PALACKÝ UNIVERSITY OLOMOUC

FACULTY OF MEDICINE AND DENTISTRY

INTERACTION OF MITOGEN-ACTIVATED PROTEIN KINASES INHIBITORS WITH AhR-SIGNAL PATHWAY

DISERTATION THESIS

Olomouc 2009 Pavla Henklová

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Supervisor: Doc. RNDr. Zdeněk Dvořák, PhD.

Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry

Department of Cell Biology and Genetics, Faculty of Science

I hereby declare the presented thesis is based on my own research carried out at the Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Olomouc, in the period September 2006 – September 2009. Co-authors agree with the inclusion of published results.

Olomouc ..... …..……..

Mgr. Pavla Henklová

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SOUHRN

Receptor pro aromatické uhlovodíky (AhR) hraje důležitou roli v metabolismu léčiv, chemicky vyvolané karcinogenesi, vývoji, diferenciaci a v mnoha dalších důležitých fyziologických pochodech. U člověka je tento receptor exprimován převážně v plicích, thymu, ledvinách, střevu, kůži, placentě a játrech. Jelikož je fosforylace důležitým krokem v regulaci AhR, hrají v jeho funkci také důležitou roli mitogeny aktivované protein kinasy (MAPKs), které fosforylují důležité transkripční faktory. Tyto kinasy se uplatňují např. při imunitních a nádorových onemocněních, zánětech či selhání ledvin. Inhibitory výše zmíněných kinas se jeví jako vhodný terapeutický nástroj a některé z těchto inhibitorů jsou již ve fázi klinického testování. Značnou nevýhodou inhibitorů pro MAPKs je jejich interakce s jinými buněčnými cíly, což poukazuje na důležitost jejich bližšího výzkumu.

Před samotnou studií vlivu inhibitorů pro MAPKs na signální dráhu AhR byly provedeny experimenty s vybranými „klasickými“ aktivátory (epidermální růstový faktor, sorbitol a anisomycin), a to v HepG2 buňkách a primárních kulturách lidských hepatocytů. Všechny tyto aktivátory vykazovaly nespecifickou aktivaci MAPKs jak v lidských hepatocytech, tak v HepG2 buňkách, jelikož jeden aktivátor aktivoval více než jednu kinasu. V této části experimentů byl také zkoumán vliv dioxinu (TCDD, což je typický ligand a aktivátor AhR) na aktivaci kinas. Přestože v literatuře byl prokázán jeho aktivující vliv na kinasy, ovšem na jiných buněčných modelech, v HepG2 buňkách tento účinek prokázán nebyl.

V dalších experimentech bylo zaměřeno studium inhibitorů (SB203580, SP600125 a U0126) na sledování aktivace kinas. Vzhledem k tomu, že SB203580 vyvolalo aktivace dvou kináz, bylo studium této problematiky zaměřeno především na tento inhibitor. Byla pozorována aktivace ERK a JNK vyvolaná SB203580 v lidských hepatocytech a aktivaci ERK v HepG2. Jelikož byly viděny aktivované rozdílné kinasy u těchto dvou buněčných modelů, byl vyšetřen účinek SB203580 ještě v dalších liniích (konkrétně v SaOS-2, HaCat a HL-60). Obdobně jako u HepG2 buněk byla u ostatních linií prokázána aktivace pouze ERK. Pro ověření, zda nejsou tyto rozdílné výsledky způsobeny metabolity SB203580, byly provedeny HPLC analýzy. SB203580 bylo metabolizováno v lidských hepatocytech, ovšem v HepG2 buňkách nebyly nalezeny zádné metabolity. Aktivace JNK byla tedy nejspíše způsobena metabolity SB203580.

Další studium bylo zaměřeno na zjištění vlivu SP600125, SB203580 a U0126 na expresi mRNA a proteinů CYP1A a transaktivaci plasmidů pDRE-luc a p1A1-luc. U0126 způsobilo, že exprese CYP1A1 mRNA byla indukována v závislosti na velikosti koncentrace tohoto inhibitoru v HepG2 buňkách. U0126 také způsobilo transaktivaci plazmidu pDRE-luc (plasmid obsahující 2 sekvence responsivního úseku pro xenobiotika; po stimulaci aktivátory AhR indukuje expresi luciferázy) a plazmidu p1A1-luc (obsahuje fyzilogickou promotorovou oblast pro lidský CYP1A1 gen a taktéž po stimulaci aktivátory AhR indukuje expresi luciferázy), které byly přechodně natransfekovány v HepG2 buňkách. U SB203580 byla pozorována nejvyšší indukce CYP1A1 na úrovni mRNA u 10 μM testované koncentrace v HepG2. Ovšem v reportérové studii se vliv SB203580 na aktivaci pDRE-luc a p1A1-luc plazmidů nepotvrdil. SP600125 indukovalo CYP1A mRNA v lidských hepatocytech. V HepG2 buňkách byla CYP1A1 mRNA indukována také po aplikaci SP600125, ovšem indukce byla u obou typů buněk menší v porovnání s indukcí, která byla způsobena typickým AhR agonistou (TCDD) a všechny tyto indukce byly inhibovány resveratrolem (což je popsaný antagonista tohoto receptoru). Na druhé straně SP600125 inhibovalo indukci CYP1A mRNA, která byla vyvolána TCDD v lidských hepatocytech. Abychom potvrdili naši hypotézu, že SP600125 je parciální agonista AhR, studovali jsme dále jeho vliv za využití genové reportérové studie. SP600125 transaktivovalo pDRE-luc a p1A1-luc plazmidy, které byly přechodně transfekovány do HepG2 buněk. Naopak transaktivace, která byla způsobená TCDD, byla SP600125 zainhibována. Všechna tato data ukazují na to, že SP600125 je parciální agonista AhR. Pro ověření, zda nejsou tyto rozdílné výsledky způsobeny metabolity SP600125, byly provedeny HPLC analýzy. SP600125 bylo metabolizováno v lidských hepatocytech, ovšem v HepG2 buňkách nebyly nalezeny zádné metabolity. Z těchto studií se ukazuje, že výsledky, které jsme obrdželi za použití SP600125 nejsou způsobeny metabolity tohoto inhibitoru. Dále jsme studovali vliv U0126 inhibitoru na úrovni proteinů. U0126 indukovalo expresi CYP1A na úrovni protenů v lidských hepytocytech a jen velmi slabě u 25 μM koncentrace v HepG2 buňkách. SB203580 bylo také studováno na úrovni proteinů, kde byla pozorována indukce CYP1A způsobená SB203580 v obou buněčných modelech, avšak v lidských hepatocytech byla indukce slabá v porovnání s indukcí v HepG2 buňkách.

Souhrnně rečeno, účinek inhibitorů a aktivátorů MAPKs se liší v nádorových linií a primárních buňkách. Tyto výsledky ukazují na to, že je důležité provádět studie jak na nádorových buněčných liniích, tak na primárních buňkách, abychom lépe porozuměli

mechanismům, které probíhají v obou buněčných modelech. Všechny testované inhibitory SP600125, SB203580 a U0126 interagují se signální dráhou AhR a jejich užití je ve studiu tohoto receptoru rozporuplné. Kromě těchto poznatků se ještě ukazuje, že inhibitor SB203580 aktivuje ERK kinasu, a tudíš jeho využití ve studiu MAPKs vede k nejednoznačným výsledkům.

Klíčová slova: signální dráhy, receptor pro aromatické uhlovodíky, HepG2, lidské hepatocyty, JNK, SP600125, ERK, U0126, p38 kinasa, SB203580.

SUMMARY

Aryl hydrocarbon receptor (AhR) plays an important role in drug metabolism, chemically-induced carcinogenesis, developement, differentiation and other essential physiological functions. In man, AhR is expressed in particular in the lungs, thymus, kidney, intestine, skin, placenta and liver. Since phosphorylation is an important step in the regulation of these receptor, mitogen-activated protein kinases (MAPKs) which phosphorylate important transcriptional factors play also important role in function of AhR. These kinases are involved e.g. in immune and oncological diseases, inflammation or renal failure. Mitogen-activated protein kinases inhibitors seem as appropriate therapeutic tools and some of them are undergoing clinical trials. Interaction of MAPKs inhibitors with other cellular targets is their main disadvantage. Thus the research of these pharmacological inhibitors is important.

