihydroxyvitamin D3 (VD3; 50 nM) increased luciferase activity in average by 47-fold, as compared to vehicle-treated cells. Tested complexes did not influence basal or VD3-inducible transcriptional activity of VDR, and the decrease in luciferase activities by high concentrations of the complexes was rather due to their cytotoxicity (Fig. 2C).

3.2. Effects of resveratrol on transcriptional activity of AhR in AZ-AHR cells and the expression of CYP1A1 mRNA in LS180 cells

Since we observed induction of luciferase in AZ-AHR cells by tested compounds, we tested whether these effects are AhR-dependent. For this purpose, we incubated AZ-AHR and LS180 cells for 24 h with tested complexes (1 μM), dioxin and vehicle, in the presence or in the absence of resveratrol (RVT; 100 μM), which is an antagonist of AhR. Dioxin increased luciferase activity in three consecutive passages of AZ-AHR cells 1305-fold, 387-fold and 487-fold. Complexes **1**, **2** and **3** induced luciferase in average 48-fold, 20-fold and 3-fold, respectively. An induction of luciferase by complexes and TCDD was significantly ($p < 0.05$) abolished by RVT (Fig. 3; upper panel), implying the involvement of the AhR in the process.

Incubation of LS180 cells with TCDD caused strong induction of CYP1A1 mRNA in three consecutive passages by factors 787-fold, 528-fold and 314-fold. Complexes **1**, **2** and **3** induced CYP1A1 mRNA (20-fold to 40-fold) in three independent experiments. An induction of CYP1A1 mRNA was drastically diminished by RVT, affirming AhR-dependent mechanism of the induction (Fig. 3; lower panel).

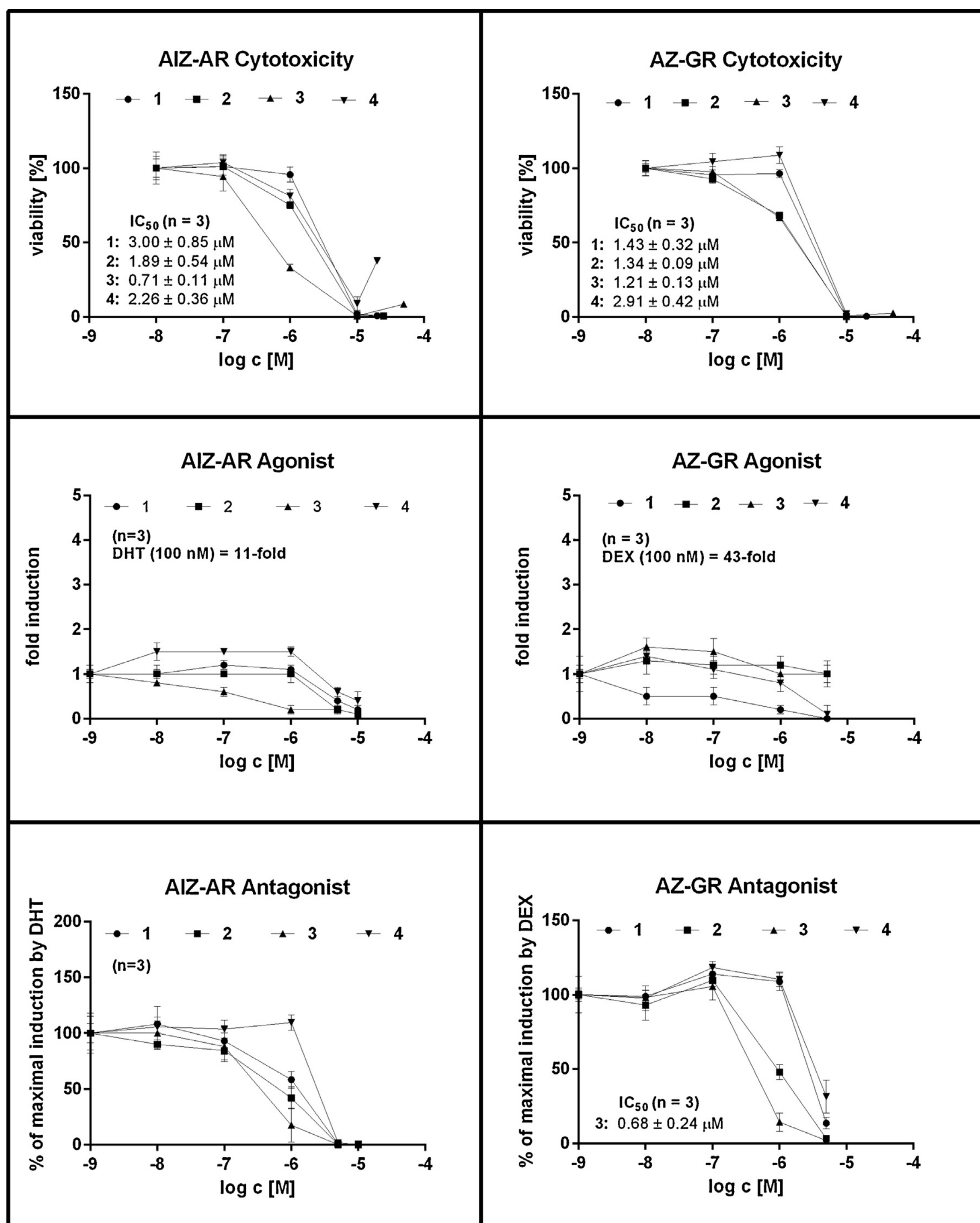


Fig. 2. Effects of the copper(II) complexes (**1**–**4**) on transcriptional activities of nuclear receptors, steroid receptors and xenoreceptors in gene reporter assays. Cells were stabilized for 16 h and then incubated for 24 h with vehicle (UT; 0.1% DMF v/v) and the complexes **1**, **2**, **3** and **4** in the concentrations ranging from 1 nM to 50 μM, in the presence or absence of the model ligands for the receptors, using 96-well culture plates. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. Treatments were carried out in triplicates. Student's *t*-test was used for statistical analysis of the data and EC_{50}/IC_{50} values were calculated where appropriate using GraphPad Prism 6. *Upper panels*: MTT test—the data are expressed as percentage of viability of control cells. Representative viability assays are shown. *Middle panels*: Agonist mode—cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Representative gene reporter assays and average fold-inductions by model ligands are shown. *Lower panels*: Antagonist mode—cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of maximal induction attained by model ligands. Representative gene reporter assays are shown. *Part A*: AIZ-AR cells (androgen receptor; 5α-dihydrotestosterone DHT 100 nM) and AZ-GR cells (glucocorticoid receptor; dexamethasone DEX 100 nM). *Part B*: AZ-AHR cells (aryl hydrocarbon receptor; dioxin TCDD 5 nM) and LS180 cells transiently transfected with *p3A4-luc* reporter and pregnane X receptor PXR (rifampicin RIF 10 μM). *Part C*: PZ-TR transgenic cells (thyroid hormone receptor; 3,3′,5-triiodo-L-thyronine T3; 10 nM) and LS180 cells transiently transfected with *pCYP24-luc* reporter (vitamin D receptor; 25-hydroxyvitamin D3; VD3; 50 nM).

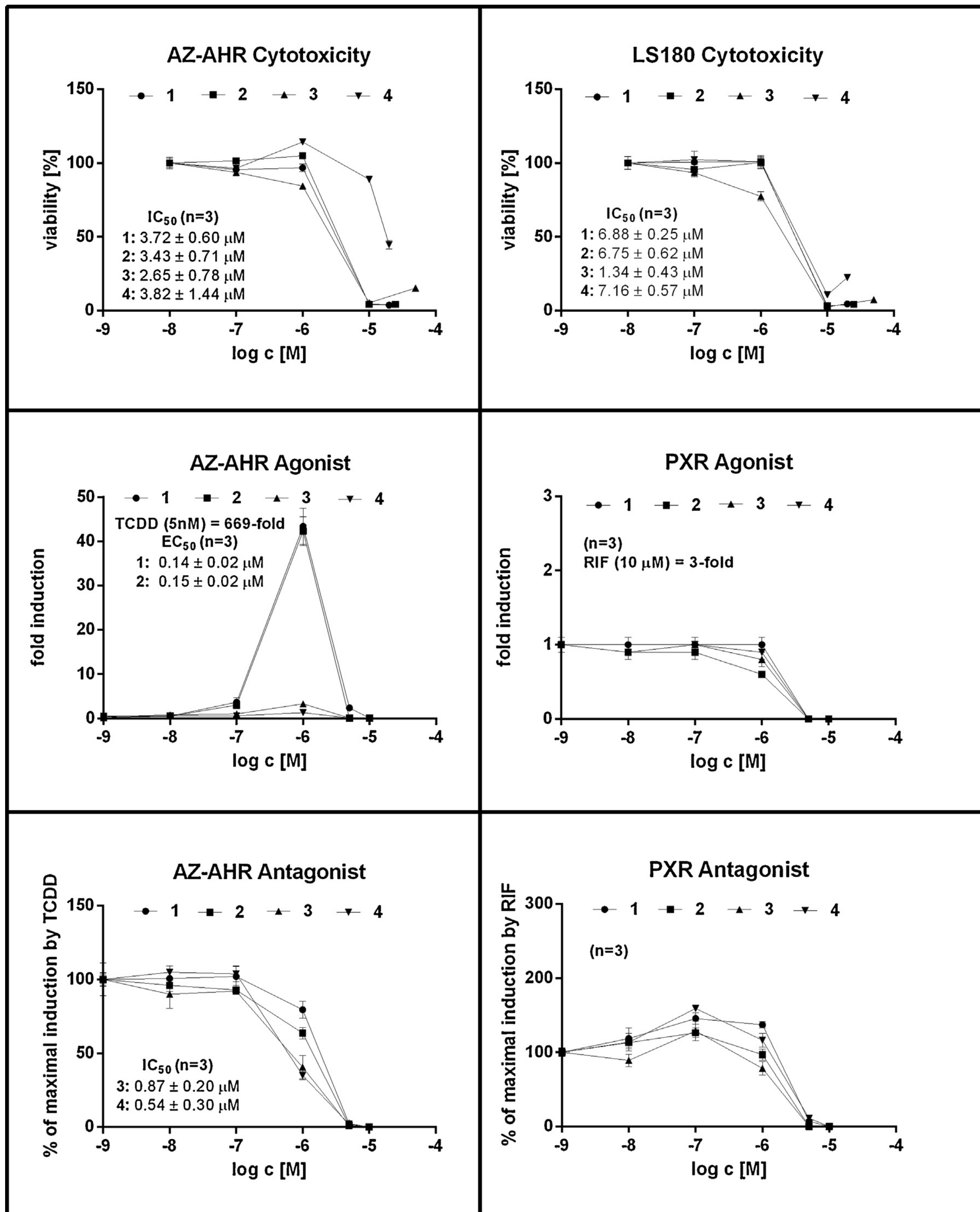
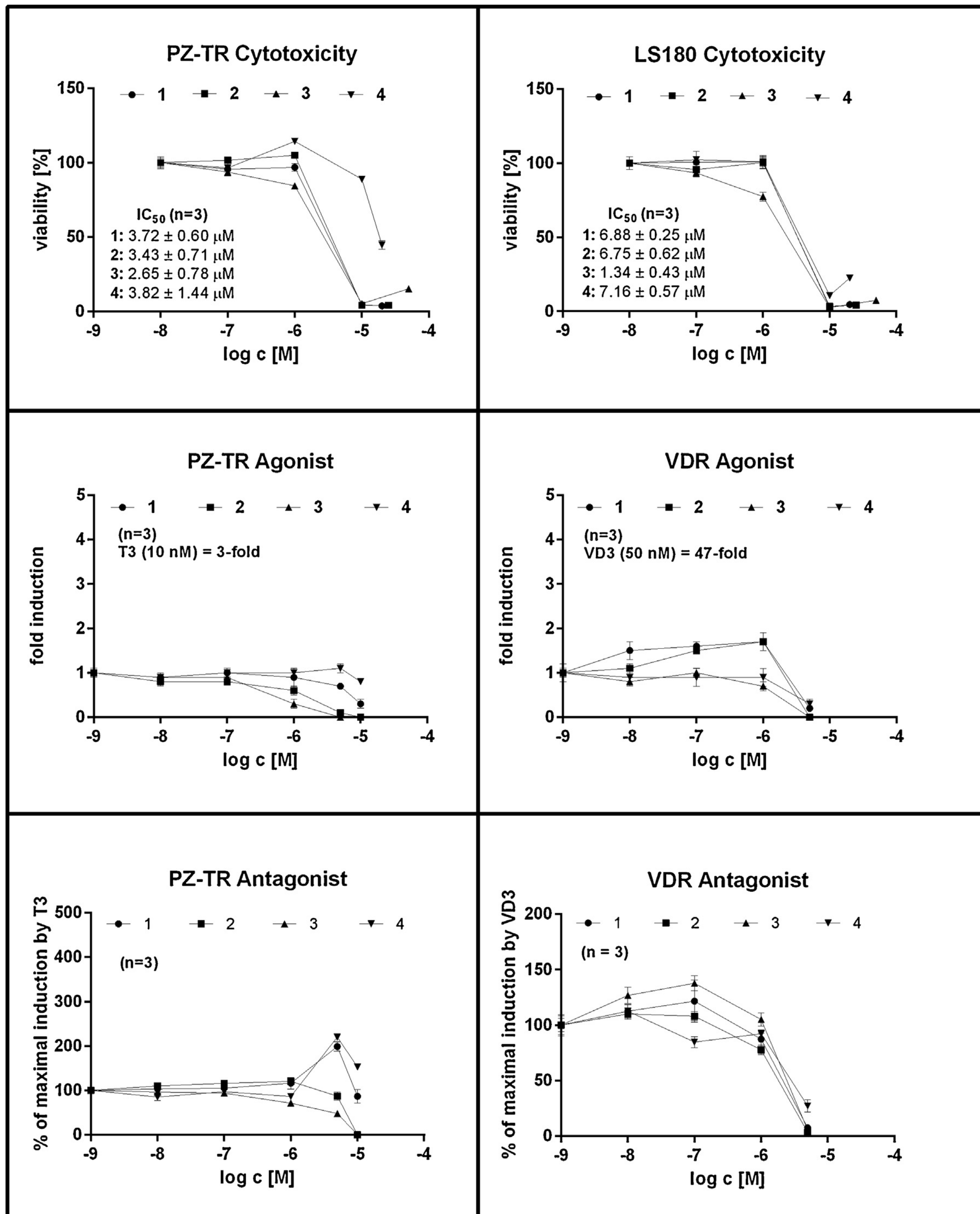


Fig. 2. (Continued)



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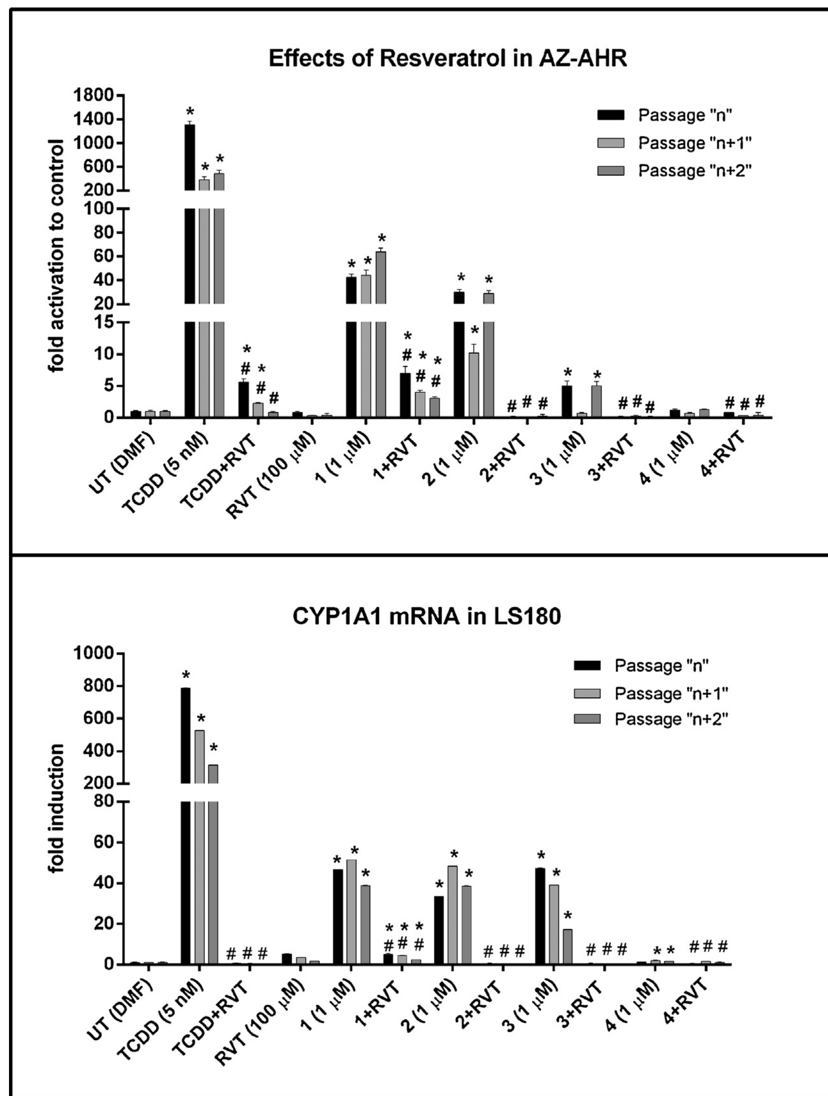

Fig. 3. Effects of resveratrol on transcriptional activity of AhR in AZ-AHR cells and the expression of CYP1A1 mRNA in LS180 cells. Cells were stabilized for 16 h and then treated for 24 h with the complexes (**1**–**4**; 1 μM), vehicle (UT; 0.1% DMF v/v), dioxin (TCDD; 5 nM) and resveratrol (RVT; 100 μM). Showed are results from three consecutive cell passages. The data are expressed as the mean ± SD from triplicate measurements and are expressed as a fold induction over the DMF-treated cells. * = values significantly different from DMF-treated cells ($p < 0.05$). # = significant difference between cells incubated in presence or absence of RVT ($p < 0.05$). *Upper panel*: Gene reporter assays in AZ-AHR cells. *Lower panel*: RT-PCR analyses of CYP1A1 mRNAs in LS180 cells. The data were normalized to GAPDH mRNA levels.

3.3. Copper(II) mixed-ligand complexes induce formation of AhR-DNA complex

In the next series experiments, we to test whether copper(II) complexes can transform human AhR to its DNA-binding form. We treated MCF7 cells with tested complexes and we performed electrophoretic mobility shift assay (EMSA) in nuclear extracts. Strong formation of AhR-DNA complex was observed in extracts from cells incubated with complex **2**, while complex **1** had only moderate effect and complexes **3** and **4** were inactive (Fig. 4).

3.4. Effects of the copper(II) mixed-ligand complexes on the expression of drug-metabolizing cytochromes P450 in primary human hepatocytes

In final series of experiments, we examined the ability of the copper(II) complexes to induce CYP1A1 and CYP1A2 enzymes in primary cultures of human hepatocytes, which are the golden standard for P450 induction studies. We also measured the expression of GR-dependent gene tyrosine aminotransferase (TAT)

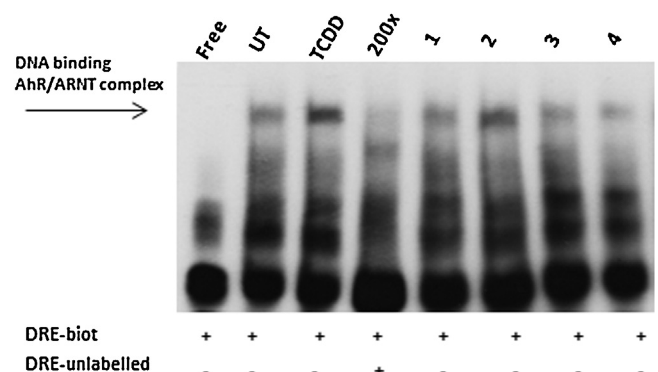

Fig. 4. Electrophoretic mobility shift assay (EMSA). MCF-7 cells at 100% confluence were treated for 2 h with DMF as a negative control (UT; 0.1% v/v), TCDD (5 nM), the complexes (**1**–**4**; 1 μM). Nuclear extracts were incubated with a biotin-labeled probe containing AhR binding site and electrophoresed on 5% polyacrylamide gel as described under "Section 2." Experiments were performed in three independent cell passages and representative EMSA is showed.

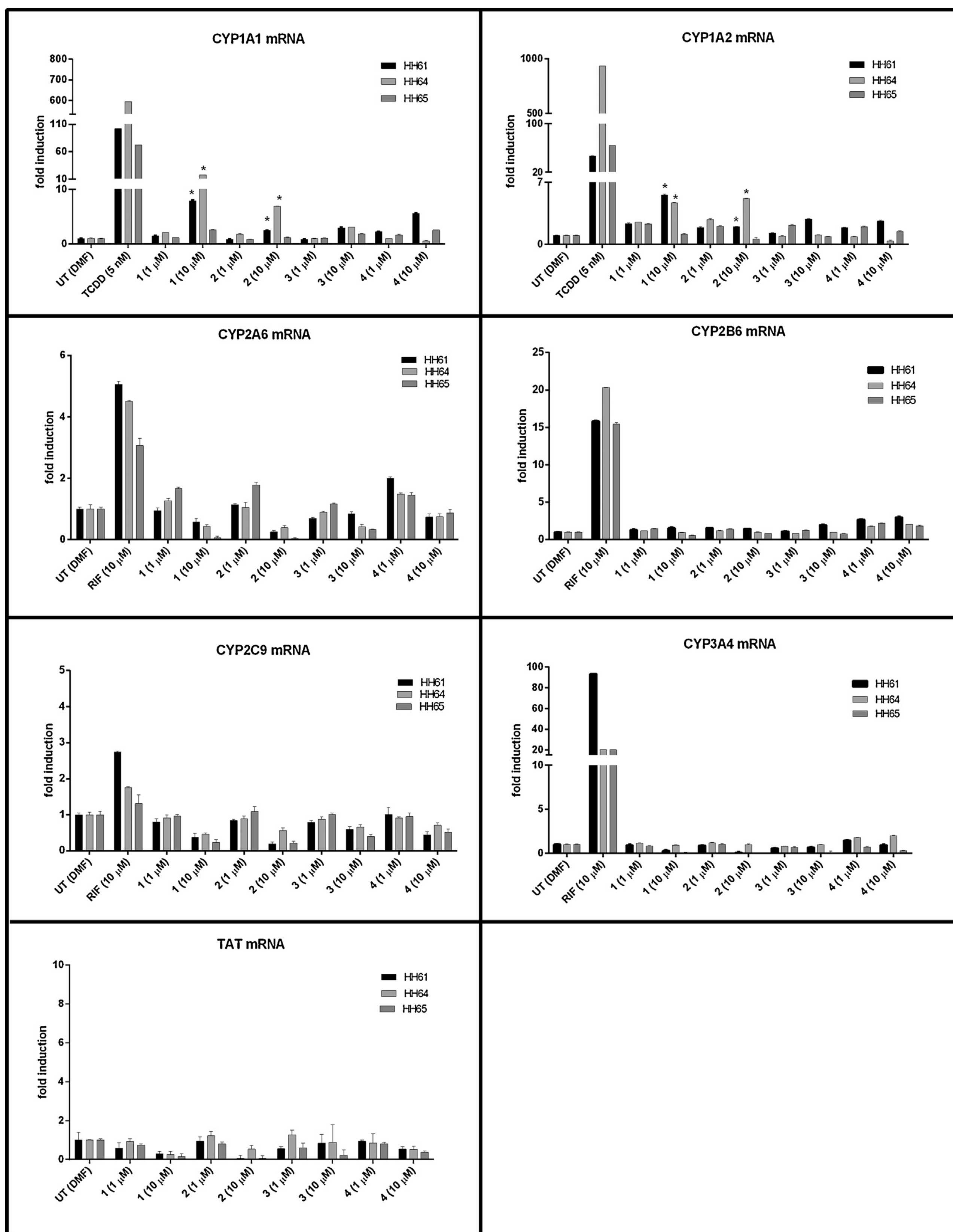

Fig. 5. Effects of the copper(II) complexes (**1**–**4**) on the expression of drug-metabolizing cytochromes P450 and tyrosine aminotransferase TAT in primary human hepatocytes. Human hepatocytes from three different donors (HH61, HH64, HH65) were treated for 24 h (mRNA) and 48 h (protein) with vehicle (UT; 0.1% DMF v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), and copper(II) complexes (**1**–**4**; 1 μM and 10 μM). The data are expressed as the mean ± SD from triplicate measurements and are expressed as a fold induction over the vehicle-treated cells. *Panel A*: Bar graphs show RT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4 and TAT mRNAs. The data were normalized to GAPDH mRNA levels. *Panel B*: Bar graphs show CYP1A1 and CYP1A2 proteins analyses by Simple Western SallySue. The data were normalized to β-actin levels.

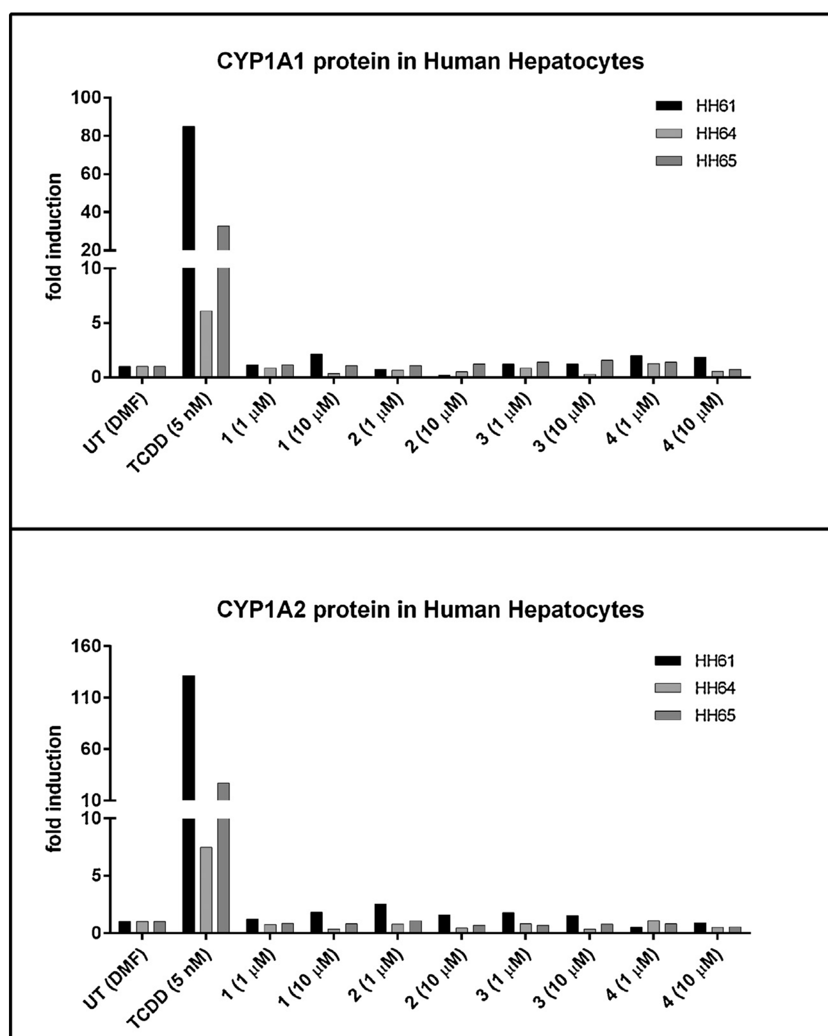

Fig. 5. (Continued)

and other xenobiotics-inducible drug-metabolizing cytochromes P450, including CYP2A6, CYP2B6, CYP2C9 and CYP3A4. We incubated three primary human hepatocytes cultures (HH61, HH64, HH65) for 24 h and 48 h with copper(II) complexes (1 μM; 10 μM), and model inducers, including dioxin (TCDD; 5 nM) and rifampicin (RIF; 10 μM).

The expression of CYP1A1 and CYP1A2 mRNAs and proteins was strongly induced by TCDD in all human hepatocytes cultures. Consistently with data from gene reporter assays and mRNA analyses in LS180 cells, complexes **1** and **2** weakly and dose-dependently induced CYP1A1 and CYP1A2 mRNAs (Fig. 5A). On the other hand, there was not induction of CYP1A1/2 proteins by any of the complexes tested (Fig. 5B). Rifampicin induced PXR-regulated CYP2A6, CYP2B6 and CYP3A4 mRNAs in all human hepatocytes cultures. Induction of CYP2C9 mRNA was achieved only in one culture, probably due to the high constitutive levels of this gene. Tested compounds slightly altered the expression of CYP2A6, CYP2B6, CYP2C9 and CYP3A4 mRNAs, but these effects were inconsistent, and they reflected rather inter-individual variability between liver donors (Fig. 5A).

Finally, since we observed weak anti-glucocorticoid effects of complexes in gene reporter assays, we also measured the expression of GR-regulated TAT gene, in the presence of dexamethasone (constituent of culture medium). While we observed some diminution of TAT mRNA by tested compounds, the effects were not in correlation with data from AZ-GR cells, revealing rather about cytotoxicity of complexes against AZ-GR cells (Fig. 5A).

4. Discussion

In the present paper we investigated the effects of four copper (II) mixed-ligand complexes on transcriptional activities of several nuclear receptors, steroid receptors and xenoreceptors. Tested compounds did not influence basal or ligand-inducible activities of AR, GR, TR, PXR and VDR receptors. Surprisingly, complexes (**1**,**2**) activated AhR and induced AhR dependent genes, which is supported by following: (i) AhR-dependent luciferase activity in transgenic AZ-AHR cells was dose dependently increased by (**1**,**2**) and the induction was reverted by AhR antagonist resveratrol; (ii) Expression of CYP1A1 mRNA in LS180 cells was induced by (**1**,**2**,**3**) and resveratrol abolished this induction; (iii) Complexes (**1**,**2**) dose-dependently induced CYP1A1 and CYP1A2 mRNAs in primary cultures of human hepatocytes; (iv) Transformation of AhR to its DNA-binding form was induced by (**1**,**2**) as revealed by EMSA in MCF-7 cells.

Soluble intracellular receptors that act as ligand-activated transcriptional factors, including nuclear receptors, receptors for steroid hormones and xenoreceptors, play a variety of cell functions. They are involved in cell cycle regulation, differentiation, development, apoptosis, immune response, intermediary metabolism and homeostasis, detoxification etc. Therefore, perturbation,

in particular chronic one, of cell signal pathways by soluble receptors may result in pathological states referred as to endocrine disruption. Since copper(II) mixed-ligand complexes examined in the current paper did not influence transcriptional activities of AR, GR, TR, PXR and VDR receptors, it may be anticipated that the endocrine disrupting potential of those complexes is none or low with regard to the receptors under examination.

In contrast, an activation of AhR by complexes (**1**,**2**) is considered undesired in terms of potential for endocrine disruption or chemically-induced carcinogenesis. The values of EC_{50} in gene reporter assays were about 150 nM for both complexes, indicating medium to high potency, as compared to that of TCDD. However, the magnitude of AhR activation (efficacy) by complexes (**1**,**2**) was only about 6% of that by TCDD. The fold inductions of CYP1A1 and CYP1A2 mRNAs by complexes (**1**,**2**) in human hepatocytes ranged from 2% to 7% of those by TCDD, implying rather low potential to give a rise to drug–drug interactions through these two enzymes. In addition, other major drug-metabolizing enzymes including CYP2B6, CYP2C9 and CYP3A4 were not induced by any tested complex, implying low or no potential for drug–drug interactions. We found slight inconsistency in case of complex **3**, which induced CYP1A1 in LS180 cells, but not in human hepatocytes. A plausible explanation could be a metabolic inactivation of complex **3** in human hepatocytes that are fully equipped with detoxification enzymes unlike LS180 cells.

The difference between AhR activation and CYP1A genes induction by individual complexes may be explained on structural basis. The common structural feature of the complexes is the presence of substituted 1,10-phenanthroline and quinoline in their molecules. Both chelating (1,10-) and non-chelating (1,7- and 4,7-) phenanthrolines were described as partial agonists or competitive antagonists of rat AhR (Mahon and Gasiewicz, 1992). Moreover, quinoline-derived compounds, such as kynurenic acid were identified as potent endogenous ligands of AhR (DiNatale et al., 2010). The most active complexes **1** and **2** contain in their structure either unsubstituted or 5-methylated (small molecule) 1,10-phenanthroline, respectively. Inactive complex **3** is 4,7-diphenyl substituted, and two large aromatic ring may account for loss of AhR activation by the complex. While inactive complex **4** has unsubstituted 1,10-phenanthroline in its molecule, it contains large substituents at positions 2 and 7 in quinoline-based part. Taken together, we can speculate that it is both substituted 1,10-phenanthroline and quinoline ligands that account co-operatively for AhR activation.

In conclusion, based on the *in vitro* data presented in the current paper, tested copper(II) mixed-ligand complexes may be considered safe in terms of potential disruption of signalling pathways by nuclear receptors, steroid receptors and xenoreceptors. When intended for pharmacotherapy, weak activation of AhR and induction of AhR-dependent genes by complexes **1** and **2** should be taken in account. It cannot be generalized, whether AhR activators increase or decrease the toxicity of the xenobiotics. Since AhR is also transcriptional regulator of phase II conjugation enzymes, and many other genes, it may happen that (auto)-induction of AhR-target genes results in either augmented (Loaiza-Perez et al., 2004) or attenuated cytotoxicity (Ho and Lee, 2002). However, *in vitro* and *in vivo* data may substantially differ; therefore, *in vivo* experiments on rodents would enriche the knowledge about toxicokinetics and toxicodynamics of studied compounds.

Conflict of interests

We declare no conflict of interest.

Acknowledgement

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APPENDIX II.

Kubesova K., Travnicek Z., Dvorak Z. (2016). Pleiotropic effects of gold(I) mixed-ligand complexes of 9-deazahypoxanthine on transcriptional activity of receptors for steroid hormones, nuclear receptors and xenoreceptors in human hepatocytes and cell lines. *Eur J Med Chem 121*, 530-540. **[IF_{2016} - 4.519]**

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Research paper

Pleiotropic effects of gold(I) mixed-ligand complexes of 9-deazahypoxanthine on transcriptional activity of receptors for steroid hormones, nuclear receptors and xenoreceptors in human hepatocytes and cell lines

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ABSTRACT

Development of metal-based compounds is an important research avenue in anti-cancer and anti-inflammatory drug discovery. Here we examined the effects of three gold (I) mixed-ligand complexes with the general formula $[Au(L_n)(PPh_3)]$ (**1**, **2**, **3**) involving triphenylphosphine (PPh_3) and a deprotonated form of *O*-substituted derivatives of 9-deazahypoxanthine (L_n) on the transcriptional activity of aryl hydrocarbon receptor (AhR), androgen receptor (AR), glucocorticoid receptor (GR), thyroid receptor (TR), pregnane X receptor (PXR) and vitamin D receptor (VDR), employing gene reporter assays. In addition, we measured mRNA (RT-PCR) and protein (western blot) expression of target genes for those receptors, including drug-metabolizing P450s, in primary human hepatocytes and cancer cell lines LS180 and HepG2. The tested compounds displayed anti-glucocorticoid effects, as revealed by inhibition of dexamethasone-inducible transcriptional activity of GR and down-regulation of tyrosine aminotransferase. All the compounds slightly and dose-dependently activated PXR and AhR, and moderately induced CYP3A4 and CYP1A1/2 genes in human hepatocytes and LS180 cells. The complexes antagonized basal and ligand-activated AR and VDR, indicating inverse agonist behaviour. Both basal and thyroid hormone-inducible transcriptional activity of TR was dose-dependently increased by all tested compounds. In contrast, the expression of SPOT14 mRNA was decreased by tested compounds in human hepatocytes and HepG2 cells. In conclusion, if intended for human pharmacotherapy, the potential of the complexes **1**–**3** to influence studied receptors should be taken in account.

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1. Introduction

Metals-containing compounds were used for medicinal applications from ancient times, yet. The examples are the use of mercurous chloride as a diuretic or the arsenical Salvarsan in the treatment of Syphilis. A pioneer metal-based drug introduced to human pharmacotherapy was anti-cancer drug *cis*-platinum, followed by plethora of compounds containing platinum central atom. Since then, a myriad of metal-based drugs (containing Li, Mg, Al, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Rb, As, Sr, Mb, Ag, Ba, Bi etc.) were approved for human medicinal (anti-cancer, anti-infective, anti-arthritic etc.) and diagnostic purposes (contrast agents, positron emission tomography etc.) [1–4]. Among the above-mentioned metals, a due attention is focused on Au-complexes owing to their use in the treatment of rheumatoid arthritis, such as sodium

Abbreviations: AhR, aryl hydrocarbon receptor; AR, androgen receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; DR4, direct repeat separated by four nucleotides; DEX, dexamethasone; DHT, dihydrotestosterone; DMF, *N*,*N*-dimethylformamide; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; MTT, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan; PXR, pregnane X receptor; RARs, retinoic acid receptors; RIF, rifampicin; RVT, resveratrol; RXRs, retinoid X receptors; T3, 3,3′,5-triiodo-L-thyronine; TAT, tyrosine aminotransferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TR, thyroid hormone receptor; VD3, 25-hydroxyvitamin D3; VDR, vitamin D receptor.