Before the effects of MAPKs inhibitors on AhR signalling were tested, the selected „classical“ activators (epidermal growth factor, sorbitol and anisomycin) were studied in HepG2 cells and primary cultured human hepatocytes. All these activators displayed nonspecific activation of MAPKs both in human hepatocytes and HepG2 cells since one activator activated more than one MAPKs. In addition, dioxin (TCDD, a knonw AhR ligand and activator) did not activate kinases (c-Jun *N*-terminal kinase, extracellular regulated kinase and p38 kinase) in HepG2 though it was demonstrated in literature as MAPKs activator in other cells.

Next the MAPKs inhibitors (SP600125, SB203580 and U0126) were studied, specially SB203580, since it activated ERK and JNK in human hepatocytes and ERK in HepG2. Since different effects of SB203580 were observed in human hepatocytes and HepG2 cells, another measurements were proceed in SaOS-2, HaCat and HL-60 cells. As with HepG2 cells only activation of ERK was observed. To test whether or not all effects of SB203580 were due to its metabolic transformation, HPLC analyses were performed. SB203580 was metabolized in human hepatocytes but no metabolites of SB203580 were observed in HepG2 cells. Thus it is likely that activation of JNK in human hepatocytes was caused by metabolites of SB203580.

Another part of experiments was focused on examination of SP600125, SB203580 and U0126 effects on CYP1A mRNA, protein expression and plasmids (pDRE-luc and p1A1-luc) transactivation. U0126 caused induction of expression of CYP1A1 mRNA in a

dose-dependent manner in HepG2 cells. Moreover, dose-dependent transactivation of pDRE-luc and p1A1-luc plasmids transfected in HepG2 cells mediated by U0126 was observed. SB203580 induced mRNA CYP1A1 in HepG2 cells. The 10 μM tested concentrations of SB203580 caused maximal induction on CYP1A1 mRNA level. However, the gene reporter assay did not confirm inducing effect of SB203580 on pDRE-luc and p1A1-luc plasmids. SP600125 induced CYP1A mRNA in human hepatocytes. CYP1A1 was induced by SP600125 in HepG2 cells but the induction in both type of cells was smaller compared to induction mediated by a typical AhR agonist TCDD and this induction was abolished using resveratrol (referred AhR antagonist). On the other hand TCDD-mediated CYP1A induction of both mRNA and proteins was abolished using SP600125 in human hepatocytes. To verify our hypothesis that SP600125 is a partial agonist of AhR, the effects of SP600125 were tested using gene reporter assay. All obtained results imply that SP600125 is a partial agonist of AhR. To test whether all effects of SP600125 were not due to their metabolic transformation, HPLC analyses were performed. SP600125 was metabolized in human hepatocytes but no metabolites of SP600125 were observed in HepG2 cells. Thus the effects of SP600125 were not mediated by its metabolites. Next, the effects of U0126 inhibitor were examined on protein level. U0126 induced expression of CYP1A proteins in human hepatocytes and very slightly in HepG2 cells. SB203580 was also studied on protein level. SB203580 caused slight induction of expression CYP1A proteins in human hepatocytes and induction of CYP1A in HepG2 cells.

Taken together, effects of MAPKs inhibitors and activators differed in cancer cell lines and primary cells. These results imply that it is important to carry out studies both in cancer cell lines and primary cells to better understand the mechanisms that proceed in both types of cells. All tested inhibitors SP600125, SB203580 and U0126 interact with AhR and their use in studies of these receptor is doubtful. In addition SB203580 activates ERK and its use in MAPKs studies is of uncertain outcome too.

Keywords: Signalling pathways, Aryl hydrocarbon receptor, HepG2, Human hepatocytes, JNK, SP600125, ERK, U0126, p38 kinase, SB203580.

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ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon nuclear translocator
B[*a*]P	Benzo[*a*]pyrene
CYP	Cytochrome P450
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin-responsive element
EC_{50}	Concentration required to induce a 50% effect
ERK	Extracellular regulated kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HepG2	Human hepatoma cells
HPLC	High Performance Liquid Chromatography
Hsp90	Heat-shock protein 90 kDa
IC_{50}	Concentration that reduces the effect by 50%
JNK	c-Jun *N*-terminal kinase
MAPKs	Mitogen-activated protein kinases
mRNA	Messenger ribonucleic acid
p38	p38-Mitogen-activated protein kinase
PAHs	Polycyclic aromatic hydrocarbons
PCR	**P**olymerase **c**hain **r**eaction
PKC	Protein kinase C
RNA	Ribonucleic acid
RT-PCR	**R**everse **t**ranscription **p**olymerase **c**hain **r**eaction
RVT	Resveratrol
SB203580	4-(4-Fluorophenyl)-2-(4- methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole
SP600125	1,9-Pyrazoloanthrone
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
U0126 ethanolate	1,4-Diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene

1. INTRODUCTION

Environmental pollutants can affect signal transduction pathways. For example polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins (2,3,7,8- tetrachlorodibenzo-*p*-dioxin; TCDD) cause acute and chronic toxicity in exposed animals and human and some of them (i.e. benzo[*a*]pyrene, naphthalene, TCDD) are carcinogens. In addition TCDD can cause formation of reactive oxygen species via cytochrome P450 (CYP1) induction [1]. A lot of these compounds induce the expression of xenobiotic-metabolising enzymes (such as cytochrome P450 CYP1A1, CYP1A2, CYP1B1 [2, 3]) and phase II enzymes (UDP-glucuronosyl transferase UGT1A6 [4]) via the aryl hydrocarbon receptor (AhR) [5-9]. CYP1A1 and 1B1 are involved in the biotransformation of PAHs to ultimate carcinogenes [10]. This biotransformation process is believed to be associated with the development of lung cancer. A high CYP1A1 activity is also associated with colorectal cancer. AhR plays a key role in both chemical toxicity (exogenous ligands dependent) and as an important component of normal development (adaptive role, independent of exogenous ligands) [11]. AhR is a ligand-activated transcriptional factor. The inactivated form of the receptor is found in cytoplasm. When a ligand is bound, AhR translocates to the nucleus and forms a complex with aryl hydrocarbon nuclear translocator (ARNT) that binds to the DNA sequence called xenobiotics response element and activates expression of certain genes (CYP1A1 and CYP1A2). Since phosphorylation is an important step in regulation of AhR, mitogen-activated protein kinases (MAPKs) which phosphorylate important transcriptional factors play also an important role in function of AhR. More informations about AhR interactions with MAPKs have appeared recently (summarized in [12]). MAPKs are also involved in a lot of important biological processes such as cell growth and differentiation such as extracellular regulated kinase (ERK), cellular signalling, in the immune system, cancerogenesis and in the pathogenesis of diabetes such as c-Jun *N*-terminal kinase (JNK), inflammatory response, apoptosis and cell cycle such as p38-Mitogen-activated protein kinase (p38). Recently specific pharmacologic MAPKs inhibitors have been discovered such as 1,9-pyrazoloanthrone (SP600125), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) and 1,4-Diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene ethanolate (U0126) for JNK, p38 and ERK, respectively. However, the major problem is their interference with other cellular pathways and targets. For instance SP600125 is partial agonist of AhR (found by our

group) [13], U0126 is partial agonist of AhR [14] and SB203580 activates ERK and JNK in primary cultures of human hepatocytes [15]. Our studies were predominatly carried out on primary cultures of human hepatocytes which are quiescent non-transformed cells. This research on primary cells is very important because the majority of *in vitro* studies have been carried out in cancer cell lines.

2. THEORETICAL PART

2.1. Mechanism of cellular signalling

Receptors both for polypeptides and steroids have several (at least two) functional domains. A recognition one which binds the hormone, and a second one which generates a signal.

Signal transduction occurs in two general ways. The first signal transduction occurs via receptors located in the plasma membrane that bind peptides (endorfins), protein hormones (insulin, glucagon), catecholamines (adrenalin), gamma-aminobutyric acid (GABA), glutamate, glycine, serotonine, histamine etc. The second type of signal transduction pathways occurs via intracellular receptors that bind streroid hormones (glucocorticoids, mineralocorticoids, estrogens and androgens), thyroid hormones (T3, T4), retinoids, vitamine D, cholesterol etc. These receptors are members of a large superfamily and many related members have no known ligand and are called orphan receptors (e.g. pregnane X-receptor (PXR) and constitutive androstane receptor (CAR)). Aryl hydrocarbon receptor (AhR) is another one intracellular receptor that belongs to the family of important transcriptional factors. Another group of receptors is connected to intermedial metabolism such as cholesterol-sensing liver X receptor (LXR), bile acid-activated farnesoid X receptor (FXR), peroxisome proliferator-activated receptors (PPAR).