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aurothiomalate (Myochrysin®, sodium ((2-carboxy-1-carboxylatoethyl)thiolato)gold(I)), aurothioglucose (Solganol®, {(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)-oxane-2-thiolato}gold(I) and Auranofin (Ridaura®, triethylphosphine-(2,3,4,6-tetra-*O*-acetyl-β-D-thiopyranosato)gold [5–7]. Recently, we published a series of four works dealing with the study of anti-inflammatory and anti-cancer properties of gold(I) complexes having the composition of $[Au(L)(PPh_3)]$, where L stands for a deprotonated form of 6-benzylaminopurine [8,9], 9-deazahypoxanthine [10] and hypoxanthine derivatives [11]. Some of the presented compounds overcame the activity of Auranofin (in the case of anti-inflammatory activity) and cisplatin (in the case of anti-cancer activity) as reference compounds. A plethora of gold(I) complexes were studied by other groups for their cytotoxicity and interactions with numerous cell targets. For instance, gold(I) alkynyl complexes with phosphane ligands were shown as activators of extracellular regulated kinase ERK and inhibitors of thioredoxin reductase [12]. Also adamantane-azole gold(I) complexes were identified as potent inhibitors of thioredoxin reductase [13]. High levels of cytotoxicity were observed for alkynyl(triphenylphosphine)gold(I) complexes in human cancer cell lines [14].

Various cellular functions, such as energy production or storage, intermediary metabolism, xenobiotics metabolism, cell cycle control, immune response, apoptosis or differentiation are regulated by the whole array of transcriptional factors, including nuclear receptors (e.g. vitamin D receptor – VDR, thyroid hormone receptor – TR, retinoic acid receptors – RARs, retinoid X receptors – RXRs), receptors for steroid hormones (e.g. glucocorticoid receptor – GR, androgen receptor – AR, estrogen receptor – ER) and xenoreceptors (e.g. aryl hydrocarbon receptor – AhR, constitutive androstane receptor – CAR, pregnane X receptor - PXR). Formerly, nuclear and steroid receptors were considered as pivotal regulators of physiological and endocrine functions, while xenoreceptor as key factors controlling the expression of genes in detoxification pathways, including phase I enzymes, conjugation enzymes and drug transporters. There is an increasing body of evidence for mutual and multiple cross-talks between xenoreceptors and receptors for steroid hormones and nuclear receptors [15]. For instance, xenoreceptors AhR [16–18] and PXR [19–22] were demonstrated to be involved in regulation of glucose and lipid homeostasis. On the other hand, nuclear receptors and steroid receptors including VDR [23], GR [24], ER [25], RXR [26] are important transcriptional regulators of xenobiotics-metabolizing enzymes. The interactions between drugs (xenobiotics) and ligand-activated intracellular receptors may have three aspects relevant for human health: (i) Endocrine disruption – interactions of xenobiotics with homeostasis of steroid hormones and ligands for nuclear receptors, mostly involving xenoestrogen and antiandrogen activities, may result in complex perturbation of intermediary metabolism with serious health consequences [27]; (ii) Drug-drug interactions – a phenomenon, which occurs when pharmacokinetic on drug “A” is influenced in the presence of drug “B”, a situation also referred as victim-perpetrator interaction [28]; (iii) Food-drug interactions – a situation when a food-born chemical interacts with pharmacokinetic of the drug [29].

In the current paper, we examined the effects of three gold (I) mixed-ligand complexes with the general formula $[Au(L_n)(PPh_3)]$ (**1**, **2**, **3**) on the transcriptional activities of AhR, AR, GR, TR, PXR and VDR, employing gene reporter assays, RT-PCR and western blot. We found differential activities of the compounds against transcriptional activities of evaluated receptors and the expression of their target genes. We concluded that if intended for human pharmacotherapy, the potential of tested compounds to influence studied receptors should be taken in account in both positive and negative meaning.

2. Materials and methods

2.1. Compounds and reagents

N,*N*-Dimethylformamide (DMF), hygromycin B, dexamethasone (DEX), rifampicin (RIF), dihydrotestosterone (DHT), 3,3′,5-triiodo-L-thyronine (T3) and resveratrol (RVT) were purchased from Sigma–Aldrich (Prague, Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was from Ultra Scientific (Rhode Island, USA). 25-hydroxyvitamin D3 (VD3) was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The preparation, characterization and biological activity study of gold(I)-triphenylphosphine complexes, $[Au(L_n)(PPh_3)]$, **1** (L_1 = 6-isopropyloxy-9-deazapurine); **2** (L_2 = 6-phenethyloxy-9-deazapurine); **3** (L_3 = 6-benzyloxy-9-deazapurine) (Fig. 1) were described in the literature [10]. ^1H and ^{13}C NMR spectra of complexes **2** and **3** can be found in Supplementary material (see Figs. S1–S4), while the same spectra of complex **1** are depicted in the literature [10]. Luciferase lysis buffer and FuGENE® HD Transfection Reagent were obtained from Promega (Madison, WI, CA). Oligonucleotide primers used in RT-PCR reactions were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). LightCycler 480 Probes Master and 480 SYBR Green I Master kit were from Roche Diagnostic Corporation (Intes Bohemia, Czech Republic). All other chemicals were of the highest quality commercially available.

2.2. Primary cultures of human hepatocytes

Human hepatocytes were isolated from human liver obtained from two sources: (i) multiorgan donors HH61 (M; 64 years), HH64 (M; 73 years), HH65 (M; 34 years) and HH66 (M; 65 years). Tissue acquisition protocol and the use of liver cells was approved by “Ethical committee at the Faculty Hospital Olomouc”, and it was in accordance with Transplantation law #285/2002 Sb.; (ii) from the liver biopsy - *long-term human hepatocytes in monolayer* Batch HEP220879 (F; 65 years) (Biopredic International, Rennes, France). Cells were plated at collagen-coated dishes in hormonally and chemically defined medium as previously described [30,31] and stabilized for 24 h before the treatment. Hepatocytes were incubated in a serum-free medium for 24 h (for mRNA analyses) and 48 h (for protein analyses) with vehicle (UT; 0.1% DMF v/v), TCDD (5 nM), RIF (10 μM), T3 (10 nM) and the tested complexes. Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

2.3. Human cancer cell lines

Human Caucasian colon adenocarcinoma cells LS180 (ECACC No. 87021202) were purchased from European Collection of Cell Cultures (ECACC). Cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 4 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were cultured at 37 °C and 5% CO_2 in a humidified incubator. Stably transfected gene reporter cell lines AZ-AHR [32], AZ-GR [33], AIZ-AR [34], and PZ-TR [35] were described elsewhere.

2.4. Cell viability assay (MTT)

The human cancer cell lines AIZ-AR, AZ-AHR, AZ-GR, PZ-TR and LS180 were treated with the tested compounds, vehicle (UT; 0.1% v/v DMF) and Triton X-100 (1%, v/v) for 24 h, using 96-well culture plates. The gold(I) complexes (**1, 2, 3**) were applied to the cells in

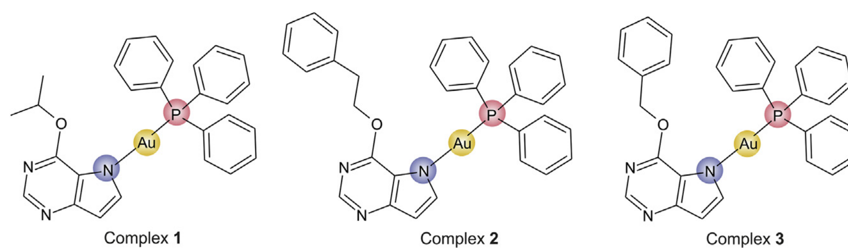

Fig. 1. Structural formulas of the studied gold(I) mixed-ligand complexes (1–3). Three gold(I) complexes with the general formula [Au(L_n)(PPh_3)] involving triphenylphosphine (PPh_3) and a deprotonated form of *O*-substituted derivatives of 9-deazahypoxanthine (L_n), namely, 6-isopropyloxy-9-deazapurine (**1**); 6-phenethyloxy-9-deazapurine (**2**) and 6-benzyloxy-9-deazapurine (**3**) are shown.

concentrations ranging from 1 nM to 50 μM (unless the solubility was lower). MTT assay was performed and absorbance was measured spectrophotometrically at 570 nm on Infinite M200 (Schoeller Instruments, Prague, Czech Republic). The data were expressed as the percentage of cell viability. Half-maximal inhibitory concentrations (IC_{50}) were calculated using GraphPad Prism 6 software (GraphPad Software, San Diego, USA). The tested concentrations causing the decline in viability no greater than 20% were considered as non-toxic for further experiments.

2.5. Gene reporter assays

Stably transfected human luciferase gene reporter cell lines used in the study were as follows: AZ-AHR cells were derived from human hepatocyte carcinoma HepG2 cells transfected with a construct containing several aryl hydrocarbon receptor (AhR) binding sites [32]. The AZ-GR cells were derived from human cervix carcinoma HeLa cells transfected with three copies of glucocorticoid receptor (GR) response elements [33]. The AIZ-AR cells were derived from human prostate carcinoma 22Rv1 cells transfected with a construct containing four copies of androgen receptor (AR) binding sites [34]. The PZ-TR was derived from human hepatocyte carcinoma HepG2 cells transfected with a construct containing two copies of tandem thyroide response elements TREs oriented as direct repeats separated by four nucleotides – DR4 [35].

Transiently transfected gene reporter cell lines used for assessment of transcription activity pregnane X receptor (PXR) and vitamin D receptor (VDR) were described elsewhere [36].

The cells were incubated for 24 h with the tested compounds, TCDD (5 nM), DEX (100 nM), DHT (100 nM), T3 (10 nM), RIF (10 μM), VD3 (50 nM) and/or vehicle (UT; 0.1% DMF v/v). After the treatments, the cells were lysed using Reporter Lysis Buffer (Promega, Madison, WI, USA) according to manufacturers' instructions and the luciferase activity was measured spectrophotometrically on Infinite M200 (Schoeller Instruments, Prague, Czech Republic). Dose-response curve fittings and calculation of half-maximal effective concentrations EC_{50} and/or half-maximal inhibitory concentrations EC_{50} values were determined using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

2.6. Quantitative Reverse Transcriptase PCR (qRT-PCR)

The total RNA was isolated using TRI Reagent® (Molecular Research Center, Ohio, USA). After the treatment with DNase I, the cDNA was synthesized, according to the common protocol, using M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, Massachusetts, USA) and random hexamers (New England Biolabs). The qRT-PCR was carried out on Light Cycler 480 II instrument (Roche Diagnostic Corporation, Prague, Czech Republic). The levels of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, TAT, SPOT14 and GAPDH mRNAs were determined as described elsewhere [37]. The sequences of primers are shown in Table 1. The measurements were performed in triplicates. The gene expression levels were normalized to the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Data were processed by delta-delta method.

2.7. Simple western blotting by Sally Sue™

The cells were incubated for 48 h with vehicle (UT; 0.1% DMF v/v), TCDD (5 nM), RIF (10 μM) and the tested compounds. The total protein extracts were isolated as described previously [38]. Reagents for simple western by *Sally Sue™*, capillaries, containing a proprietary UV-activated chemical linked reagent, 384-well plates, antibody diluent and goat anti-mouse secondary antibody were purchased from ProteinSimple (San Jose, California) and samples were prepared according to manufacturer's instructions (www.proteinsimple.com). CYP1A1 (goat polyclonal, sc-9828, G-18), CYP1A2 (mouse monoclonal, sc-53614, 3B8C1), CYP3A4 (mouse monoclonal; sc- 53850, HL3) primary antibodies and rabbit anti-goat secondary antibody (sc-2768, H0712) were purchased from Santa Cruz Biotechnology Inc. β-actin (mouse monoclonal; 3700S,

Table 1
List of Used Primers for PCR. Primer sequences with appropriate Universal Probes Library (UPL) numbers.

Gene symbol	Forward primer sequence	Reverse primer sequence	UPL probe Number
CYP1A1	CCAGGCTCCAAGAGTCCA	GATCTTGGAGGTGGCTGCT	33
CYP1A2	ACAACCCTGCCAATCTCAAG	GGGAACAGACTGGGACAATG	34
CYP2A6	CCACGGGACTTCATTGACTC	CCCAATGAAGAGGTTCAACG	63
CYP2B6	TTCACTCATCAGCTCTGTATTCG	GCCCCAGGAAAGTATTTCAA	106
CYP2C9	GAAGCACCCAGAGGTCACAG	CTCGTGCACCACAGCATC	92
CYP3A4	TGTGTTGGTGAGAAATCTGAGG	CTGTAGGCCCCAAAGACG	38
TAT	GCACCCCTAGAAGCTAAGGAC	CAGGTCTTGGAACCAGGATG	37
SPOT14	CATGCACCTCACCGAGAA	TGTCTTCTATCATGTGAAGGGATCT	79
GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC	60

8H10D10) primary antibody was from Cell Signalling Technology (Denvers, Massachusetts, USA). Target proteins were identified using primary antibodies and immunoprobed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was detected and quantified by the Compass Software version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the signals were normalized to β-actin as a loading control.

2.8. Statistical analyses

The statistical significance of the data was evaluated using Student's *t*-test and a level of probability was at $p < 0.05$. Dose-response curve fittings and IC_{50}/EC_{50} values were determined using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

3. Results

3.1. Effects of the gold(I) complexes on transcriptional activity of steroid receptors, nuclear receptors and xenoreceptors

In the first series experiments, we examined the effects of the gold(I) complexes on transcriptional activity of: (i) receptors for steroid hormones, i.e. androgen receptor (AR) and glucocorticoid receptor (GR), as the representatives of receptors for sex hormones and corticoids, respectively; (ii) xenoreceptors as main regulators of drug-metabolizing genes, i.e. pregnane X receptor (PXR) and aryl hydrocarbon receptor (AhR); (iii) nuclear receptors, i.e. receptor for thyroid hormone (TR) and vitamin D receptor (VDR). For this purpose, stably or transiently transfected human cell lines were incubated for 24 h with complexes (**1, 2, 3**) and vehicle in the absence (*agonist mode*) or in the presence (*antagonist mode*) of model agonist for individual receptor. Following the treatments measurements of luciferase activity and/or MTT test were performed. Half-maximal effective concentrations (EC_{50}) and half-maximal inhibitory concentrations (IC_{50}) were calculated where appropriate.

Androgen receptor: Transcriptional activity of AR was evaluated in stably transfected AIZ-AR cells. Dihydrotestosterone (100 nM), a model ligand and agonist for AR, increased luciferase activity in average by 11-fold as compared to vehicle-treated cells. All tested complexes dose-dependently antagonized both basal and ligand-inducible transcriptional activity of AR, revealing about inverse agonist behaviour. The values of IC_{50} were 1.8 ± 0.3 μM and 4.0 ± 0.1 μM for **2** and **3**, respectively. Inhibitory effects of **1** were rather due to its cytotoxicity against AIZ-AR cells (Fig. 2A; left).

Glucocorticoid receptor: Transcriptional activity of GR was evaluated in stably transfected AZ-GR cells. Dexamethasone (100 nM), a model ligand and agonist for GR, increased luciferase activity in average by 43-fold as compared to vehicle-treated cells. Basal activity of GR was not influenced by any tested complex, while dexamethasone-inducible transcriptional activity of GR was dose-dependently decreased by **1, 2** and **3** with IC_{50} values 2.9 ± 1.9 μM, 5.0 ± 0.1 μM and 2.5 ± 0.1 μM, respectively (Fig. 2A; right). Since the drop in luciferase activity was not due to intrinsic cytotoxicity of Au(I) complexes, their antagonist effects against GR are assumed.

Aryl hydrocarbon receptor: Transcriptional activity of AhR was evaluated in stably transfected AZ-AhR cells. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5 nM), a model ligand and agonist for AhR, increased luciferase activity in average by 669-fold as compared to vehicle-treated cells. Compounds **1, 2** and **3** dose-dependently increased luciferase activity with similar potency, and average EC_{50} values were 5.2 ± 0.1 μM, 5.0 ± 0.1 and 3.8 ± 1.7 μM, respectively. The efficacy of complexes was very weak and it ranged from 0.5% to 1.5% of that by TCDD in order **2**~**3** > **1**. Ligand-dependent transcriptional activity of AhR was not altered by any of tested complexes (Fig. 2B; left).

Pregnane X receptor: Transcriptional activity of PXR was evaluated in transiently transfected LS180 cells. Rifampicin (10 μM), a model ligand and agonist for PXR, increased luciferase activity in average by 3-fold as compared to vehicle-treated cells. Complexes **1, 2** and **3** dose-dependently increased luciferase activity and EC_{50} values ranged from 0.4 μM to 1.0 μM. The potency of complexes was similar to that by rifampicin in order **2** > **1**>**3**. Ligand-inducible activity of PXR was dose-dependently inhibited by **1** and **3**, but slightly augmented by **2** (Fig. 2B; right).

Thyroid hormone receptor: Transcriptional activity of TR was evaluated in stably transfected PZ-TR cells. 3,3′,5-triiodo-L-thyronine (10 nM), a model ligand and agonist for TR, increased luciferase activity in average by 3-fold as compared to vehicle-treated cells. All complexes dose-dependently increased luciferase activity with EC_{50} values of 3.4 ± 1.7 μM, 3.9 ± 1.7 μM and 4.6 ± 0.8 μM for **1, 2** and **3**, respectively. The magnitude of these inductions was about 70–100% of induction attained by T3. Interestingly, combined treatment of PZ-TR cells with T3 and the complexes displayed strong and dose-dependent additive effect on T3-inducible luciferase activity. The most drastic effect was achieved by **1** (10 μM), which in combination with T3 caused approx. 5-times stronger luciferase induction as compared to T3 and/or **1** itself. The apparent potencies EC'_{50} were estimated to range from 5.6 μM to 5.9 μM (Fig. 2C; left).

Vitamin D receptor: Transcriptional activity of VDR was evaluated in transiently transfected LS180 cells. 25-hydroxyvitamin D3 (50 nM), a model ligand and agonist for VDR, increased luciferase activity in average by 47-fold as compared to vehicle-treated cells. All tested complexes dose-dependently antagonized both basal and ligand-inducible transcriptional activity of VDR, revealing about inverse agonist behaviour. The values of IC_{50} were 2.5 ± 0.1 μM and 2.7 ± 0.9 μM for **1** and **3**, respectively. Inhibitory effects of **2** were rather due to its cytotoxicity against LS180 cells (Fig. 2C; right).

3.2. Effects of the gold(I) complexes on the expression of CYP1A1, CYP3A4 and SPOT14 mRNAs in human cancer cell lines

Since we observed activation of AhR, PXR and TR by tested compounds, we evaluated the expression of their target genes, i.e. CYP1A1, CYP3A4 and SPOT14, respectively. Therefore, human colon adenocarcinoma LS180 cells and PZ-TR cells were treated for 24 h with dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), 3,3′,5-triiodo-L-thyronine (T3; 10 nM), vehicle (UT; 0.1% DMF v/v) and the gold(I) complexes at concentrations 1 μM, 5 μM and 10 μM. Dioxin induced CYP1A1 mRNA by factors 1113-fold, 1300-fold and 1280-fold in three consecutive passages of LS180 cells. All tested complexes significantly induced CYP1A1 mRNA in three independent experiments (Fig. 3; upper panel), which is consistent with AhR activation by these compounds (Fig. 2B; left). Rifampicin induced CYP3A4 mRNA approx. 2.5-fold, but there was no CYP3A4 mRNA induction by any tested complex (Fig. 3; middle panel), which is inconsistent with PXR activation by these complexes (Fig. 2B; right). Triiodo-L-thyronine T3 induced SPOT14 mRNA approx. 2.5-fold in PZ-TR cells. All tested complexes decreased both basal and T3-inducible SPOT14 mRNA expression (Fig. 3; lower panel), which is opposite to their effects on TR transcriptional activity in gene reporter assays (Fig. 2C; left).

3.3. Effects of the gold(I) complexes on the expression of drug-metabolizing cytochromes P450, TAT and SPOT14 in primary human hepatocytes

In final series of experiments, we examined the ability of the

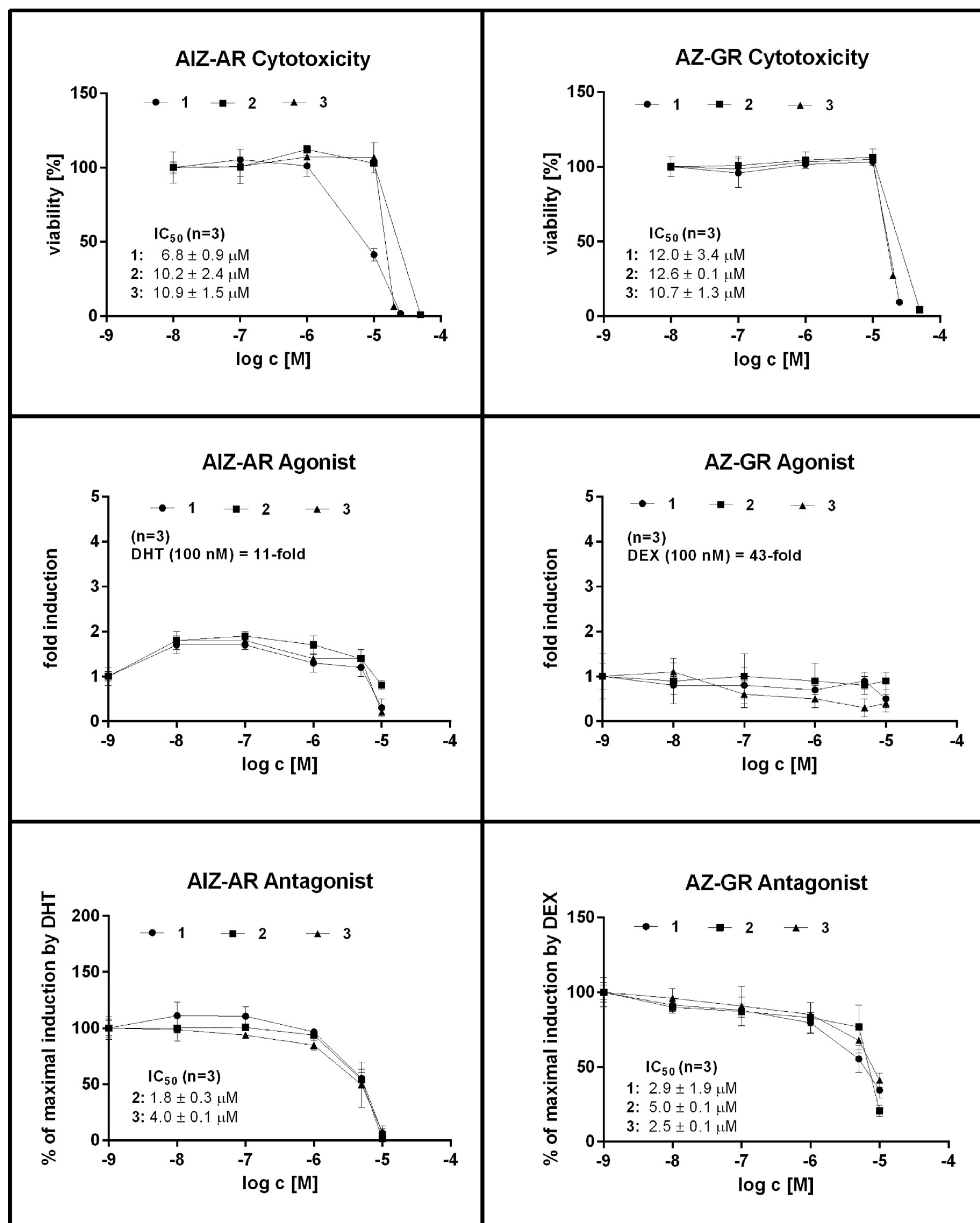

Fig. 2. Effects of the gold(I) mixed-ligand complexes (1–3) on transcriptional activities of nuclear receptors, steroid receptors and xenoreceptors in gene reporter assays. Cells were stabilized for 16 h and then incubated for 24 h with vehicle (DMF; 0.1% v/v) and the complexes1, 2 and 3 in the concentrations ranging from 1 nM to 50 μM, in the presence or absence of model ligands for the receptors, using 96-well culture plates. The data are expressed as the mean ± SD from experiments performed in three consecutive passages of cells. Treatments were performed in triplicates. Student's *t*-test was used for statistical analysis of the data and EC_{50}/IC_{50} values were calculated where appropriate using GraphPad Prism 6. *Upper panels*: MTT test - the data are expressed as percentage of viability of control cells. Representative viability assays are shown. *Middle panels*: Agonist mode - cells were incubated with tested compounds in the absence of model ligands for the receptors. Data are expressed as a fold induction of luciferase activity over control cells. Representative gene reporter assays are shown. *Lower panels*: Antagonist mode - cells were incubated with tested compounds in the presence of model ligands for the receptors. Data are expressed as a percentage of maximal induction attained by model ligands. Representative gene reporter assays are shown. *Part A*: AIZ-AR cells (androgen receptor; 5α-dihydrotestosterone DHT 100 nM) and AZ-GR cells (glucocorticoid receptor; dexamethasone DEX 100 nM). *Part B*: AZ-AHR cells (aryl hydrocarbon receptor; dioxin TCDD 5 nM) and LS180 cells transiently transfected with *p3A4-luc* reporter and pregnane X receptor PXR (rifampicin RIF 10 μM). *Part C*: PZ-TR transgenic cells (thyroid hormone receptor; 3,3′,5-triiodo-L-thyronine T3; 10 nM) and LS180 cells transiently transfected with *pCYP24-luc* reporter (vitamin D receptor; 25-hydroxyvitamin D3; VD3; 50 nM).

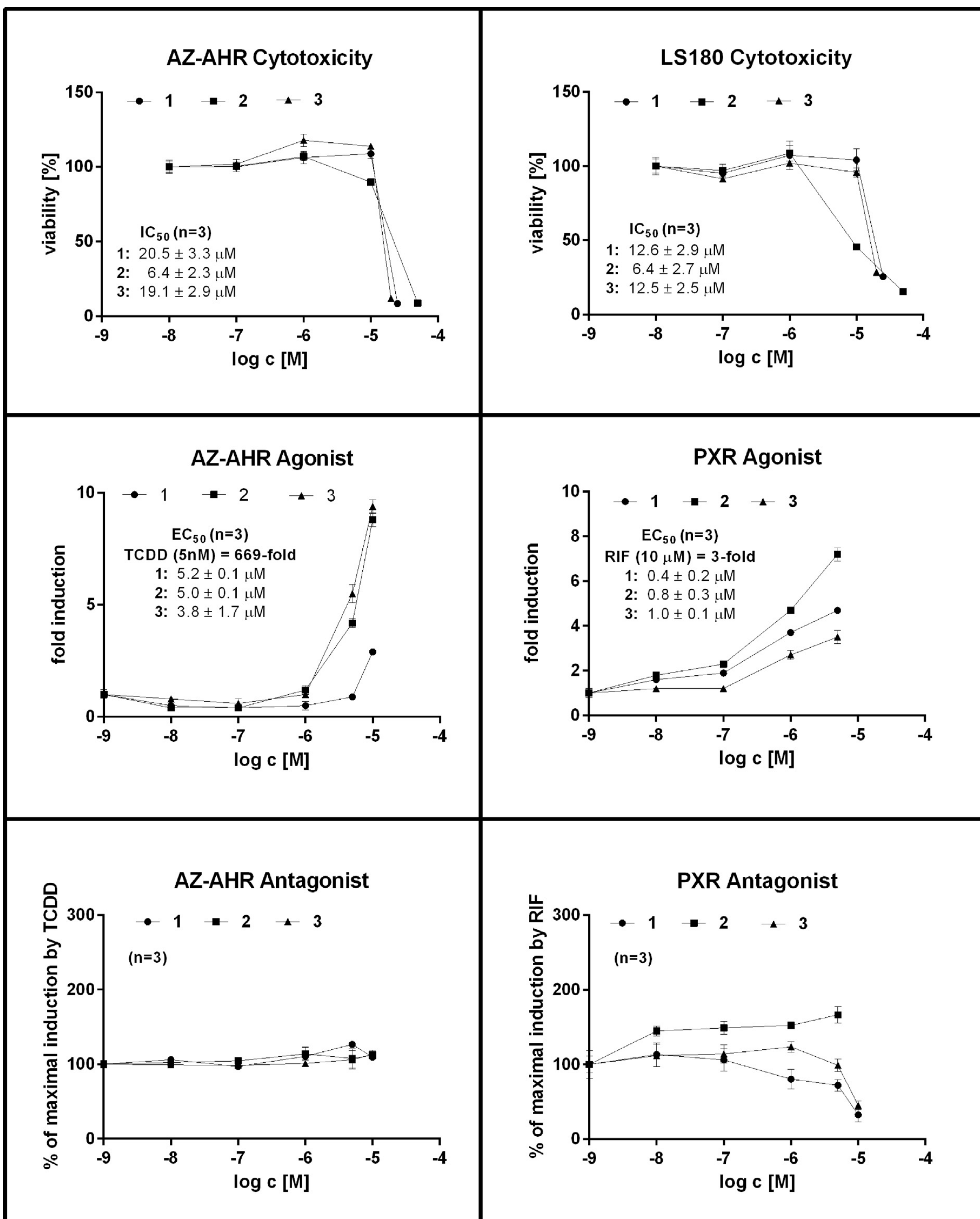

Fig. 2. (*continued*).

gold(I) complexes to induce selected target genes for steroid receptors, nuclear receptors and xenoreceptors in primary cultures of human hepatocytes, which are golden standard for toxicological and pharmacological studies. We measured the expression of tyrosine aminotransferase TAT, SPOT14, and xenobiotics-inducible drug-metabolizing cytochromes P450 CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9 and CYP3A4. We incubated five primary human hepatocytes cultures (HH61, HH64, HH65, HH66, Hep220879) for 24 h and 48 h with.

gold(I) complexes (1 μM; 10 μM), and model inducers for target genes, including dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), 3,3′,5-triiodo-L-thyronine (T3; 2 nM or 10 nM) and vehicle (UT; 0.1% DMF v/v).

The expression of CYP1A1 and CYP1A2 mRNAs and proteins was

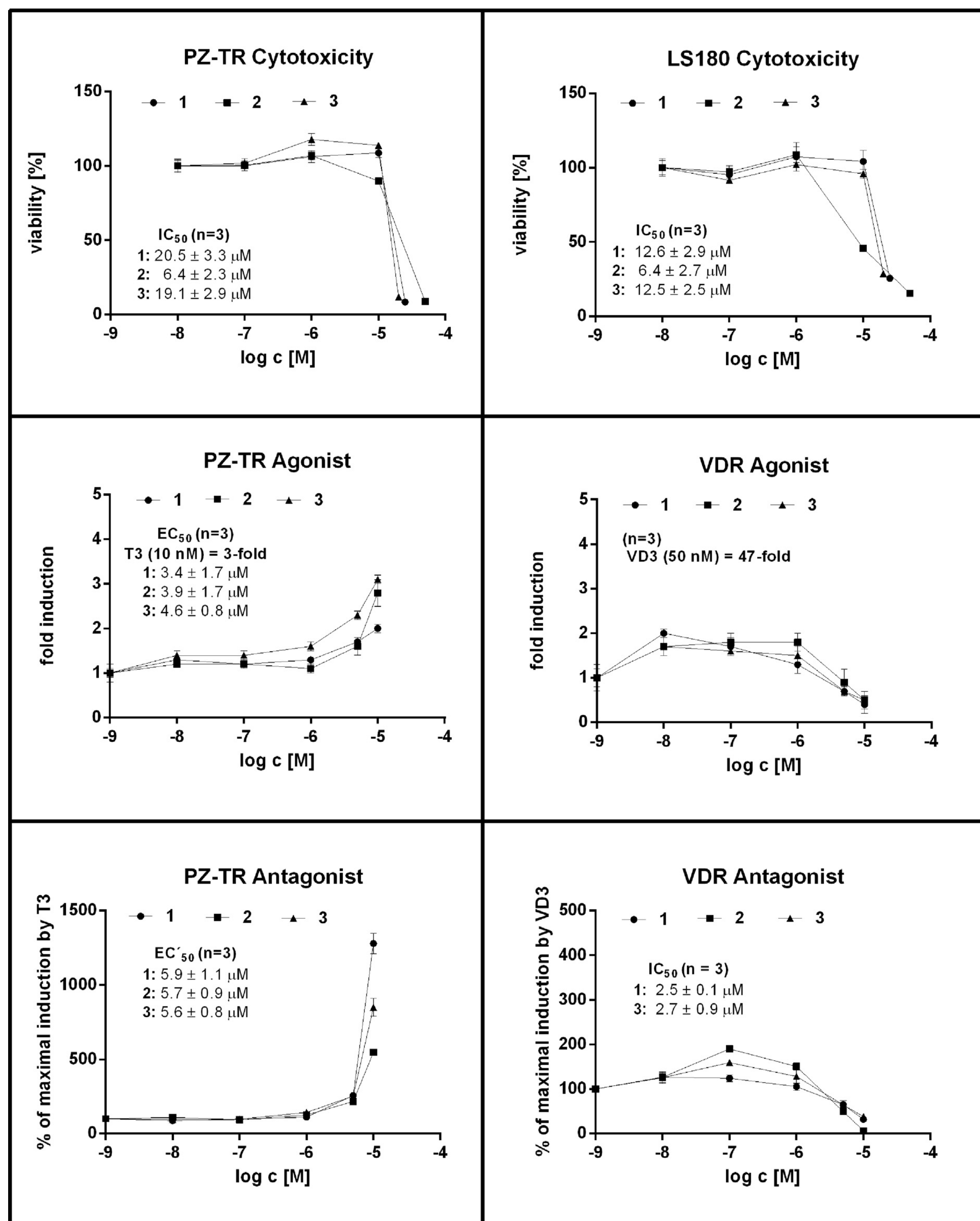

Fig. 2. (*continued*).

strongly induced by AhR activator TCDD in human hepatocytes cultures obtained from five different donors. Consistently with CYP1A1 mRNA induction in LS180 cells and AhR activation in gene reporter assays, gold(I) complexes **1, 2** and **3** weakly induced CYP1A1/CYP1A2 mRNAs and proteins. Rifampicin induced PXR-regulated CYP2A6, CYP2B6, CYP2C9 and CYP3A4 mRNAs in all human hepatocytes cultures. Tested compounds weakly induced CYP2A6, CYP2B6, CYP2C9 and CYP3A4 mRNAs, which is consistent with PXR activation in gene reporter assays. The induction profiles were highly inconsistent between individual cultures and for individual compounds, probably due to the inter-individual variability between the donors (Fig. 4). The expression of CYP3A4

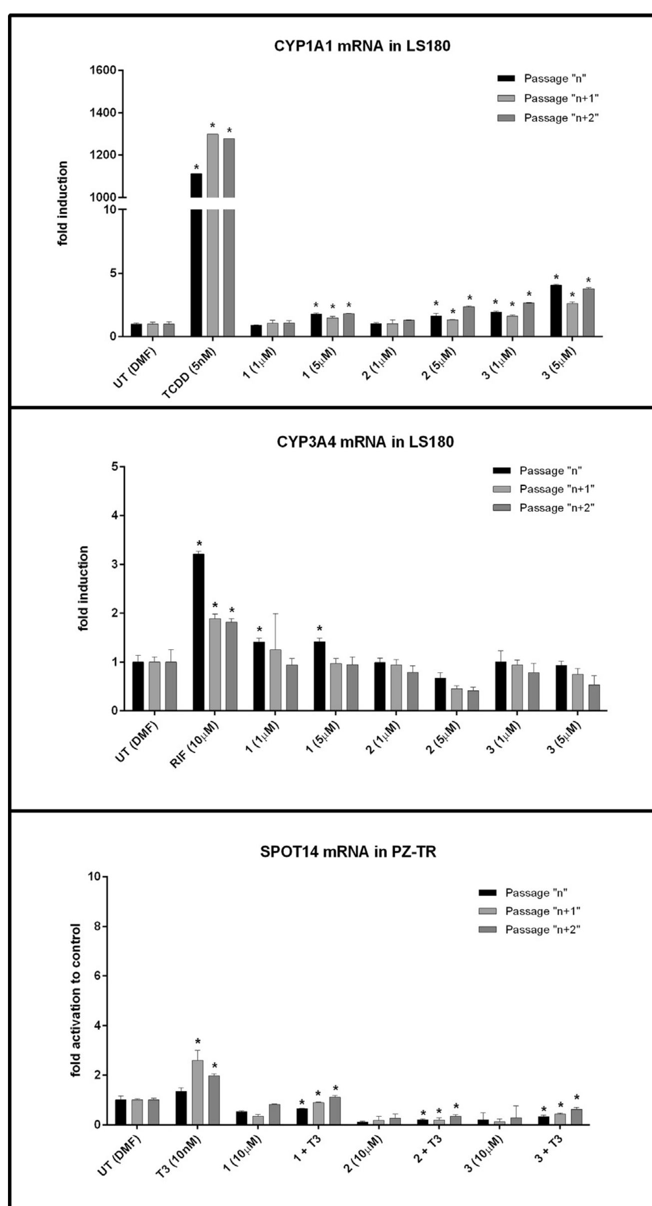

Fig. 3. Effects of the gold(I) mixed-ligand complexes (1–3) on the expression of CYP1A1, CYP3A4 and SPOT14 mRNAs in cell lines LS180 and PZ-TR. Cells were stabilized for 16 h and then incubated for 24 h with vehicle (UT; 0.1% DMF v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), 3,3′,5-triiodo-L-thyronine (T3; 10 nM) and gold(I) complexes (1 μM, 5 μM, 10 μM). Bar graphs of RT-PCR analyses of CYP1A1, CYP3A4 and SPOT14 mRNAs are shown. The data were normalized to GAPDH mRNA levels. The data are expressed as the mean ± SD from triplicate measurements from three consecutive passages and are expressed as the fold induction over the DMF-treated cells. * = values significantly different from the DMF-treated cells ($p < 0.05$).

protein was not significantly influenced by any tested compound (Fig. 5). The expression of GR-regulated TAT was dose-dependently decreased by complexes **1, 2** and **3** (Fig. 4), which is consistent with anti-glucocorticoid effects observed in gene reporter assays. The expression of TR-regulated SPOT14 mRNA was dose dependently decreased by all tested complexes, which further confirms the data from PZ-TR cells (Fig. 4).