2.2. Aryl hydrocarbon receptor

Aryl hydrocarbon receptor (AhR) is a member of the bHLH/PAS (basic helix-loop-helix / PER ARNT Sim) family of proteins that are transcriptional factors. AhR is activated by ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin). However, there was also described a way of AhR activation, i.e. via cAMP-mediated phosphorylation [16] or by compounds (e.g. omeprazol) that do not bind to AhR but activate it [17].

AhR plays an important role in various processes such as drug metabolism [18], chemically-induced carcinogenesis [19], development, differentiation and other essential

physiological functions [20] or in the regulation of matrix remodeling [21]. In man, AhR is expressed in particular in the lungs, thymus, kidney, intestine, skin, placenta or liver (reviewed in [22, 23]).

The inactive form of the AhR (in the absence of a ligand) resides in the cytoplasm and forms a complex with two molecules of Hsp90 [24], the Hsp90-interacting protein p23 [25] and the immunophilin-like protein XAP2 (also referred to as AIP or ARA9) [26]. The stability of the AhR/Hsp90 complex is important for the stability of the AhR protein [27]. Hsp90 interacts with bHLH region and masks the nuclear localization signal (NLS). When a ligand is bound, conformation of AhR complex changes [28] and AhR translocates to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) [29-33], which is AhR-related nuclear protein. The activated AhR/ARNT complex binds to dioxin response elements (DREs), which are located in the enhancer/promoter region of TCDD responsive genes and the expression of AhR target genes such as CYP1A1 [34] and CYP1A2 [30] is activated (Fig. 1). Another important protein termed AhR repressor (AhRR) resides in the nucleus. AhRR interacts with ARNT and competes with AhR. This process serves as a negative regulation since AhRR/ARNT also recognizes DRE but is not transcriptionally active [35].

The AhR activity is altered by variety of cellular factors (e.g. co-activators and co-repressors). Steroid receptor coactivator (SRC-1), nuclear activator 2 (NcoA-2), p300/cAMP-responsive element-binding protein cointegrator protein (p/CIP) are some of co-activators with histone acetylation that facilitate gene activation by interacting with AhR or ARNT. On the other hand silencing mediator of retinoid acid and thyroid hormone receptors (SMRT) and small heterodimer partner (SHP) inhibit transcriptional activity of AhR/ARNT complex (reviewed in [36]).

Protein degradation is regulated by the ubiquitin-proteasome system. The AhR degradation can proceed by ligand dependent pathway (TCDD) and ligand independent pathway (geldamycin) [37]. After exposure to the ligand and finally nuclear export, the AhR protein is degraded via the 26S proteasome pathway both *in vitro* (in different cell lines) and *in vivo* [38, 39]. However Giannone et al. (1998) found that the AhR mRNA levels were not altered [40]. The degree of degradation differs (65-95%) in various tissues and cells [37] and it works as a negative regulatory feedback loop that prevents permanent activation of the receptor. The degradation of AhR is blocked by 26S proteasome inhibitors (MG132 or lactycystin) [37, 38, 41]. Recently, Ohtake et al. [42] have published that AhR is involved as a component of CUL4B-based E3 ubiquitin ligase

complex for ubiquitination of sex steroid receptors (estrogen and androgen receptors). The function of this ubiquitin ligase complex is dependent on the presence of ligands [42, 43].

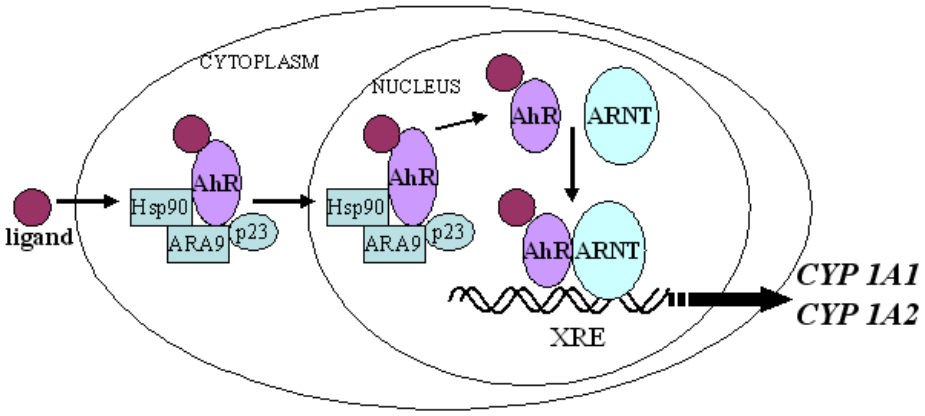


Fig. 1: **Activation of AhR** (accordig to [44]). When no ligand is present, AhR resides in a complex with chaperones (Hsp90 - Heat shock protein, Hsp90-interacting protein p23, ARA9 - aryl hydrocarbon receptor associated protein 9) in the cytoplasm. After ligand binding, AhR translocates to the nucleus and dissociate from multiprotein complex. Next AhR heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This activated AhR/ARNT complex binds to dioxin response elements (DREs) and then expression of AhR target genes (i.e. CYP1A1 and CYP1A2) is activated.

2.2.1. Phosphorylation of AhR

The cellular proteins in cells undergo covalent modifications, such as methylation, adenylation, and phosphorylation/dephosphorylation, ubiquitination, acetylation, etc. Phosphorylation of proteins is a reversible modification that modulates protein conformation and/or catalytic activity, leading to altered DNA-binding activity, protein-protein interaction and nuclear translocation. An important step in AhR signalling is its phosphorylation (Fig. 2) which controls transcriptional activity, subcellular distribution and stability of AhR and also other shuttling proteins.

Importantly, AhR and ARNT are both phosphoproteins [45, 46]. The activation of AhR can proceed by cAMP-mediated phosphorylation [16]. Transforming of unliganded AhR into functionally active AhR/ARNT heterodimer is regulated also by phosphorylation [47] which proceeds in the *C*-terminal part of the peptide *in vitro* [48].

Ikuta et al. [49] suggested a two-step model for AhR ligand-dependent nuclear translocation in which phosphorylation/dephosphorylation plays an important role. After ligand binding to the AhR, the nuclear localization signal (NLS) domain is unmasked and this facilitates interaction of NLS with nuclear import component. At the second step the phosphorylation of NLS at Ser12 or Ser36 mediated by protein kinase C abolishes AhR ligand-dependent translocation and on the other site dephosphorylation facilitates interaction of NLS with receptors and AhR subsequently accumulates in nucleus. The NLS is composed of two basic amino acid segments: AhR (a.a. residues 13–16:Arg-Lys-Arg-Arg) and AhR (a.a. residues 37–39:Lys-Arg-His) [50]. However, TCDD-induced cytoplasmic diminution/nuclear accumulation of AhR was not affected by the presence of protein kinase C activator or inhibitor [51]. Thus, it seems that PKC does not affect AhR-mediated transcription directly but via some another transcriptional factor in the nucleus.

Following the nuclear import of AhR and its functions in gene transcription, AhR is exported to cytosol. This process involves nuclear export signal (NES) [50], presented by aminoacid residues 55–75 near the NH_2-terminal region of AhR. Importantly, the phosphorylation of Ser68 in NLS region was demonstrated [49].

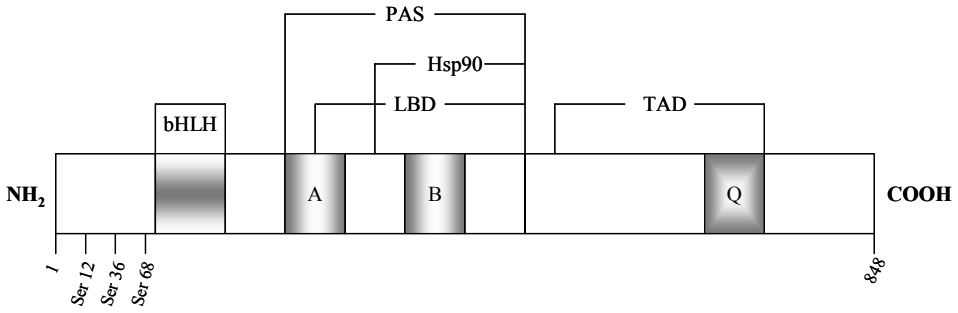


Fig. 2: Functional domains of human AhR with known phosphorylation sites. A and B, repeated nucleotide sequences in PAS domain; ARNT, AhR nuclear translocator; bHLH, basic helix-loop-helix; HSP90, heat-shock protein 90 kDa binding domain; Q, glutamine-rich domain; PAS, period single-minded and human protein ARNT domain; TAD, transcriptional activation domain.