4. Discussion

In the current paper we investigated the effects of gold(I) complexes towards transcriptional activity of nuclear receptors, receptors for steroid hormones and xenoreceptors. We found differential activities of tested compounds against transcriptional activities of evaluated receptors and the expression of their target genes. While gene reporter assays are potent and efficient tool for assessment of transcriptional activities *in vitro*, there are several factors that may substantially influence the output of the assay, and therefore, the data must be interpreted with prudence. Since the residues of tested compounds may persist in reaction mixture when measuring luciferase activity, we routinely perform the screening for inhibitory activity of the analytes against catalytic activity of luciferase. The decline higher than 20% from control activity is considered as disqualifying condition for testing compound in that particular concentration. Some compounds are cytotoxic against cell lines used in gene reporter assays, therefore, in parallel with luciferase measurement, we also perform MTT test and where appropriate, we normalize the relative luciferase units *per* cell viability.

Basal transcriptional activity of GR in stably transfected AZ-GR cells was not influenced by any tested complex, but ligand-inducible transcriptional activity of GR was dose-dependently decreased by **1, 2** and **3**. Taking in account no decline in cell viability by compounds up to 10 μM, their antagonist effects against GR are assumed. The anti-glucocorticoid effects of gold(I) complexes were further confirmed by down-regulation of GR-controlled TAT mRNA in human hepatocytes. All tested complexes dose-dependently antagonized both basal and ligand-inducible transcriptional activity of AR and VDR, revealing about inverse agonist behaviour. The effects of **1** and **2** could be also partly due to its cytotoxicity against AIZ-AR and LS180 cells, respectively. From medicinal chemistry points of view, inverse agonist activity of potential drugs is considered pharmacologically advantageous over antagonist behaviour. A condition *sin qua non* is that targeted receptor exerts intrinsic transcriptional activity in the absence of the ligand. Such constitutive activity is then diminished by inverse agonists, which may be a case of AR and VDR by tested complexes. Collectively, gold(I) complexes examined in the current paper are potent tool for simultaneous inhibition of AR, VDR and GR transcriptional activities by distinct mechanisms.

Compounds **1, 2** and **3** dose-dependently activated AhR, but the efficacy of complexes was very weak. Ligand-dependent transcriptional activity of AhR was not altered by any of tested complexes. All complexes weakly induced AhR-regulated CYP1A1 mRNA in LS180 cells and CYP1A1/CYP1A2 mRNAs and proteins in five primary human hepatocytes cultures. Complexes JG12, JG14 and JG15 dose-dependently increased PXR transcriptional activity. Ligand-inducible activity of PXR was dose-dependently inhibited by **1** and **3**, but slightly augmented by **2**. There was no CYP3A4 mRNA induction by any tested complex, in LS180 intestinal cancer cells. On the other hand, tested compounds weakly induced CYP2A6, CYP2B6, CYP2C9 and CYP3A4 mRNAs, in human hepatocytes. The induction profiles highly varied between individual cultures and for individual compounds, probably due to the inter-individual variability between the donors. The inconsistency of CYP3A4 mRNA data from LS180 cells as compared to gene reporter assays and human hepatocytes may reveal about cell type specific and tissue specific regulation of CYP3A4 [39]. All complexes dose-dependently increased both basal and T3-inducible luciferase activity in. PZ-TR cells. In contrast, gold(I) complexes decreased both basal and T3-inducible SPOT14 mRNA expression in LS180 cells and basal SPOT14 mRNA in human hepatocytes. The plausible explanation for that inconsistency would be a complex transcriptional regulation of SPOT14, which could involve other regulators besides TR. Indeed, hepatospecific regulation of SPOT14 by CAR and PXR

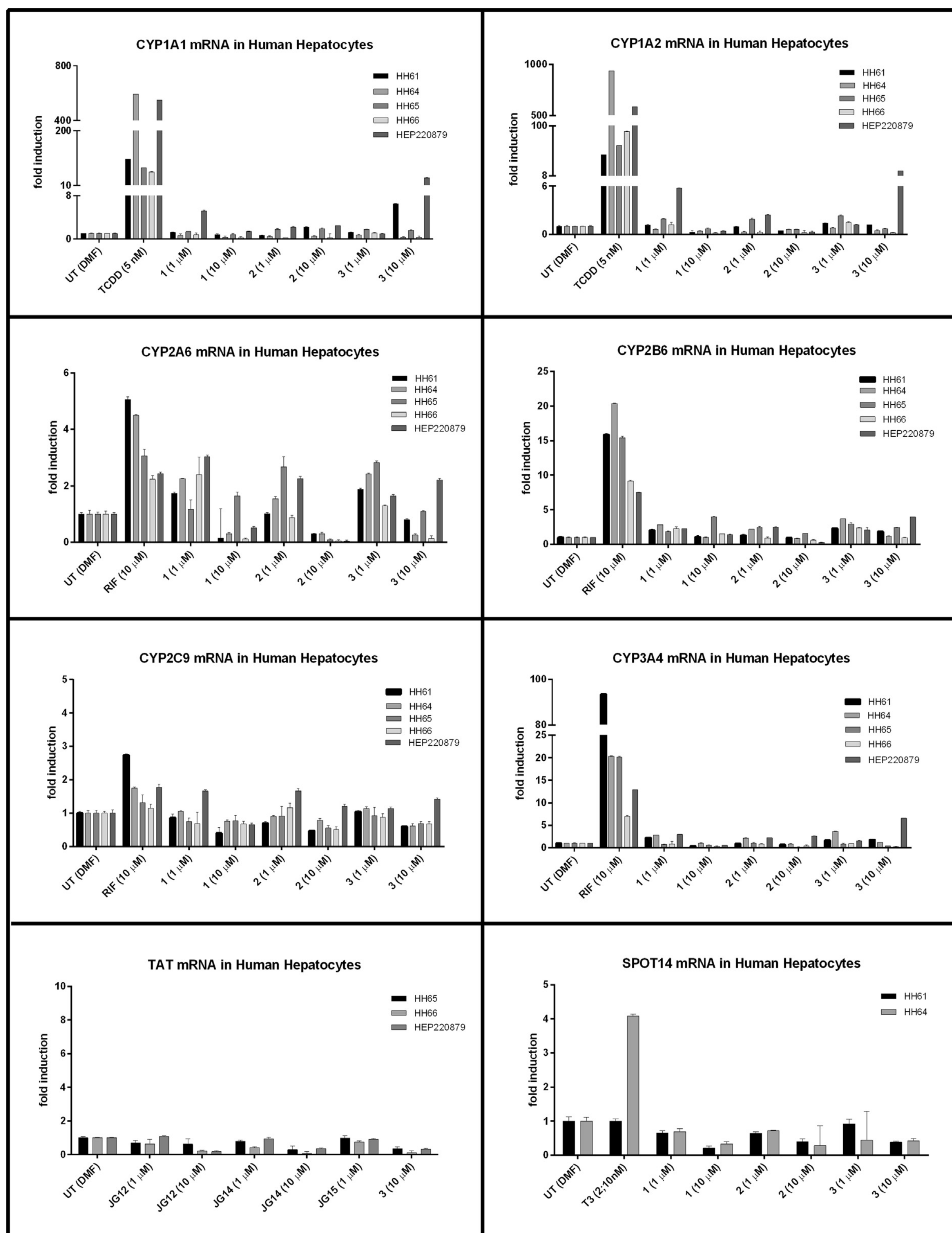

Fig. 4. Effects of the gold mixed-ligand complexes on the expression of drug-metabolizing cytochromes P450, tyrosine aminotransferase TAT and SPOT14 mRNAs in primary human hepatocytes. Human hepatocytes from five different donors (HH61, HH64, HH65, HH66, Hep220879) were incubated for 24 h with vehicle (UT; 0.1% DMF v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM), 3,3′,5-triiodo-L-thyronine (T3; 10 nM) and gold(I) complexes (1 μM, 10 μM). Bar graphs show RT-PCR analyses of CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP3A4, SPOT14 and TAT mRNAs. The data were normalized to GAPDH mRNA levels. The data are expressed as the mean ± SD from triplicate measurements and are expressed as the fold induction over the DMF-treated cells.

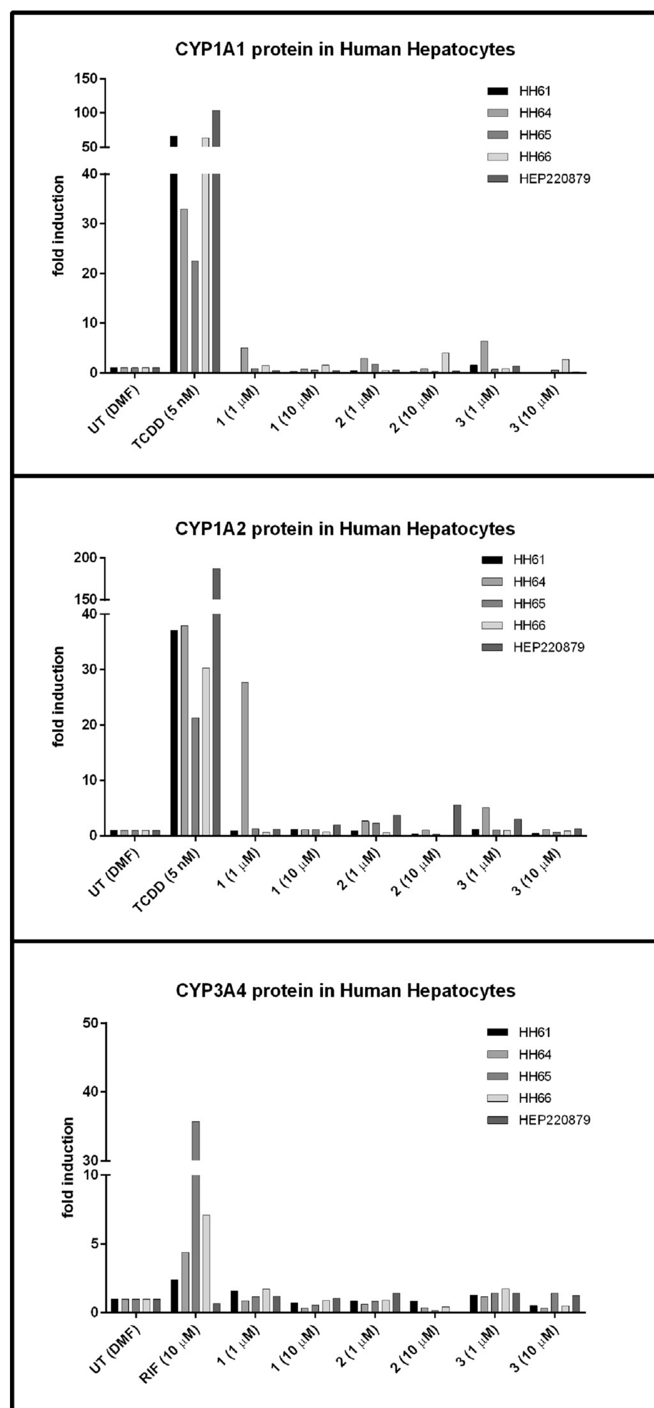

Fig. 5. Effects of the gold(I) mixed-ligand complexes (1–3) on the expression of CYP1A1, CYP1A2 and CYP3A4 proteins in primary human hepatocytes. Human hepatocytes from five different donors (HH61, HH64, HH65, HH66, Hep220879) were incubated for 48 h with vehicle (UT; 0.1% DMF v/v), dioxin (TCDD; 5 nM), rifampicin (RIF; 10 μM) and gold(I) complexes (1 μM, 10 μM). Quantified data from Simple Western blots of CYP1A1 are shown as bar graphs. The data are expressed as a fold induction over vehicle-treated cells and normalized to β-actin levels.

was demonstrated [40].

In conclusion, we described activation of AhR and PXR and induction of their target genes by gold(I) complexes; in addition we demonstrated anti-androgen and anti-glucocorticoid activities of studied compounds.

Conflict of interest

We declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.05.064.

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PALACKÝ UNIVERSITY OLOMOUC

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IN VITRO BIOLOGICAL ACTIVITY OF SELECTED COMPLEXES OF TRANSITION METALS

Ph.D. Thesis Summary

P1527 Biology

Mgr. Kateřina Kubešová, Dis.

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The Ph.D. thesis is an original work and based on my own research carried out within framework of Ph.D. study program P1527 Biology in the Department of Cell Biology and Genetics, Faculty of Science, Palacký University, Olomouc, from September 2013 to May 2017.

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SUMMARY

Metal compounds have an invaluable and vital role in medicinal chemistry. The D-block elements form a unique group, referred to as transition metals, in the periodic table. These elements use the *d* orbitals of their valence layer, which are gradually filled, to form chemical bonds. These elements have different oxidation states and could bind to different molecules or anions (referred to as ligands), to form coordination compounds. Metal atoms are often coordinated by negatively charged biomolecules (e.g. proteins and nucleic acids). Moreover, significant progress has reportedly been observed in the use of transition metal complexes for the treatment of human diseases, including carcinomas, lymphomas, anti-inflammatory, anti-diabetic, anti-infective, and neurological disorders. The current study enabled us to understand the mechanism of action and pharmacological effect of selected coordination compounds. Copper(II)/gold(I) mixed-ligand complexes containing quinolinone and *N*-donor heterocyclic ligands and/or triphenylphosphine and deprotonated forms of *O*-substituted derivatives 9-deazahypoxanthine in their structure were studied, respectively. Their effects on the transcriptional activity of receptors for steroid hormones (androgen receptor, glucocorticoid receptor), xenoreceptors (aryl hydrocarbon receptor, pregnane X receptor) and nuclear receptors (thyroid receptor, vitamin D receptor) were evaluated by employing the gene reporter assay, electrophoretic mobility shift assay (EMSA), qRT-PCR, and simple western blotting. The results revealed that gold(I) mixed-ligand complexes of 9-deazahypoxanthine exhibited pleiotropic effects against the panel of tested receptors, and that mixed-ligand copper(II) complexes activated the aryl hydrocarbon receptor AhR, and induced CYP1A gene expression in human hepatocytes and human cancer cell lines.

SOUHRN

Sloučeniny kovů mají neocenitelnou a zásadní úlohu v lékařské chemii. Prvky *d-bloku* reprezentují v periodickém systému unikátní skupinu označovanou jako přechodné kovy. Tyto prvky využívají k tvorbě chemických vazeb postupného zaplňování *d* orbitalů jejich valenční vrstvy. Kromě toho se vyskytují v různých oxidačních stavech a mohou na sebe vázat různé molekuly nebo anionty (označované jako ligandy) za vzniku koordinačních sloučenin. Atomy kovu jsou často koordinovány záporně nabitými biomolekulami (např. proteiny a nukleovými kyselinami). Výzkumné studie navíc ukázaly významný pokrok ve využití komplexů přechodných kovů pro léčbu lidských onemocnění, jako jsou karcinomy, lymfomy, protizánětlivé, anti-diabetické, anti-infekční a neurologické onemocnění. Důvodem pro vypracování současné studie bylo porozumění mechanismu účinku a popis farmakologické účinnosti vybraných koordinačních sloučenin. Z tohoto důvodu byly studovány měďnaté/zlatné komplexy obsahující ve své struktuře chinolinon a *N*-donorové heterocyklické ligandy a/nebo trifenylfosfin a deprotonovanou formu *O*-substituovaných derivátů 9-deazahypoxantinu. Hodnotily se jejich účinky na transkripční aktivitu receptorů pro steroidní hormony (androgenní receptor, glukokortikoidní receptor), xenoreceptorů (aryl uhlovodíkový receptor, pregnanový X receptor) a jaderných receptorů (thyroidní receptor, receptor pro vitamin D), za využití reportérové eseje a gelové retardační analýzy (EMSA), qRT-PCR a western blotu. Hlavní zjištěné poznatky jsou takové, že zlatné komplexy s deriváty 9-deazahypoxantinu vykazují pleiotropní účinky vůči panelu testovaných receptorů a že měďnaté komplexy aktivují aryl uhlovodíkový receptor a indukují expresi genu CYP1A v lidských hepatocytech a lidských nádorových buněčných liniích.

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1 AIMS

The current study aimed to investigate the effects of the selected copper(II) and gold(I) mixed-ligand complexes on the signalling pathways of human nuclear receptors, steroid receptors, and xenoreceptors, evaluate the following:

1. The transcriptional activities of androgen receptor, glucocorticoid receptor, receptor for thyroid hormone, vitamin D receptor, aryl hydrocarbon receptor, and pregnane X receptor in human cancer cell lines, by means of reporter gene assays and EMSA.
2. The expression of target genes for the above-listed receptors at the mRNA and protein levels in primary cultures of human hepatocytes and human cancer cell lines. These genes included *CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP3A4*, tyrosine aminotransferase (*TAT*) and *SPOT14*.

2 INTRODUCTION

Humans are permanently exposed to a variety of exogenous chemicals (xenobiotics), such as food additives, metals, pesticides, alkaloids, environmental pollutants, and drugs. If the human body is exposed to xenobiotics, biochemical regulatory pathways might be disrupted, which could lead to an imbalance in the organism, resulting in the onset of disease. Physiological (sex, age, nutrition, pregnancy, and polymorphisms) and pathophysiological (inflammation, infection, environmental pollutants, oxidative stress, and hypoxia) factors also play a crucial role in the regulation of xenobiotic metabolism. Therefore, it is important to understand the molecular basis of interactions between xenobiotics and the signalling pathways involved in eubiotic and xenobiotic metabolism.

Cytochromes P450 (CYPs) are enzymes that play a pivotal role in oxidative xenobiotic biotransformation. CYPs could be transcriptionally activated by various xenobiotics and endogenous substrates through receptor-dependent mechanisms. CYP enzyme inhibition is a principal mechanism that enables metabolism-based drug-drug interactions (DDIs) to occur. The use of many chemotherapeutic drugs could cause DDIs to occur owing to their ability to either inhibit or induce the CYP enzyme system. Genetic polymorphisms and epigenetic changes in CYP genes might be responsible for inter-individual and inter-ethnic variations in disease susceptibility and the therapeutic efficacy of drugs.

Organometallic compounds have received a great amount of attention in the past three decades because they are potential chemotherapeutic agents (Brabec and Novakova, 2006; Liu and Sadler, 2011). Moreover, it has been unambiguously demonstrated that these compounds might also exhibit anti-inflammatory, anti-microbial (Berners-Price and Filipovska, 2011), or anti-viral activity (e.g. anti-HIV) (Lewis et al., 2011) in both *in vitro* and *in vivo* systems, depending on the central atom (metal) and ligand type. The discovery of the anti-proliferative properties of *cis*-diammine-dichloridoplatinum(II), *cis*-$[Pt(NH_3)_2Cl_2]$, commonly known as cisplatin, in the late 1960s had sparked an interest in metal-based anticancer drugs (Rosenberg et al., 1967). Currently, this platinum-based chemotherapeutic agent remains the most widely used drug (Kelland and Farrell, 2000) in oncology patients. Though it is primarily used in the treatment of cancer (e.g. cancer of the ovaries or testicles), it is also used against other types of tumours (sarcomas, lymphomas) (Zhang and Lippard, 2003). Unfortunately, the use of cisplatin is limited because of a number of drawbacks (e.g. nephrotoxicity, neurotoxicity, and myelosuppression) (Wang and Lippard, 2005). Additionally, there is a risk of the patient developing drug resistance to cisplatin. Consequently, tremendous efforts have been devoted to the research and development of new anti-tumour active coordination compounds with improved and/or selective pharmacological properties (Alessio, 2011). A number of transition metal complexes have been tested to estimate their biological activity, particularly complexes of platinum, but also complexes of copper, zinc, iron, cobalt, and nickel, etc. However, only a few cisplatin derivatives have been approved for treatment (Tiekink and Gielen, 2005). Therefore, designing new metal complexes is one possible way to discover new biologically active coordinating compounds. Numerous studies have shown that coordination with a suitable metal can cause a change or increase in the biological activity of the complex, as compared to that of parental molecule (Vanco et al., 2017).

The present study focused on gold(I) and copper(II) mixed-ligand complexes. Gold(I), the central atom, was coordinated with an *O*-substituted derivative of 9-deazahypoxanthine. In addition, a triphenylphosphine molecule was introduced into the structure. The central atom of copper(II) was coordinated with bidentate *N*-donor heterocyclic ligands, quinolinone, and quinolinone-7-carboxamide derivatives. Their complexes were also extensively studied, particularly with respect to cancer therapy (Tardito and Marchio, 2009). Their activities of several biologically active complexes of transition metals at nuclear receptors and the expression of their target genes were investigated.

3 METAL-BASED COMPLEXES

The d-block elements, called transition metals (e.g. platinum, copper, gold, zinc, iron, cobalt, nickel), have a characteristic ability to exist in many different oxidation states, which could be very important to form very stable metal-based complexes. Organometallic compounds have received a great amount of attention in the past three decades because they are potential chemotherapeutic agents (Brabec and Novakova, 2006; Liu and Sadler, 2011). In addition, the transition metal complexes may be also used for treatment of human diseases such as carcinomas, lymphomas, anti-inflammatory, anti-diabetic, anti-infective, anti-viral and neurological disorders (Berners-Price and Filipovska, 2011; Lewis et al., 2011), depending on the central atom (metal) and the ligand type. The present study focused on gold(I) and copper(II) mixed-ligand complexes.

Copper is a biogenic element that is indispensable for the human organism, and therefore appears to be suitable for the preparation of biologically active non-platinum compounds. Structurally, copper(II) mixed-ligand complexes under study containing quinolin-4(1*H*)-one skeleton, known for their biological activities (Figure 1A), and *N*, *N'*-donor heterocyclic ligands. Specifically, four copper(II) mixed-ligand complexes with the general composition [Cu(*qui1*)(L)]Y·*x*H_2O (**1**-**3**) and [Cu(*qui2*)(phen)]Y (**4**), where *Hqui1* = 2-phenyl-3-hydroxy-4(1*H*)-quinolinone (Figure 1B), *Hqui2* = 2-(4-amino-3,5-dichlorophenyl)- *N*-propyl-3-hydroxy-4(1*H*)-quinolinone-7-carboxamide (Figure 1C), Y = NO_3 and L = 1,10-phenanthroline (phen) (**1**), 5-methyl-1,10-phenanthroline (mphen) (**2**), and bathophenanthroline (bphen) (**3**) (Figure 2; **1**-**4**) were evaluated to investigate their ability to influence the expression of major drug-metabolizing enzymes and transcriptional activities of steroid receptors, nuclear receptors, and xenoreceptors.

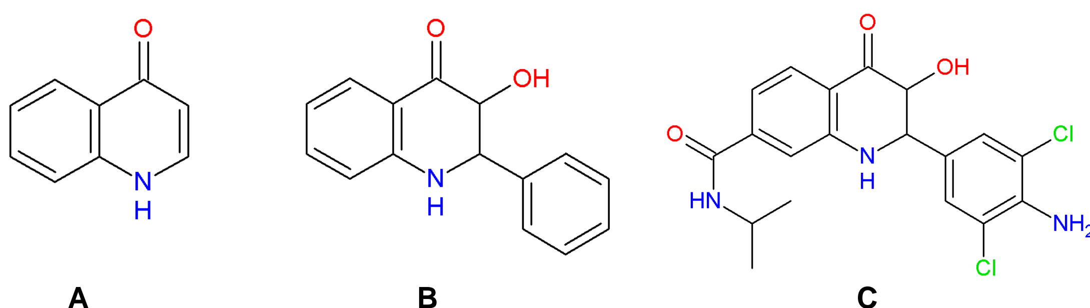


Figure 1. The structural formulas ligands under study of copper(II) mixed-ligand complexes. *The structures of quinolin-4(1H)-one skeleton (A), 2-phenyl-3-hydroxy-4(1H)-quinolinone (Hqui1) (B) and 2-(4-amino-3,5-dichlorophenyl)-N-propyl-3-hydroxy-4(1H)-quinolinone-7-carboxamide (Hqui2) (C) are shown (ACD/ChemSketch).*

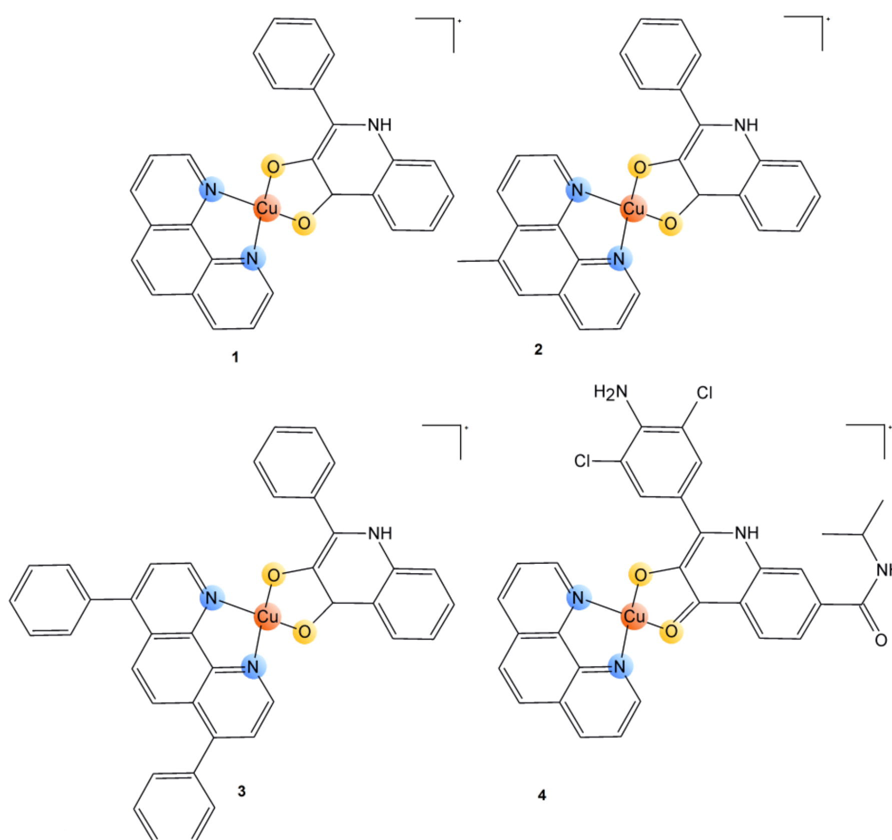


Figure 2.
The structural formulas of copper(II) mixed-ligand complexes:
Four structure of copper(II) complexes [Cu(qui1)(phen)]$NO_3.H_2O$ (1), [Cu(qui1)(mphen)]$NO_3.H_2O$ (2), [Cu(qui1)(bphen)]$NO_3.H_2O$ (3) and [Cu(qui2)(phen)]NO_3 (4) are shown. Copper(II) quinolinone mixed-ligand complexes were designed and prepared at the Department of Inorganic Chemistry, Palacký University, Olomouc (Buchtik et al., 2012; Buchtik et al., 2011; Krikavova et al., 2016; Travnicek et al., 2014).

The 1,10-phenanthroline-based ligands (known as Casiopeinas®) were the first reported copper(II) complexes that inhibited tumour growth *in vivo* (Dwyer et al., 1965). Since then, many copper(II) complexes containing 1,10-phenanthroline (phen) and other bidentate *N*-donor heterocyclic ligands (N-N) have been synthesized and studied to determine if they have anticancer properties.

In general, copper complexes are an object of interest because of their demonstrated anti-oxidant (Vanco et al., 2004), anti-diabetic (Murata et al., 1998), anti-inflammatory (Weder et al., 2002), anti-microbial (Psomas et al., 2006), anti-parasitic (Gokhale et al., 2003), and cancerostatic effects (Giovagnini et al., 2008). All these derivatives have a common mechanism of action, i.e. they target DNA-gyrases. These enzymes (topoisomerase II and IV) are involved in DNA replication, and quinolones act as potent topoisomerase II inhibitors (Pommier et al., 2010).

Since ancient times, gold has been known as an element that has medicinal effects (Higby, 1982). Interest in gold compounds increased after the discovery of the use of gold cyanide $K[Au(CN)_2]$ as an anti-tuberculosis drug by Robert Koch in 1890 (Koch, 1890). Structurally, gold(I) mixed-ligand complexes under study containing triphenylphosphine (Figure 3A) and deprotonated form of *O*-substituted 9-deazahypoxanthine (Figure 3B). The complexes of gold with *O*-substituted 9-deazahypoxanthine were found to be highly biologically active (Galikova and Travnicek, 2015; Vanco et al., 2017).

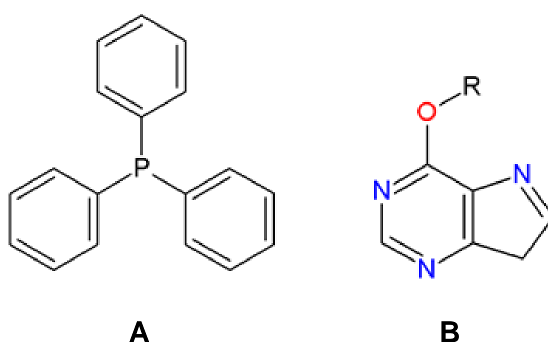


Figure 3. The structural formulas ligands under study of gold(I) mixed-ligand complexes. *Structure of triphenylphospine (A) and deprotonated form of O-substituted derivatives of 9-deazahypoxanthine (B) are shown (ACD/ChemSketch).*

The gold(I) complexes having the composition $[Au(L_n)(PPh_3)]$ exhibited selective activity against some human cancer cell lines. Moreover, these complexes also showed anti-inflammatory activities that were similar to that of a commercially used anti-arthritis drug, Auranofin (Vanco et al., 2014). Therefore, we studied gold(I) phosphine mixed-ligand complexes having the general formula $[Au(L_n)(PPh_3)]$ (**1-3**), where HL_n stands for various *O*-substituted derivatives of 9-deazahypoxanthine suchas 6-isopropyloxy-9-deazahypoxanthine (HL_1) (**1**), 6-phenethyloxy-9-deazahypoxanthine (HL_2) (**2**), 6-benzyloxy-9-deazahypoxanthine (HL_3) (**3**) (Figure 6; **1-3**), and PPh_3 which represents triphenylphosphine (Figure 5A). These compounds were evaluated for their capability to influence the expression of major drug-metabolizing enzymes and transcriptional activities of steroid receptors, nuclear receptors and xenoreceptors.

It is generally known that the substitution of organic ligands, such as the aforementioned N6-benzyladenines, which constitute the structures of transition metal complexes, modulate the biological activity of such complexes (Lemoine et al., 2004; Sorenson, 1976). This fact motivated us to focus on gold(I) triphenylphosphine complexes during the present study. Gold(I) complexes containing organic phosphine

and heterocyclic *N*-donor ligands have received attention because of their biological activity, predominantly because of their cytotoxic (Lima and Rodriguez, 2011), biocidal (Navarro, 2009) and anti-inflammatory activities (Travnicek et al., 2012). Gold(I) coordination compounds are known to play several biochemical roles (Gielen et al., 2005). It has been shown that the gold(I) complexes that exhibit the cytotoxic and antitumor activities do not primarily target DNA; their main targets are the components of the proteasome. In addition, it was shown that gold(I) compounds interact with the active site of cytosolic and mitochondrial thioredoxin reductases (TrxR); acting as potent TrxR inhibitors (Buac et al., 2012).

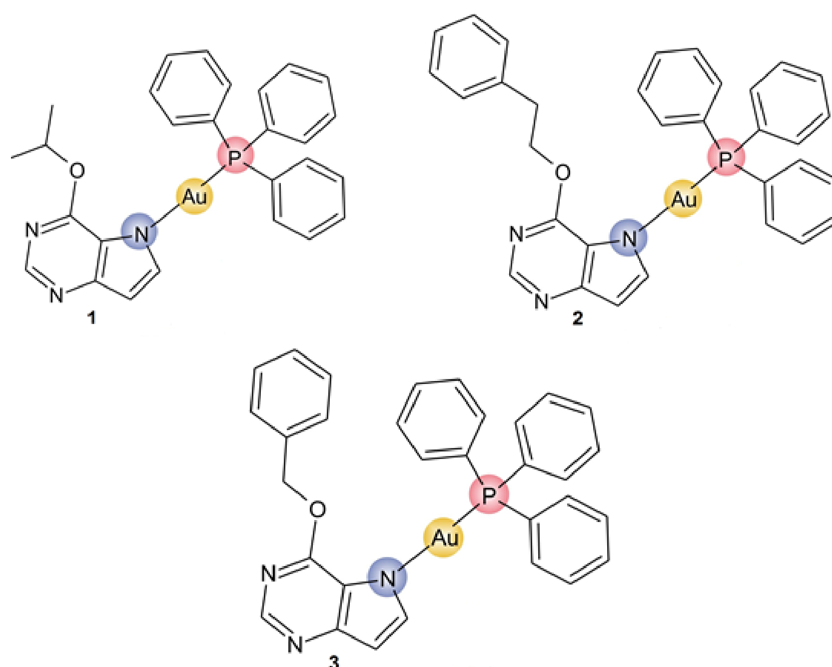


Figure 4.
The structural formulas of gold(I) mixed-ligand complexes.
Three structure of gold(I) complexes [Au(HL$_1$)(PPh$_3$)] (1); [Au(HL$_2$)(PPh$_3$)] (2); [Au(HL$_3$)(PPh$_3$)] (3) are shown.Gold(I) – phosphine mixed-ligand complexes have been designed and prepared at Department of Inorganic Chemistry, Palacký University, Olomouc (Krikavova et al., 2014; Vanco et al., 2014).

4 MATERIALS AND METHODS

4.1 BIOLOGICAL MATERIAL

4.1.1 Human hepatocytes

Primary human hepatocyte cultures were obtained from two sources: (i) *long-term human hepatocytes in monolayer "*Batch HEP220879" (female; 65 years) were isolated after liver biopsy at Biopredic International (France); (ii) liver segments were resected from the multiorgan donors HH61 (male; 64 years), HH64 (male; 73 years), HH65 (male; 34 years), and HH66 (male; 65 years) at Faculty Hospital, Olomouc. The tissue acquisition protocol and the use of liver cells was approved by the "Ethical committee at Faculty Hospital, Olomouc", and the protocol was in accordance with the Transplantation Act #285/2002 Coll. Cells were seeded in collagen-coated plates in a hormonally and chemically defined medium consisting of a mixture of William's E and Ham's F-12 [1:1 (v/v)] (Isom et al., 1985; Pichard-Garcia et al., 2002) and were stabilized for 24 h before treatment. Cultures were maintained in a humidified incubator (37 ºC; 5% CO_2). Hepatocytes were treated with the studied complexes, inducers, and/or the vehicle (DMF; 0.1%, v/v) for 24 h (RNA) and 48 h (protein).

4.1.2 Human cancer cell lines

Human Negroid cervix epithelioid carcinoma cells or HeLa cells (ECACC No. 93021013), human Caucasian hepatocellular carcinoma cells or HepG2 cells (ECACC No. 85011430), human Caucasian colon adenocarcinoma cells or LS180 cells (ECACC No. 87021202), human Caucasian breast adenocarcinoma cells or MCF7 cells (ECACC No. 86012803) and human prostate carcinoma epithelial cells or 22Rv1 cells (ECACC No. 05092802) were purchased from the European Collection of Authenticated Cell Cultures (ECACC). Cell lines HeLa, HepG2, LS180, and MCF7 were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 µg/mL penicillin, 100 U/mL streptomycin, 1% non-essential amino acids, 4mM L-glutamine, and 1 mM sodium pyruvate. The cell line 22Rv1 was cultured in RPMI 1640 Medium (RPMI) enriched with the same components as DMEM, except for the non-essential amino acids. Cell cultures were maintained at 37 ºC and 5% CO_2 in a humidified incubator. AZ-GR (Novotna et al., 2012), AZ-AHR (Novotna et al., 2011) PZ-TR (Illes et al., 2015) and AIZ-AR cells (Bartonkova et al., 2015) were incubated under the same conditions that were provided while culturing as parental cells.