Mouse AhR contains 23 tyrosine residues, 19 of which are conserved in several species, including humans. These regions include 5 phosphotyrosines within residues 368–605 (Tyr372, Tyr408, Tyr462, and Tyr532) and 639–759 (Tyr698) and they have been shown to be highly phosphorylated *in vivo* [45]. In addition, *N*-terminal Tyr9 is required for AhR binding to DRE in DNA and for transcriptional activity of the receptor [52]. Involvement of protein tyrosine kinases (PTKs) in the AhR-mediated response has been investigated as well [53]. It seems that ligand-independent activation of AhR involves PTKs. Phosphorylation of AhR co-chaperones is also important in regulating receptor functions. It has been suggested that the phosphorylation of Hsp90 modulate the formation of the functional cytosolic AhR multiprotein complex [54]. These authors determined the site-specific phosphorylation of the steady-state cytosolic mouse AhR complex and they identified phosphorylations of the Hsp90 subunits within the AhR complex at Ser225 and Ser254 of Hsp90ß and Ser230 of Hsp90α, respectively. Moreover, serine/threonine phosphatase inhibitors have been shown to increase gene transcription mediated by the AhR [55]. This implies that serine/threonine kinases may be directly or indirectly involved in the regulation of AhR/ARNT functions. The most prominent members of the serine/threonine family kinases are mitogen-activated protein kinases (MAPKs). Therefore, I focused the presented PhD thesis on the investigation of MAPKs inhibitors interactions with AhR-CYP1A signalling pathway.

2.2.2.Ligands and activators of AhR

AhR activity is regulated or modulated by endogenous and exogenous factors (chemical agents such as TCDD, benzo[*a*]pyrene, dexamethasone etc. and biological factors such as serum, lipopolysaccharide etc.). AhR ligands, xenobiotics, hormones, growth factors, cytokines and cell density are examples of the many factors that regulate AhR [36].

2.2.2.1. Exogenous AhR ligands

The majority of AhR ligands with high affinity (also called classical ligands) belongs to category that includes planar hydrophobic halogenated aromatic hydrocarbons

(HAHs) (e.g. polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, biphenyls) and polycyclic aromatic hydrocarbons (PAHs) (e.g. 3-methylcholanthrene, benzo[*a*]pyrene). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 2,3,7,8-tetrachlorodibenzofuran, 3-methylcholanthrene, benzo[*a*]pyrene, ß-naphthoflavone can serve as an example of classical AhR ligands and CYP1A1 and CYP1A2 inducers (reviewed in [30]). However the effect of these ligands can differ between studied models. For example TCDD lowered the AhR protein level in mouse hepatoma cells and contrary the AhR protein level in rat liver was increased (reviewed in [36]). New AhR ligands M50354 and M50367 (which are selective inhibitors of TH2 cells differentiation with high efficacy toward reducing atopic allergic symptoms) were found to be full agonists [56]. It was reported that the c-Jun *N*-terminal kinase inhibitor SP600125 is a ligand and antagonist of the AhR [57] but we showed that SP600125 is a partial agonist of human AhR [13]. Omeprazole, H^+/K^- -ATPase inhibitor, activates AhR without direct binding to AhR protein [58]. Murray et al. [58] demonstrated that omeprazole-stimulated activation of AhR (nonligand binding) is necessary for induction of human insulin-like growth factor binding protein-1 which exhibits multiple functions (such as participation in glucose homeostasis that means that *in vivo* its level is increased by glucocorticoids and glucagon hormones via its second messenger cAMP and decreased by insulin, these alterations are correlated with *IGFBP-1* gene transcription).

Naturally occurring (including dietary) AhR ligands are mainly flavonoids (i.e. flavones - chrysin, flavanols - quercetin, isoflavanones – resveratrol). These ligands comprise both agonists and antagonists of AhR [59]. Importantly, resveratrol was reported as AhR antagonist [60], whereas we demonstrated that resveratrol acts as partial agonist of AhR in human hepatocytes and HepG2 cells [13].

2.2.2.2. Endogenous AhR ligands

While AhR was considered for a long time as an orphan receptor, the endogenous ligands of AhR were identified. Endogenous AhR ligands comprise indoles (the most of these AhR ligands are formed from tryptophan), indigoids (e.g. indigo and indirubin), tetrapyrroles, arachidonic acid and heme metabolites, tryptophan metabolites or interestingly 7-ketocholesterol etc. [30, 44, 61, 62].

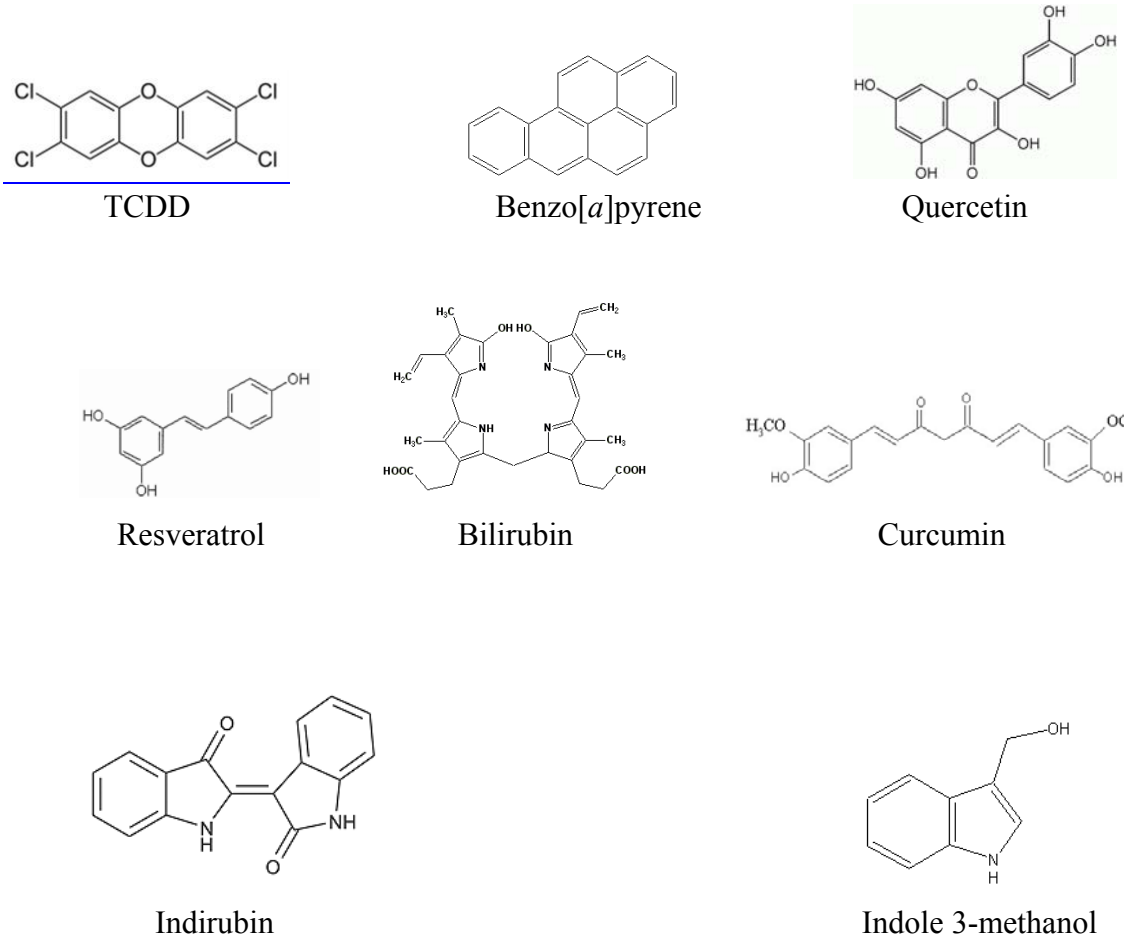


Fig. 3: The structures of several exogenous and endogenous AhR ligands (description in text).

2.3. Mitogen-activated protein kinases

MAPKs, serine/threonine kinases, are members of the signal transduction system involved in various processes in eukaryotic cells. The main roles of MAPKs are: (i) regulation of the activity of down-stream Ser/Thr kinases (e.g. ribosomal S6 kinase [63]), (ii) regulation of gene expression [64], and (iii) regulation of cytoskeletal function [65].