4.2 COMPOUNDS AND REAGENTS

N,N´-dimethylformamide (DMF), dimethylsulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hygromycin B, dexamethasone (DEX), rifampicin (RIF), dihydrotestosterone (DHT), 3,3´,5-triiodo-*L*-thyronine (T3), resveratrol (RVT), culture medium (DMEM, RPMI), non-essential amino acids, L-glutamine, penicillin and streptomycin were purchased from Sigma-Aldrich (Czech Republic). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from Ultra Scientific (USA). 25-hydroxyvitamin D3 (VD3) was purchased from Santa Cruz Biotechnology Inc. (Germany). TRI Reagent® was acquired from the Molecular Research Center (USA). M-MuLV Reverse Transcriptase and random hexamers were purchased from New England Biolabs (USA). The oligonucleotide primers used in qRT-PCR reactions were synthesized by Generi Biotech (Czech Republic). Light-Cycler® 480 Probes Master and protease/phosphatase inhibitor cocktails were purchased from Roche Diagnostic Corporation (Czech Republic). Luciferase lysis buffer and FuGENE® HD Transfection Reagent were obtained from Promega (USA). Reagents used for Simple Western Blotting by Sally Sue™, antibody diluent, a goat anti-rabbit IgG antibody, and a goat anti-mouse IgG antibody were purchased from ProteinSimple (California). Primary antibodies against CYP1A1 (goat polyclonal; sc-9828, G-18), CYP1A2 (mouse monoclonal; sc-53614, 3B8C1), CYP2A6 (mouse monoclonal; sc-53615, F16P2D8), CYP2B6 (rabbit polyclonal; sc-67224, H-110), CYP3A4 (mouse monoclonal; sc-53850, HL3), a rabbit anti-goat IgG secondary antibody (sc-2768, H0712), secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology Inc. (Germany). A β-actin antibody (mouse monoclonal; 3700S, 8H10D10) was obtained from Cell Signaling Technology (USA). A Light Shift Chemiluminescent EMSA Kit was purchased from Thermo Scientific (USA). All other chemicals purchased were of the highest quality commercially available.

Gold(I) triphenylphosphine complexes with the general compositions of $[Au(L_n)(PPh_3)]$, **1** (L_1 = 6-isopropyloxy-9-deazahypoxanthine); **2** (L_2 = 6-phenethyloxy-9-deazahypoxanthine); **3** (L_3 = 6-benzyloxy-9-deazahypoxanthine) were synthesized and characterized at the Department of Inorganic Chemistry, Palacký University, Olomouc as described previously (Vanco et al., 2014). The copper(II) quinolinone complexes with general formulas $[Cu(qui1)(L_n)]NO_3{\cdot}H_2O$ and $[Cu(qui2)(phen)]NO_3$, where *qui1* = 2-phenyl-3-hydroxy-4(1H)-quinolinone, *qui2* = 2-(4-amino-3,5-dichlorophenyl)-N-propyl-3-hydroxy-4(1H)-quinolinone-7-carboxamide, L_1 = 1,10-phenanthroline (phen), L_2 =

5-methyl-1,10-phenanthroline (mphen), L_3 = bathophenanthroline (bphen) were synthesized and characterized at the Department of Inorganic Chemistry, Palacký University, Olomouc as described previously (Buchtik et al., 2011; Krivavova et al., 2016; Travnicek et al., 2014).

4.3 METHODS

4.3.1 Cell viability assay

The human cancer cell lines 22Rv1, HeLa, HepG2, and LS180 were seeded in the 96-well culture plates (3×10^5 cells per well). After 16h of pre-incubation, the cells were treated with copper(II) and gold(I) complexes, in concentrations ranging from 1 nM to 50 µM (unless the solubility was lower) for 24 h. In parallel, the cells were treated with the vehicle (DMF; 0.1%, v/v; negative control) and Triton X-100 (TX100; 1%, v/v; positive control) to assess the minimal and maximal cell damage, respectively. Thereafter, the medium was removed, the cells were washed with phosphate buffered saline 1× (PBS) and incubated with MTT (100 µL; 0.3 mg/ml) for 1 h at 37°C. The reaction was stopped by removing MTT solution and adding 40-100 µl of DMSO. Absorbance was measured spectrophotometrically at 570 nm, using Infinite M200 (Schoeller Instruments, Czech Republic). The data were expressed as a percentage of cell viability, where 100% and 0% represented negative and positive controls, respectively. The data were acquired from three independent experiments (cell passages). The concentrations that caused a decline in viability that was no greater than 20% were considered to be non-toxic, and were used for further experiments.

4.3.2 Plasmids

A chimeric p3A4-luc reporter construct containing the basal promoter (-362/-53), in which the proximal PXR response element (ER6) and distal xenobiotic-responsive enhancer module (-7836/-7208) of the CYP3A4 gene 5´-flanking region was inserted to the pGL3-Basic reporter vector (Promega, UK) has been described (Goodwin et al., 1999). The expression plasmid for human PXR (pSG5-hPXR), that encoded human PXR was provided by Dr. S. Kliewer (University of Texas, Dallas, USA). The expression plasmid pSG5-hVDR that encoded human VDR was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). The pCYP24-luc plasmid containing the 5´-flanking region (−1200 to −22) of the human CYP24 gene subcloned

into the pGL3 plasmid upstream of the firefly luciferase reporter gene was provided by Dr. J. M. Pascussi (INSERM U632, Montpellier, France). The luciferase reporter construct pSG5-luc and pGL3 plasmid were purchased from Promega.

4.3.3 Luciferase inhibition assay

The highest concentration of tested compounds used during the previous experiments were added to cell lysates containing firefly luciferase, and luciferase activity was measured using the Tecan Infinite M2000 plate luminometer.

4.3.4 Transfection and luciferase gene reporter assay

Stably transfected human luciferase gene reporter cell lines AZ-AHR, AZ-GR, AIZ-AR, and PZ-TR were used to examine the transcriptional activities of the aryl hydrocarbon receptor (AhR), glucocorticoid receptor (GR), androgen receptor (AR), and thyroid hormone receptor (TR), respectively. Transiently transfected LS180 cells were used for the analysis of the transcriptional activity of vitamin D receptor (VDR) and pregnane X receptor (PXR) (Vrzal et al., 2011).

Reporter cell lines were seeded into to the 96-well plates (density 3×10^5 cells per well). After 16 h of incubation, the cells were treated with the tested compounds, TCDD (5 nM), DEX (100 nM), DHT (100 nM), T3 (10 nM) and/or vehicle (DMF; 0.1%; v/v). LS180 cells were transiently transfected by lipofection (FuGENE®HD Transfection Reagent) using 300 ng per well of the reporter gene (*pCYP24-luc* or *p3A4-luc*) and 100 ng per well of expression vectors (hPXR or hVDR) in 24-well plates. Prior to the treatments, cultures were stabilized for 16 h and then incubated for 24 h with the tested compounds, RIF (10 µM), VD3 (50 nM), and/or vehicle (DMF; 0.1%, v/v). After the treatments, cells were lysed (Reporter Lysis Buffer) and the luciferase activity was measured using the Infinite M200 plate luminometer (Schoeller Instruments, Czech Republic).

4.3.5 RNA isolation, reverse transcription and qRT-PCR

LS180 cells and primary human hepatocytes were seeded in 6-well plates (density: $1\text{x}10^6$ cells per well). After 16 h of incubation, the cells were treated with the tested compounds, TCDD (5 nM), RIF (10 µM), and/or vehicle (DMF; 0.1%, v/v). The total RNA was isolated using TRI Reagent®. The cDNA was synthesized from 1000 ng of total RNA, according to a common protocol that use M-MuLV Reverse Transcriptase

and random hexamers at 42 ºC for 60 min. The real-time quantitative reverse transcription polymerase chain reaction (real time qRT-PCR) was carried out using Light Cycler® 480 Probes Masteron a Light Cycler 480 II instrument (Roche Diagnostic Corporation, Czech Republic). The mRNA levels were determined using primers and the Universal Probes Library (UPL; Roche Diagnostic Corporation, Czech Republic) technology described previously (Vrzal et al., 2015).

The steps used were as follows: an activation step at 95 °C for 10 min was followed by 45 cycles of PCR (denaturation at 95 °C for 10 s; annealing with elongation at 60 °C for 30 s). The measurements were performed in triplicates. The gene expression levels were normalized to those of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Data were processed by using the delta-delta method (Pfaffl, 2001). Results are expressed in terms of the x-fold induction over DMF-treated cells.

4.3.6 Simple western blotting by SallySue™

The total protein extracts were obtained as follows: the cells were washed twice with ice-cold PBS, scraped into 1 mL of PBS, and a suspension was centrifuged (4500 rpm/5 min/4°C). The pellet was re-suspended in 150 µL of ice-cold lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.2, 0.1% (w/v) SDS, anti-protease cocktail, 1% (v/v) Triton X-100, anti-phosphatase cocktail, 1% (v/v) sodium deoxycholate, 5 mM EDTA). After homogenization, the mixture was vortexed and incubated for 10 min on ice and centrifuged (13500 rpm/15 min/4°C). The supernatant was collected, and the protein content was determined using the Bradford assay. The samples were stored at – 80°C.

The Sally Sue Simple Western System was used for the detection of CYP proteins using to the ProteinSimple manual (Sally Sue, USA). The following diluted primary antibodies were used: CYP1A1 (dilution 1:25), CYP1A2 (dilution 1:250), CYP3A4 (dilution 1:10000), and β-actin (dilution 1:100). Target proteins were identified using primary antibodies and immune-probed using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate. The resulting chemiluminescent signal was analysed and quantified by Compass Software, version 2.6.5.0 (ProteinSimple). For quantitative data analysis, the signals were normalized to those of β-actin, which was used as a loading control.

4.3.7 Electrophoretic mobility shift assay (EMSA)

MCF-7 cells (density: $2x10^6$ cells per well) were seeded in 6-well plates. After 16 h of pre-incubation, the cells were treated for 2 h with 1 µM of the copper(II) complexes (**1**–**4**), TCDD (5 nM), and vehicle, used as a negative control (DMF; 0.1%, v/v). Nuclear and cytoplasmic fractions were isolated using the Nuclear Extract Kit according to the protocol provided by the manufacturer (Active Motif). The following double-stranded
5´-biotinylated oligonucleotides containing DNA-binding sequences specific for AhR, which corresponded to the 27-bp protein binding site of DRE 3 were used (Denison et al., 1988):

sense 5′ − GATCCGGCTCTTCTCACGCAACTCCGAGCTCA − 3′;
antisense 5′ − GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG − 3′

A gel mobility shift assay was performed using the LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, Waltham, MA, USA). The nuclear protein extract (15 µg) was pre-incubated in binding buffer (final concentrations: 10 mMTris, 50 mM KCl, 1 mM DTT; pH 7.5), along with 2.5% glycerol, 0.05% NP-40, ddH_2O and non-specific competitor Poly (dI.dC); then, the biotin-labelled probe was added (10 fmol/µL). An un-labelled probe (2 pmol/µL) was added to the reaction mixture for the competitive assay. The complete reaction mixture was incubated at room temperature for 20 min. Finally, 5 µL of 5X loading buffer was added before loading the mixture in the wells of a non-denaturing 5% polyacrylamide gel for electrophoretic separation. The protein-DNA complexes were electro-blotted to a positively charged nylon membrane. Thereafter, transferred DNA was cross-linked using a UV-light cross-linker instrument. Biotin-labelled DNA was detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate contained in a Light-Shift Chemiluminescent EMSA Kit (Thermo Scientific, USA).

4.3.8 Statistical analysis

Student's *t*-test and GraphPad Prism 6 software (GraphPad Software, USA) were used to calculate the half-maximal inhibitory concentrations (IC_{50}) and half maximal effective concentrations (EC_{50}).

5 RESULTS AND DISCUSSIONS

In the present study, I investigated the effects of copper(II) and gold(I) mixed-ligand complexes on the activity of selected steroid receptors, xenoreceptors, and nuclear receptors, and the expression of their target genes. Two *in vitro* cellular models, i.e., primary human hepatocyte cultures and human cancer cell lines were used. For our biological studies, we have employed modern experimental methods such as the gene reporter assay, real-time quantitative reverse transcription polymerase chain reaction, western blotting, and electrophoretic mobility shift assay.

5.1 The effects of the copper(II) mixed-ligand complexes on regulatory pathways of human cytochrome P450s

I demonstrated that copper(II) complexes **1** and **2** significantly stimulated the AhR activity in the transgenic AZ-AHR cell line and accordingly induced the expression of the following AhR-dependent genes: *CYP1A1* mRNA in LS180 cells and *CYP1A1*/*CYP1A2* mRNAs in human hepatocytes (Kubesova et al., 2016). We found a slight inconsistency in case of complex **3**, which induced *CYP1A1* mRNA in LS180 cells, but not in human hepatocytes. Complex **4** did not stimulate AhR activity, and thus did not induce the expression of CYP1A1 and CYP1A2 in primary human hepatocytes and human cancer cell lines (Figures 6 and 7). This might be attributable to the fact that the human hepatocytes, unlike LS180 cells, are fully equipped with detoxification enzymes that might have caused the metabolic inactivation of complexes **3** and **4** (Li, 2007). The reversal of the induction of expression and AhR activation by resveratrol (AhR antagonist) revealed the involvement of AhR in the process (Figure 7). In addition, the complexes **1** and **2** induced the formation of the AhR-DNA complex (Figure 8), which is also a proof regarding the molecular activity of AhR. In general, the activation of AhR by foreign compounds is considered undesirable due to the involvement of AhR in a variety of cell functions, such as intermediary metabolism, cell differentiation, immune response, and/or chemically induced carcinogenesis (Go et al., 2015; Stejskalova and Pavek, 2011). After taking the values of EC_{50} for copper(II) complexes from gene reporter assays (~150 nM) into account, we might consider complexes **1** and **2** to have a medium to high level of potency, as compared to that of TCDD. On the other hand, the magnitude of AhR activation (efficacy) by complexes **1** and **2** was only about 6% of that by TCDD. The fold inductions of *CYP1A1* and *CYP1A2* mRNA expression by complexes **1** and **2** in human hepatocytes ranged from

2% to 7% of those by TCDD, implying that their low potential to cause AhR-based DDIs. Overall, transcriptional activity of AhR in gene reporter assays, the expression of *CYP1A1* mRNA in LS180 cells, and the expression of CYP1A1/CYP1A2 in human hepatocytes were increased by copper(II) complexes in the following (descending) order: **1** > **2** > **3** > **4**. The difference between the effects of individual complexes on AhR-mediated activity and CYP1A induction might be explained by the different structures of copper(II) complexes, in particular, the type, size, and position of substituents (Buchtik et al., 2011; Sui et al., 1998). The active complexes **1** and **2** contained either unsubstituted or 5-methylated (small molecule) 1,10-phenanthroline, respectively, in their structure. The inactive complex **3** was 4,7-diphenyl substituted, and two large aromatic rings might account for the loss of AhR activation by the complex. While inactive complex **4** contained unsubstituted 1,10-phenanthroline in its molecule, it also contained large substituents at positions 2 and 7 in the quinoline-based part of the molecule. In the past, chelating 1,10-phenanthrolines were described as partial agonists or competitive antagonists of rat AhR (Mahon and Gasiewicz, 1992), which was consistent with our findings. Unlike for AhR, we found that tested complexes did not influence the basal and ligand-inducible transcriptional activities of AR, GR, TR, PXR, and VDR receptors. Moreover, the major drug-metabolizing CYPs in human hepatocytes, including CYP2B6, CYP2C9, and CYP3A4 were not induced. Finally, the quinoline-derived compounds are clinically used in the pharmacotherapy against a number of diseases (Andriole, 2005; Barlow, 1963; Sato et al., 1995), and they act as potent inhibitors of DNA-gyrases (Pommier et al., 2010). Therefore, the present study was highly relevant and timely. The data obtained favoured the potential use of the tested compounds in human pharmacotherapy, with regard to their (no) interactions with signalling pathways via steroid receptors, nuclear receptors, and xenoreceptors, and induction of xenobiotic-metabolizing enzymes.

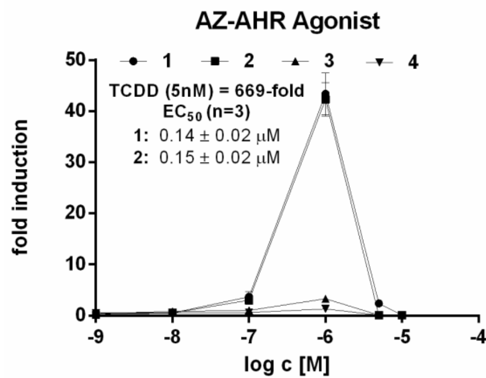


Figure 5: The agonist effects of copper(II) complexes on transcriptional activity of human AhR.

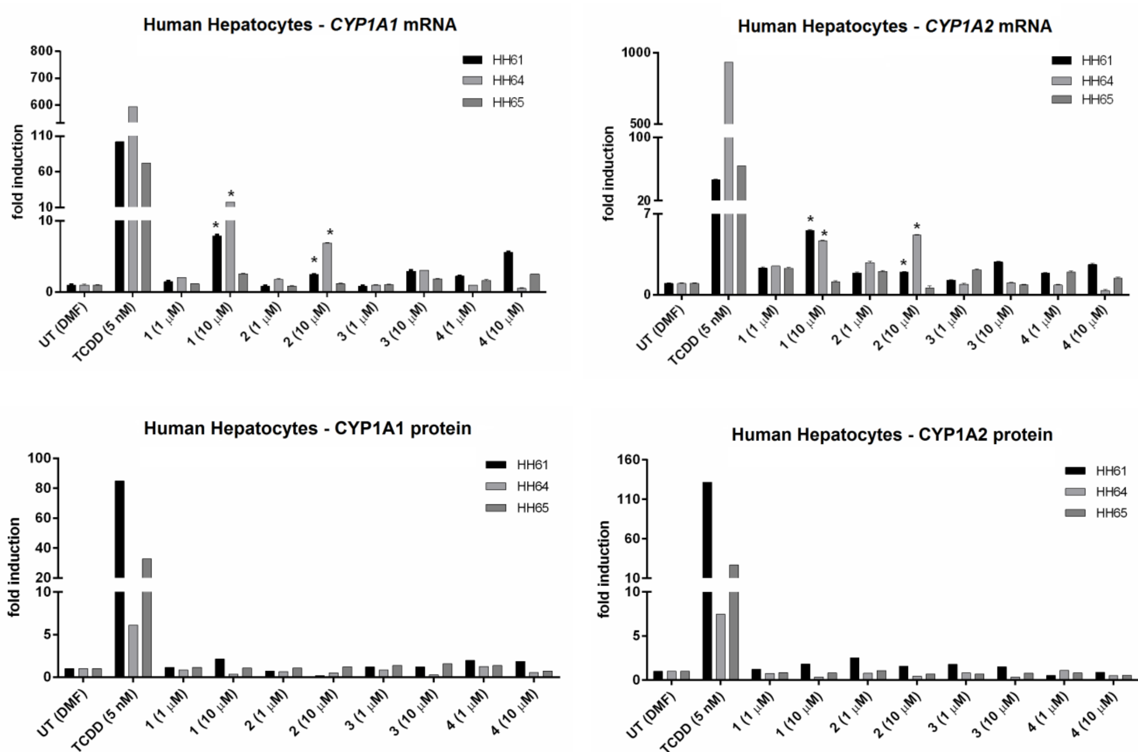
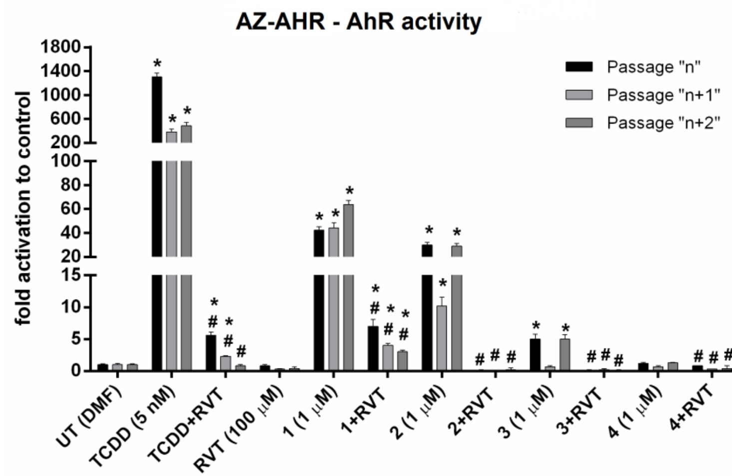


Figure 6: The effects of copper(II) complexes on CYP1A1/CYP1A2 mRNA and protein levels in human hepatocytes.



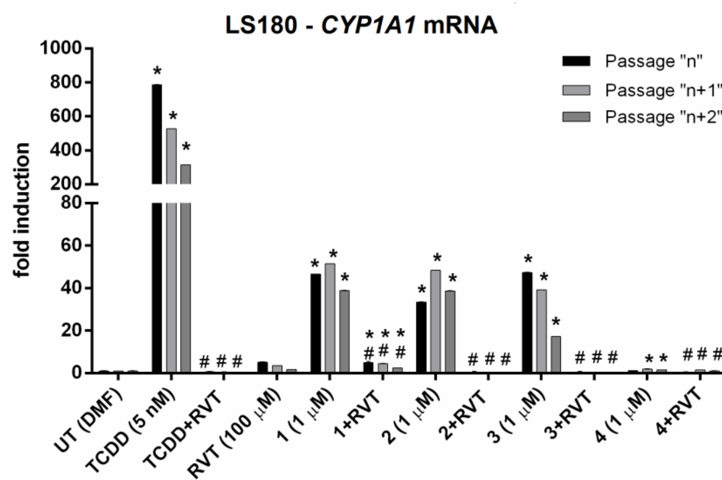


Figure 7: The effects of copper(II) complexes and resveratrol on the expression of CYP1A1 mRNA in LS180 and AhR activity in AZ-AHR cells.

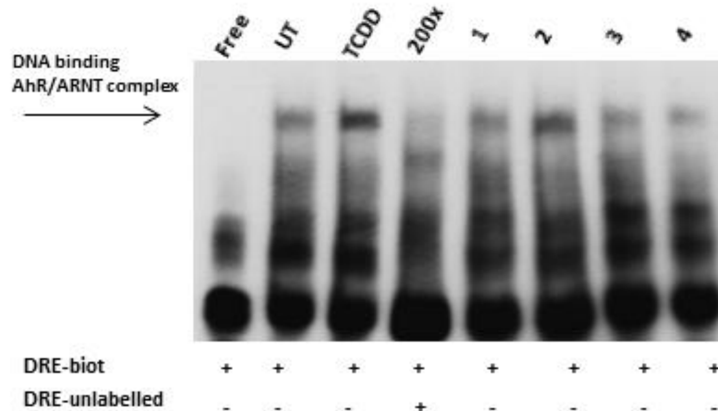


Figure 8: The effects of copper(II) complexes on the binding of AhR to DNA.

5.2 The effects of the gold(I) mixed-ligand complexes on regulatory pathways of human cytochrome P450s

I have investigated the effects of gold(I) phosphine complexes on the transcriptional activities of selected nuclear receptors, steroid receptors and xenoreceptors. I found that the tested complexes had differential effects on the transcriptional activities of the receptors and the expression of their target genes (Kubesova et al., 2016). I demonstrated that the gold(I) complexes **1**, **2** and **3** slightly and dose-dependently activated PXR and AhR, and accordingly, they moderately induced the expression of the CYP3A4 and CYP1A1/CYP1A2 genes in human hepatocytes and LS180 cells, respectively. All tested gold(I) compounds weakly induced the expression of *CYP2A6*, *CYP2B6*, *CYP2C9,* and *CYP3A4* mRNAs in primary human hepatocytes (Figure 10). In contrast, there was no induction of *CYP3A4* mRNA expression in LS180 cells by gold(I) complexes. This inconsistency might reveal the cell type-specific and tissue-specific regulation of CYP3A4 (Figures 10 and 11) (Pavek et al., 2010). All tested gold(I) complexes displayed a dose-dependent and differential inhibitory activity against GR, AR, and VDR in gene reporter assays. The transcriptional activity of GR declined in the presence of GR ligand dexamethasone, which indicates its antagonistic behaviour. The decline of the basal transcriptional activities of AR and VDR is suggestive of their inverse agonist behaviour (Figure 12). The anti-glucocorticoid effects of the gold(I) complexes (**1**-**3**) were confirmed in human hepatocytes, where these compounds down-regulated the GR-target gene *TAT* (Figure 13). Because the compounds were not cytotoxic, it is assumed that they exhibited antagonist effects against GR. A significant activation of expression of TR was observed by all gold(I) complexes; however it had a very low potency, as compared to that of T3. The magnitude of TR activation (efficacy) by these complexes was about 70-100% of the induction attained by T3. All gold(I) complexes decreased both basal and T3-inducible *SPOT14* mRNA expression levels, which is in contrast to their effects on TR transcriptional activity in gene reporter assays. This might be attributable to the complex process of the transcriptional regulation of *SPOT14*, which could involve other regulators besides TR. Indeed, the hepatospecific regulation of *SPOT14* by CAR and PXR was demonstrated (Breuker et al., 2010). Since the use of drug inverse agonists (Khilnani and Khilnani, 2011) and antagonists is of therapeutic potential and value, the data obtained here might pave the way for targeted drug research in the future (Leurs et al., 2002).

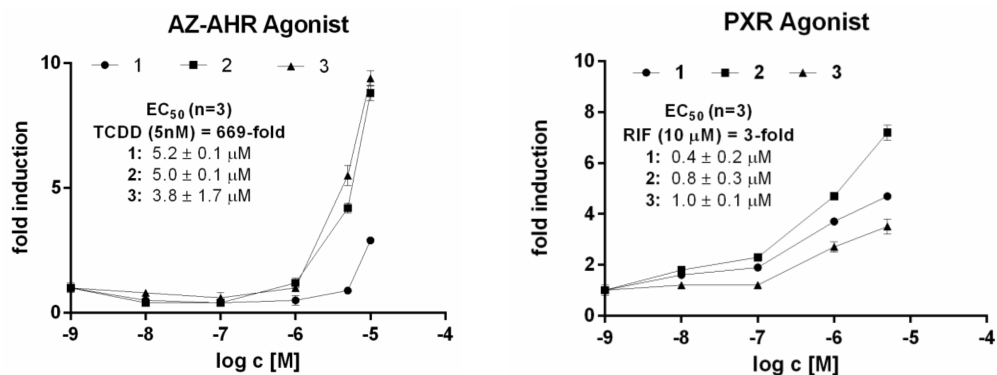


Figure 9: The agonist effects of gold(I) complexes on transcriptional activity of human AhR and PXR.

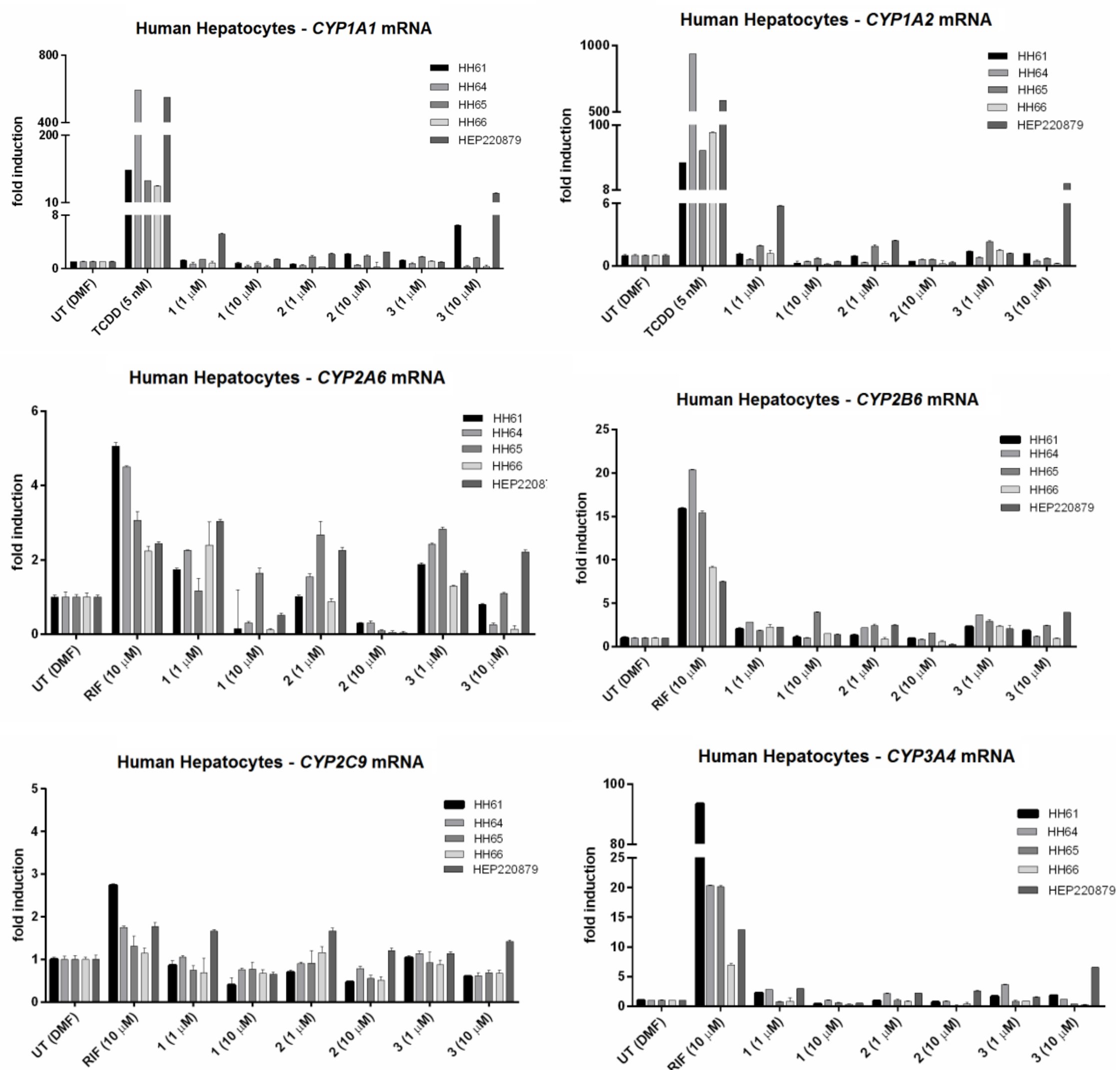


Figure 10: The effects of gold(I) complexe on the expression of drug-metabolizing CYPs mRNAs in primary human hepatocytes.

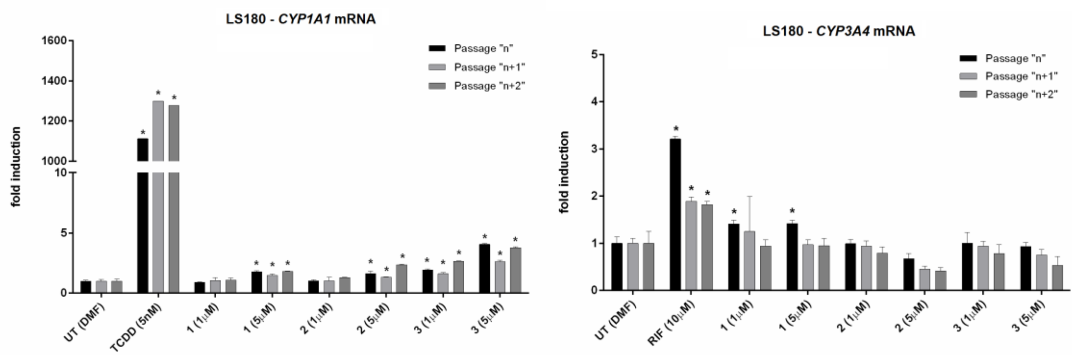


Figure 11: The effects of gold(I) complexes on the expression of CYP1A1 and CYP3A4 mRNAs in LS180 cell.

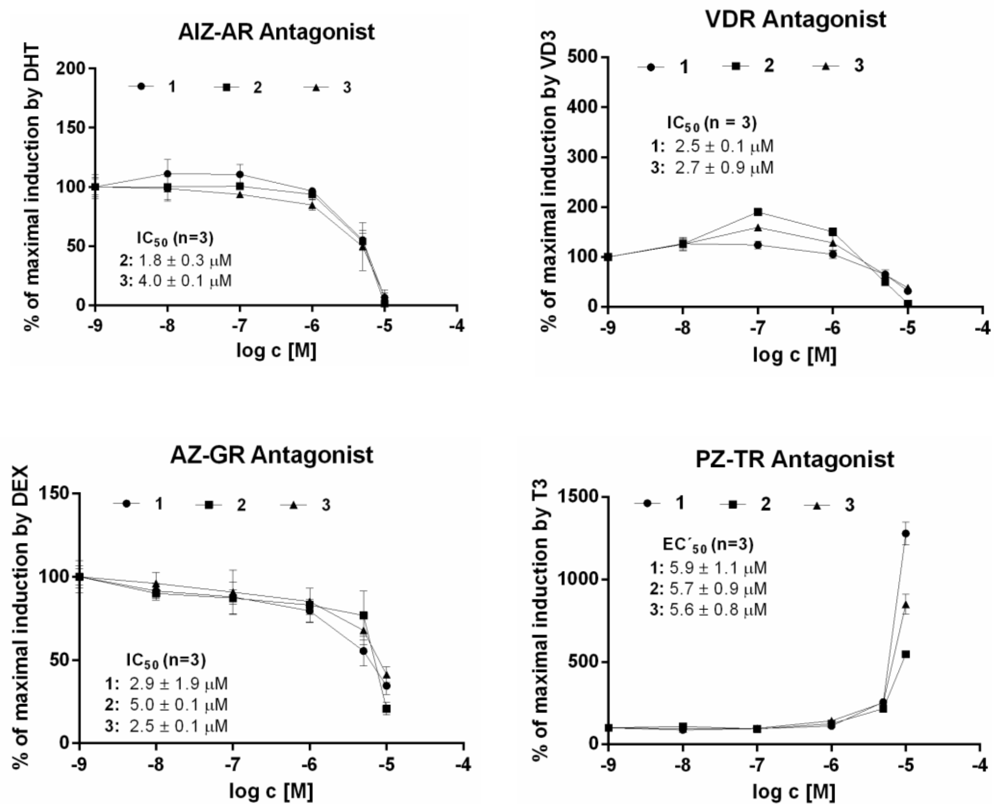


Figure 12: The antagonist effects of gold(I) complexes on the transcriptional activities of human nuclear receptors.

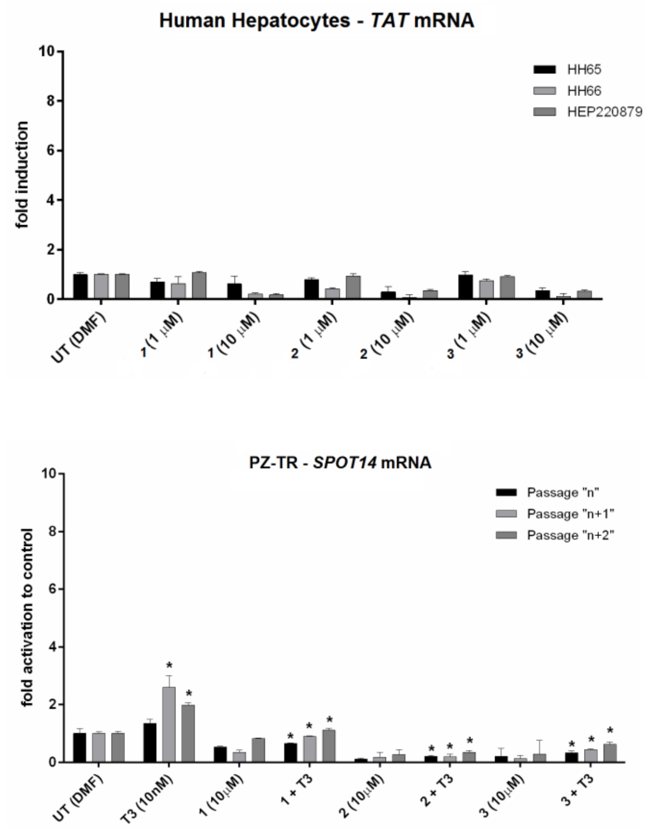


Figure 13: The effects of gold(I) complexes on the expression of TAT and SPOT14 mRNAs in human hepatocytes and PZ-TR cells.

6 CONCLUSION

The aim of this study was to determine the effects of copper(II) and gold(I) mixed-ligand complexes on the expression of selected drug-metabolizing enzymes, and on the transcriptional activities of steroid receptors (AR, GR), xenoreceptors (AhR, PXR), and nuclear receptors (TR, VDR). It was demonstrated that:

(i) Copper(II) complexes (**1**, **2**) activate AhR and induce AhR-dependent genes in human hepatocytes and cancer cell lines.
(ii) Gold(I) complexes activate AhR and PXR, and induce their target genes.
(iii) Gold(I) complexes exhibit anti-androgen and anti-glucocorticoid activities.

Overall, our results conclusively prove that gold(I) and copper(II) complexes differentially and selectively influence the activity of selected human nuclear receptors, steroid receptors, and xenoreceptors, which might have consequences for their potential therapeutic use with regard to DDIs, endocrine disruption or perturbation of intermediary metabolism.

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