MAPKs are activated by external stimuli, such as growth factors, cytokines, UV radiation, osmotic shock, genotoxic or oxidative stress [66-70]. These kinases phosphorylate important transcriptional factors (summarized in [12]) directly or via down-stream MAPK-activated protein kinases. The most important MAPKs are:

extracellular signal-regulated kinases (ERK1/2), c-Jun *N*-terminal kinases or stress-activated protein kinases (JNK/SAPK) and p38 (for review see [12, 71, 72]).

2.3.1. MAPKs pathways

The MAPKs are components of a triple kinase cascade (Fig. 4) and are involved in cell regulation. Activation of MAPKs influences various cellular responses e.g. gene expression, apoptosis, differentiation or cell cycle. Upon stimulation, MAPKKKs (MAPKs kinases kinases are also called MAP3Ks or MEKKs) are activated via phosphorylation by up-stream kinases and additional factors, such as members of Ras and Rho families of small GTPases [73-76]. Thereafter MAPKKKs activate down-stream MAPKKs by phosphorylation at Ser/Thr residues. Dual-specificity MAP kinase kinases (MAPKK or MAP2Ks) phosphorylate MAPKs both at Thr and Tyr residues (but only if followed by proline) within Thr-(Glu/Gly/Pro)-Tyr motives in ERK, p38, JNK, respectively. While ERK1/2 are involved in regulating mitogenic and developmental events, JNK and p38 rather regulate stress and inflammatory responses.

The levels of MAPKs do not change through stimulation thus it is likely that dephosphorylation by phosphatases plays an imortant role in switching-off MAPKs (e.g. it was shown recently that MAPK phosphatase-1 deactivated ERK, JNK, p38 in mice peritoneal macrophages [77]).

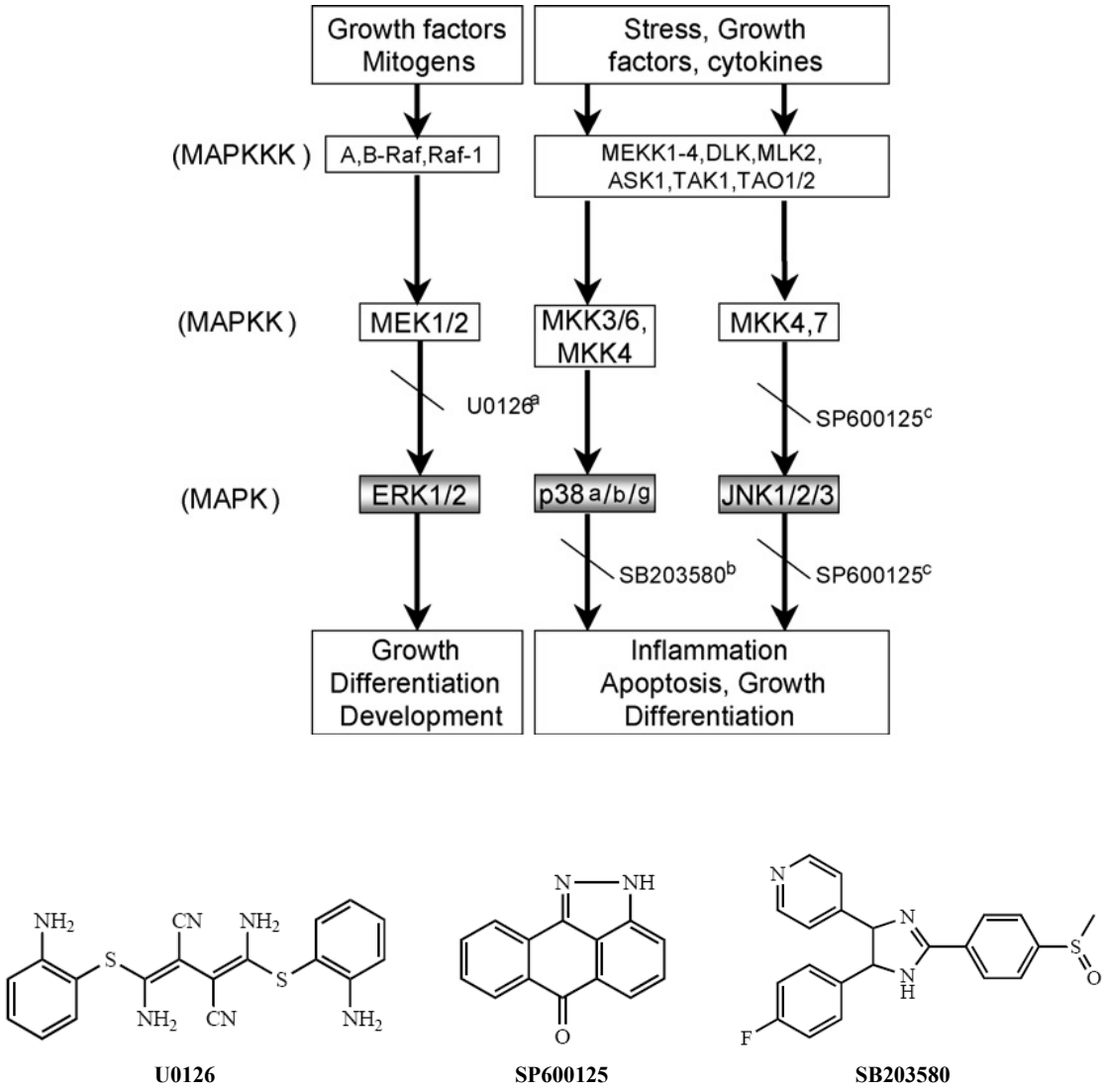


Fig. 4: Scheme of selected MAPK pathways and structures of their inhibitors. [a]Ref. [78], [b]Ref. [79], [c]Ref. [80]. ASK, apoptosis signal-regulating kinase; DLK, dual leucine zipper-bearing kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; MEK, MAP/ERK kinase; MEKK, MEK kinase; MLK, mixed-lineage protein kinase; TAK, transforming growth factor activated-β-protein kinase; TAO, 1001 amino acid; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene; SP600125, 1,9-pyrazoloanthrone.

2.3.2. Extracellular signal-regulated kinase

Several groups of MAPKs have been characterized in mammals, among which belong ERK 1/2, ERK 3/4, ERK 7/8 and ERK 5. ERK 3 and 7 are ubiquitously active, ERK 3/4 do not contain the Thr-Glu-Tyr motives, mechanism of activation ERK 4 is still unclear, ERK 5 is activated by MEK5, oxidative stress and growth factors but in literature do not exist enough information about these isoforms [81]. Extracellular signal-regulated kinases isoforms ERK1 (44 kDa) and ERK2 (42 kDa) share 83% identity in amino acid sequence and are expressed in all tissues. ERKs are activated by growth factors or mitogens [82]. These kinases are involved in mitogenic and developmental events, including cell growth, apoptosis and differentiation. ERK1 and ERK2 are activated by dual phosphorylation on Thr202/Tyr204 [83, 84]. Inhibitors of ERK pathway, PD98059 and U0126 (diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto) butadiene) act both on up-stream MEK1/2, and do not discriminate between ERK1 and ERK2 (Fig. 2). Thus, mice invalidated for either ERK1 or ERK2 were used to distinguish their roles. ERK1 knock-out (KO) mice were viable, fertile with normal size but with defective T-cell differentiation. In contrast, KO mice for ERK2 displayed embryonic lethality due to a defect in placental development (reviewed in [71]). It seems that ERK is also involved in regulation of mitosis since knock-down of B-Raf (MAPKKK, Raf kinase) resulted in damage to spindle formation in HeLa cells [85]. ERK activation was also connected with inducing of apoptosis in murine fibrosarcoma L929 cells and inhibition of ERK reduced activation of p53 [86].

2.3.3. c-Jun *N*-terminal kinase/stress-activated protein kinase

JNKs are involved in cellular signalling, in the function of the immune system (i.e. mature macrophages have a selective requirement for JNK for survival, proliferation, and differentiation [87]), stress-induced and developmentally programmed apoptosis, cancerogenesis and in the pathogenesis of diabetes (reviewed in [70]) and in regulation of histone acetylation [88].

Mammalian JNK/SAPK comprise three members; JNK1/2/3 (SAPKγ, SAPKα, SAPKß) [89, 90]. JNK has a broad tissue distribution, although JNK3 is primarily present in brain [89], heart and testis. In cells, at least 10 isoforms of JNK are expressed, which are derived from three JNK genes. Their molecular weight is approximately 46 and 55 kDa for extra-cerebral tissue isoforms [89]. JNK3 isoforms have higher MW due to the

extended NH_2-terminus. One of the down-stream targets of JNK is a transcription factor c-Jun which was believed to be phosphorylated by JNK exclusively [91]. However, it was demonstrated that p38 MAPK directly phosphorylates c-Jun within its transactivation domain at Ser63/Ser73 [92]. This only demonstrates the cross-reactivity of MAPKs, the substrates of which may be phosphorylated by any MAPK with no respect to the activation stimuli.

They are activated by osmotic, oxidative and genotoxic stress, immune stimuli (cytokines), UV radiation, growth factors, etc. [91, 93-95]. JNK up-stream kinases are designed as MKK4/7 that phosphorylate JNK/SAPK on Thr183 and Tyr185 residues, separated by proline (reviewed in [96]).

JNK down-regulation is related to MAPK phosphatase-1 and MAPK phosphatase-5 [81]. One of the most selective inhibitors of JNK is 1,9 pyrazoloanthrone, usually referred to as SP600125 [80]. We found that this specific JNK inhibitor is a partial agonist of AhR and thus interferes with AhR signalling pathway [13]. This compound was used to inhibit JNK in rheumatoid arthritis induced in rats, when its administration led to markedly lowered destruction of rodent ankles [97]. The importance of JNK *in vivo* was studied in JNK KO mice, which were fertile, normal size with defective T-cell differentiation and decreased adiposity (KO JNK1) and it was shown recently that JNK1 is required in promoting collagen [98], defective T-cell activation with protected Type I diabetes (KO JNK2) or decreased NGF deprivation induced apoptosis (KO JNK3). Embryonic lethality appeared due to a defect in brain development in double KO mice only (KO JNK1/JNK2) (reviewed in [71]). JNK activation was also required for promoting apoptosis in murine fibrosarcoma L929 cells and inhibition of JNK reduced activation of p53 [86].

2.3.4. p38 Mitogen-activated protein kinase

p38 MAPK is a 38 kDa protein which is present both in nucleus and cytoplasm in cells [81]. Cloning of p38 led to identification of four isoforms: p38α, p38ß, p38γ (alternative name ERK6, SAPK3) and p38δ (alternative name SAPK4). The tissue distribution of p38 is broad (heart, brain, placenta, kidney, etc.) [99]. All p38 MAPKs are activated by dual MAPKKs (MKK3 and MKK6 which can activates all four isoforms [81, 100] that mediate the phosphorylation of Thr180 and Tyr182, which are separated by glycine. Typical activators of the p38 pathway are similar to JNK activators such as

cytokines [99], UV radiation [68], osmotic stress, growth factors [99], lipopolysaccharide [101].

p38α and p38ß can be inactivated by MAPK phosphatase-1, MAPK phosphatase-4 and MAPK phosphatase-5 [81]. The most frequently used specific inhibitor of p38 is SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole) [79]. We showed interaction of this compound with other signalling pathways since inhibitor SB203580 activated ERK and JNK in primary human hepatocytes, and ERK in HepG2 cells, HL-60 and SaOS cells [15]. p38 kinase is involved in the inflammatory response, apoptosis [102], cell cycle, cancer, development (reviewed in [81]) and in regulation of heme oxygenase-1 as Guan et al. [103] showed in primary rat aortic smooth muscle cells. The physiological importance of p38α was demonstrated in KO mice. KO mice died due to a defect in placental development (reviewed in [71]). In contrast, KO mice for other isoforms were viable and fertile with no apparent phenotype. Among these isoforms only p38α plays a crucial role and it can compensate the absence of ß, γ and δ.

2.4. Approaches to enzyme inhibition

Enzyme inhibition can proceed at various levels. Some inhibitors bind to the enzyme at the same site *(catalytic site)* as the substrate, and others bind at different site from catalytic site *(allosteric site)*. In the case of classic competitive inhibition the chemical structure of the inhibitor is similar to that of the substrate. When both the substrate and the inhibitor are present, they compete for binding sites on the enzymes but a lot of inhibitors do not exhibit properties of pure competitive of noncompetitive inhibition. Nowadays several different methods of enzyme inhibition are beeing used. Recently the small molecules (pyrazolopyrimidines e.g. PP121 and PP487) were found. These small molecules inhibit both tyrosine kinases and phosphatidylinositol-3-OH kinases (PI(3)Ks) by interaction with the hydrophobic pocket and thus proliferation of tumor cells (e.g. glioblastoma cells) were blocked [104]. SP600125 [80], SB203580 [79] and U0126 [105] (Fig. 4) are pharmacologic inhibitors for JNK, p38 and ERK, respectively, but their interaction with other signalling pathways plays an important role and thus their application is restricted. Also cell-permeable peptide inhibitors of JNK and ERK were tested and their specifity was shown since both JNK and ERK peptide inhibitor did not affect ERK and p38 or JNK and p38, respectively. Bonny et al. [106, 107] and Holzberg [106, 107] showed a block of JNK phosphorylation of c-JUN and protection of apoptosis induced by IL-1. Kelemen et al. [108] showed the prevention of activation of ERK and ERK-mediated activation of ELK1 transcriptional activity by using peptide inhibitor. Another approach to inhibition is knockdown of expression of target gene by using small interfering RNA (siRNA) which can be used for example in silencing of genes which mediates tumorogenesis, AIDS or hepatitis [109-111]. Lin et al. [112] used siRNA in HepG2 cells and they found out nearly 100% knockdown of p53 protein expression which indicate that this approach to inhibition is effective and interaction of inhibitory compound with other signalling pathways does not occur.

2.5 AhR and MAPKs

There in an increasing body of evidence on the mutual interactions between AhR and MAPKs (Fig. 5) [113]. It has been shown that typical AhR activators, such as TCDD and benzo[*a*]pyrene (BaP) activate the MAPKs. Conversely, it has been shown that protein kinases [114], including MAPKs, are key players in regulating AhR activity, as we have reviewed recently [115-117].

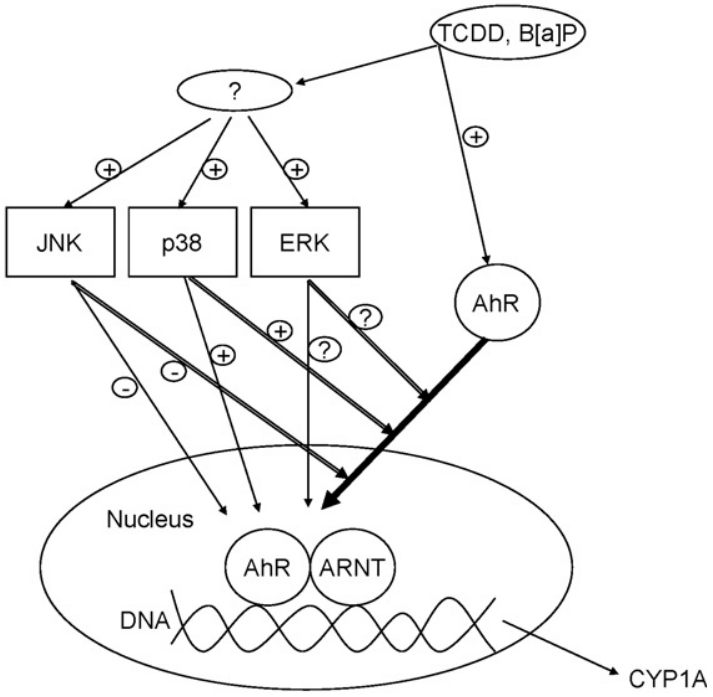


Fig. 5: JNK, p38 and ERK interaction with AhR. Ligands of AhR activate JNK, p38 and ERK in different human cells. Inhibition of translocation and transcriptional activity of AhR is mediated by activation of JNK. But activation of p38 has the opposite effect. Effect of ERK activation on AhR signalling has not been studied enough in human cells.

2.5.1. AhR and Extracellular signal-regulated kinase

The prototypic AhR ligands, TCDD and B[*a*]P, have been demonstrated to activate ERK in mouse Hepa - 1 hepatoma cell line [115], murine macrophages RAW 264.7 [118], human macrophages [119] and in human promonocytic leukemia cell line THP-1 [120]. The effects in Hepa - 1 cells proceed via an AhR-independent pathway since they occurred equally in AhR-negative as well as in AhR-positive cells [115]. In contrast,

TCDD did not induce ERK phosphorylation in rat liver epithelial WBF344 cells [121]. In addition, it has been shown that ERK is constitutively active and unaffected by TCDD treatment in human keratinocytes, while the JNK signalling pathway is modulated by TCDD in an AhR-dependent manner [122]. It was also found that epiregulin, a member of epidermal growth factor family that activates ERK [123], is induced by TCDD in an AhR-dependent manner [66]. In addition, TCDD increased raf-1, an up-stream regulator of ERK, in mouse lung tumors caused by *N*-nitrosodimethylamine (NDMA), compared with normal lung [124] and on the other site, quercetin inhibited MEK1 and raf-1 activities in JB6 mouse epidermal cells but resveratrol not affected neithet of them [125]. The different effects of quercetin and resveratrol could be due to the fact that quercetin is AhR an antagonist and resveratrol is a partial agonist of AhR. Similar findings with quercetin were observed in rat mesangial cells (SM43) [126]. It has been found that ERK is physically associated with AhR, as demonstrated by immunoprecipitation in Hepa1c1c7 cells [127]. ERK plays an important role in proteolysis of the AhR as constitutively active ERK1 facilitated AhR degradation as compared to the wild-type ERK1. Since the carboxyl region of AhR is associated with the transactivation region as well as the site for ubiquitination, it indicates that ERK-dependent phosphorylation targets the carboxyl region of the receptor [127]. Studies of the role of ERK in AhR functions are handicapped by the fact that U0126, a commonly used specific inhibitor of ERK up-stream kinase MEK1, is a partial agonist of the AhR. Typical for a partial agonist, U0126 slightly induced CYP1A1 protein in Hepa-1 cells, whereas it inhibited CYP1A1 induction by a full agonist TCDD [115]. Transiently transfected vector expressing constitutively active MEK1 was demonstrated to elevate the level of AhR-dependent luciferase activity [116]. Consistently, Andrieux et al. showed that U0126 is a ligand and agonist of AhR, since it induced CYP1A1 mRNA and protein in primary rat hepatocytes and human hepatoma B16A2 cell line. These effects were abolished by resveratrol, an established AhR antagonist [14]. Induction of CYP1A1 occurred independently of ERK1/2 activation and expression, as demonstrated by siRNA ERK2 transfection into ERK1(−/−) mouse hepatocyte [14]. In contrast to Andrieux et al., another group found U0126 not to be an AhR ligand since even at a 100 μM dose it did not displace 1 nM [^{3}H]-TCDD from binding to AhR. However, it facilitated AhR nuclear translocation and enhanced AhR/ARNT dimer binding to DRE [127]. Same as in the case of p38 and JNK, UV radiation also activates ERK1/2 in human epidermal cell line, HaCaT [70] and thus modulates AhR-dependent *CYP1* genes expression [128]. Important

AhR binding partner in the nucleus is the hypoxia-inducible factor-1 beta (HIF-1β), well known as ARNT [129]. Hypoxia also induces ERK activation in A549 and NCI-H157 cells derived from human lung adenocarcinoma and squamous cell lung cancer, respectively [130]. Thus, hypoxia-mediated inhibition of 3-methylcholanthrene-induced CYP1A1 expression [131] may be at least partialy attributed to ERK activation. Similarly to JNK and p38, ERK is activated by some cytokines [132, 133] and this activation may contribute to the suppression of CYP1A1 expression [134] during the inflammation. Acute inflammation is also mediated by TNF-α and its over-production plays a key role in many pathologic processes. Cheon et al. [120] found out that TNF-α production by TCDD in THP-1 macrophages is AhR-dependent. Similar results were obtained in human macrophages where TNF-α production were induced by TCDD and benzo[*a*]pyrene [119].

2.5.2. AhR and c-Jun *N*-terminal kinase

The activation of JNK by AhR ligands TCDD, BaP and BaP metabolites has been reported in CV-1 cells and mouse embryonic fibroblasts. Interestingly, the activation occurred in both AhR-negative and AhR-positive cells [115]. Recently, it has been demonstrated that TCDD induces morphological changes and modulates the plasticity of epithelial cells. These effects correlated with activation of JNK and were reversed by treatment with a specific JNK inhibitor [135]. Activity of JNK was also inhibited by quercetin (AhR antagonist) together with ERK but no p38 [136]. The induction of c-Jun, a down-stream target for JNK, was observed in the 5L cell line, the rat hepatoma cells expressing AhR. The induction was AhR-dependent since it was not observed in an AhR-deficient cell line. In addition, p38 kinase was involved in this process, since SB203580 (a specific p38 inhibitor) inhibited c-Jun induction by TCDD [137]. Data on the role of JNK in AhR function are scarce. Tan et al. studied JNK and ERK in the AhR-mediated induction of CYP1A1 and CYP1B1 in mice. Ablation of JNK2, together with chemically inhibited ERK, caused a significant decrease in CYP1A1 induction by TCDD in mouse thymus and testis, but not in liver. In contrast, CYP1B1 expression was unaffected in all three tissues of JNK2 knockout mice. These data suggest that JNK and ERK modulate ARNT activity and AHR/ARNT dependent gene expression [116]. We have demonstrated in our laboratory recently that activation of JNK by microtubules disruption and osmotic stress inhibits AhR transcriptional activity and nuclear translocation in primary culture of

human hepatocytes [138]. Significant progress in JNK research was made with the discovery of its specific inhibitor SP600125 [80]. However, experimental limitation of usage of these inhibitor in AhR studies has been shown. In addition to the artificial environmental stress, such as TCDD or PAHs, there are many other natural factors that may modify AhR functions. Among the daily present stimuli belong UV radiation and oxidative stress. Indeed, exposure of human skin to UV radiation results in the formation of photoreactive products of tryptophan which are weak AhR ligands and they in turn activate AhR [30, 139, 140]. This activation leads to induction of CYP1A1 and CYP1B1 mRNAs and proteins in human keratinocytes and HaCaT cultures [128]. Since it is known that JNK is activated by UV radiation [69, 141], the second mode of modulation of AhR transcriptional activity by UV radiation can be expected. Mutual interactions between JNK and AhR do not affect P450s expression only but they influence many other cellular processes, for instance, the expression of cytokines. It was described that JNK modulates the TCDD stimulated IL-1β expression in human keratinocytes [122]. Conversely, JNK is activated by interleukin-1 (IL-1) [89]. Thus, the expression of cytokines is modulated by JNK and reciprocally, JNK is activated by cytokines. The release of cytokines as a consequence of inflammation leads to inhibition of P450s' expression [142, 143] and molecular mechanism of this inhibition involves MAPKs.

2.5.3. AhR and p38

Interference between p38 and AhR signalling cascade has been proposed over the last 10 years. TCDD activated p38 MAP kinase in RAW 264.7 murine macrophages. In contrast, Tan et al. reported that AhR ligands, TCDD and B[*a*]P, activated JNK, ERK, but not p38 MAP kinase in CV-1 cells and mouse embryonic fibroblasts independently of AhR presence [115]. On the other hand Weiss et al. [137] showed activation of p38 in 5L rat cells. TCDD influenced expression of growth factors which could be possible mechanism of activation of p38. Activation of p38 substrate (Elk-1) was also observed and this activation occurred via an AhR-dependent mechanism because no activation of Elk-1 was observed in BP8-AhR$^-$ cells in contrast to BP-AhR$^+$ cells [137]. In addition, we have observed a slight increase in p38 phosphorylation in primary cultures of human hepatocytes challenged with TCDD (unpublished results). Shibazaki et al. [144] demonstrated that chemical inhibitors of p38 MAPK (SB203580 and SB202190; pyridinyl imidazole compounds) suppress CYP1A1 mRNA induction by TCDD in mouse hepatoma Hepa-1 and human hepatoma HepG2 cells. Consistently, CYP1B1 mRNA

induction by TCDD was impaired by p38 inhibitors in human breast adenocarcinoma MCF7 cells [144]. However, the transactivation of CYP1A1 reporter gene by TCDD in Hepa-1 cells was not suppressed by overexpression of the p38 MAPK dominant negative form. This implies that the suppression of CYP1A1 mRNA inducible expression by SB203580 and SB202190 is not due to inhibition of p38 activity [144]. The same authors found that SB203580 reduces spontaneous translocation of transiently overexpressed AhR in monkey kidney COS-7 cells [145]. However, an analogous compound SB202474, which is not a p38 MAPK inhibitor, did not reduce AhR translocation into the nucleus but it inhibited CYP1A1 mRNA induction by TCDD [144]. Similarly, subcellular distribution of AhR in human keratinocytes (HaCaT) was shifted towards a cytoplasmic localization after SB203580 treatment. Interestingly, a phosphorylation site within NES, Ser68, was identified [146]. The authors proposed Ser68 to be modified by p38. One-way cross-talk between p38 and ERK, where inhibition of p38 results in activation of ERK, has been described in a variety of cells including mouse fibroblasts NIH3T3 [147], human hepatoma cells HepG2 [148], rat cardiac myoblasts H9c2 [149], T-lymphocytes [150] and rat cortical neuron cultures [151]. Similarly to JNK, p38 is activated by UVR as well [68]. UV radiation-dependent activation of p38 was demonstrated by using a p38 inhibitor SB202190 on mice when the protection against sunburn-induced cell/apoptosis or inflammation was observed [152]. Thus, p38 activation could also be involved in modulation of AhR functions by UV radiation. The involvement of p38 in the expression of proinflammatory cytokines was proved by the team of Ipaktchi et al. who demonstrated the reduction of p38 by its inhibitors in mice *in vivo* [153]. Another group demonstrated modulation of hepatic CYP1A2 by Kupffer cells-derived proinflammatory cytokines during the sepsis [154]. Pro-inflammatory cytokine TNF-α was demonstrated to temporarily inhibit AhR-dependent induction of cytochrome P450 1A1 [155] and the synthesis of TNF-α culminated through activation of p38 [156].

3. OBJECTIVES

The aim of this work was to examine the effects of selected MAPKs inhibitors on AhR signalling pathway in primary cultures of human hepatocytes and cancer cell lines HepG2, with emphasis on:

- Interference of JNK specific inhibitor SP600125, p38 specific inhibitor SB203580 and U0126, an inhibitor of the up-stream MEK kinase, with AhR signalling pathway.

- Effects of model MAPKs activators on the activation of ERK, JNK and p38.

4. EXPERIMENTAL PART

4.1. BIOLOGICAL MATERIAL

4.1.1. HepG2 cells, Primary cultures of human hepatocytes, HL-60, SaOS, HaCat

Human hepatoma cells HepG2 (ECACC No. 85011430) were cultured in Dulbecco`s modified Eagle`s medium supplemented with 10% FBS, 1 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 1% non-essential amino acids, and 1 mM sodium pyruvate. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

Hepatocytes were prepared from lobectomy segments, resected from adult patients for medical reasons unrelated to our research program. Tissue acquisition protocol was in accordance with the requirements issued by local ethical commissions in France and Czech Republic. Human liver samples used in this study were obtained from patients in France (FT) and Czech Republic (LH): LH 18 (woman, 69 years); LH 19 (woman, 46 years); LH 20 (woman, 67 years), LH 21 (woman, 61 years), LH26 (man, 58 years), FT 278 (man, 71 years), FT 279 (woman, 57 years), FT 280 (man, 59 years). Hepatocytes were isolated as previously described [157]. Following isolation, the cells were plated on collagen-coated culture dishes at a density 1.4×10^5 cells/cm^2. In addition, Long-term human hepatocytes in monolayer Batch HEP220221 (Biopredict International, Rennes, France), were used. Culture medium was described previously [158] enriched for plating with 2% fetal bovine serum FBS (*v/v*). The medium was exchanged for serum-free medium the day after and the culture was allowed to stabilize for an additional 48 h – 72 h prior to treatments. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator.

Human liver microsomes were purchased as pooled, cryopreserved samples from Advancell (Barcelona, Spain) from five men and five women. Microsomes were obtained according to ethical rules of the country of origin (Spain).

Human promyelocytic leukemia cells HL-60 (ECACC no. 98070106), human primary osteogenic sarcoma SaOS-2 (ECACC no. 89050205) and immortalized non tumorigenic human keratinocytes cells HaCaT. The latter cell line was a generous gift from Dr. Miroslav Machala (Veterinary Research Institute, Brno, Czech Republic). Cell lines were cultured according to the instructions from manufacturer. Prior to the treatments, cells were deprived from serum for 4-6 h.

4.2. REAGENTS

Dulbecco`s modified Eagle`s medium (DMEM), foetal calf serum, penicillin, streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate, dexamethasone, Triton X-100 were purchased from Sigma Chemicals (St. Louis, MO, USA). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was purchased from Ultra Scientific (RI, USA). Trizol® Reagents, was purchased from GibcoBRL Life Technologies (Cergy Pontoise, France). Complete™ protease inhibitor cocktail tablets and FuGENE 6 transfection reagent were purchased from Roche Diagnostics GmbH (Mannheim, Germany). AMAXA nucleofector kit V was purchased from Lonza (Koeln, Germany). Secondary horseradish peroxidase conjugated antibody, and Western Blotting Luminol Reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anisomycin, sorbitol, epidermal growth factor, SP600125 (1,9–pyrazoloanthrone), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole), and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene ethanolate) were from Sigma-Aldrich (St Quentin Fallavier, France).

4.3. OTHER MATERIAL

Kodak X-Omat AR photographic film, Kodak GBX developer and replenisher, Kodak GBX fixer and replenisher (Eastman Kodak, USA), blotting membrane Immun-Blot™ PVDF Membrane (0.2 μm) (Bio-Rad Laboratories, USA), filtres 0.22 μm Millex®-GS (Millipore, USA), cultivation bottles Sarsdet (Sarsdet, Germany), cultivating plates, Petri dishes, sterile plastic pipettes Nunc™ (Nunc, Denmark), tubes (Eppendorf, Germany),

collagen-coated culture dishes were purchased from BD Biosciences (Le Pont de Claix, France).

4.4. METHODS

4.4.1. RNA isolation and qPCR

Total RNA was isolated using TRIZOL Reagent (Invitrogen). Cells ($1.5x10^6$ cells) were washed by PBS buffer pH 7.4 ± 0.2 (137 mM NaCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 and 2.7 mM KCl) and lysed by adding 1 ml of TRIZOL Reagent. The homogenized sample was incubated for 5 min at room temperature. Next 0.2 ml of chloroform per 1 ml of TRIZOL reagent was added. The samples were centrifugated at 12,000 x *g* for 15 min at 4°C. Following centrifugation the aquous phase was transferred to a new tube. The RNA was precipitated by adding 0.5 ml isopropyl alcohol. Sample was incubated at room temperature for 10 min and centrifuged at 12,000 x *g* for 10 min at 4°C. The pellet was washed with 1 ml of 75% ethanol. RNA pellet was briefly dried and dissolved in RNase-free water. cDNA was synthesized from 200 ng of total RNA using M-MLV Reverse Transcriptase (Invitrogen) at 37°C for 50 min in the presence of random hexamers (Amersham Biosciences). One tenth was used for qRT-PCR amplification using the Light Cycler apparatus (Roche Diagnostic Corporation, Meylan, France). The following program was used: an activation step at 95°C for 10 min was followed by 40 cycles of PCR (denaturation at 95°C for 10 seconds; annealing of 7 seconds at 65°C for CYP1A1/2 or 68°C for GAPDH; elongation at 72°C for 17 s). Primers (Generi-Biotech, Czech Republic) were as follows:

CYP1A1 – forward: 5′-TCCGGGACATCACAGACAGC-3′

CYP1A1 – reverse: 5′-ACCCTGGGGTTCATCACCAA-3’

CYP1A2 – forward: 5′-CATCCCCCACAGCACAACAA-3′

CYP1A2 – reverse: 5′-TCCCACTTGGCCAGGACTTC -3′

GAPDH – forward: 5′-CAAAGTTGTCATGGATGACC-3′

GAPDH – reverse: 5′-GGTCGGAGTCAACGGATTTGGTCG-3′

The measurements were performed in duplicates. Expression of CYP1A1 and CYP1A2, mRNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Data were processed by the delta-delta method.

4.4.2. Protein analyses, Western blotting and detection of MAPKs/AhR/ CYP1A proteins in HepG2 cells and human hepatocytes

Hepatocytes were plated on collagen-coated culture dishes. The medium was exchanged for serum-free medium the day after and the culture was allowed to stabilize for an additional 48 h – 72 h prior to treatments. HepG2 cells were seeded on 6-well dishes or on Petri dishes (100 mm I.D.) using culture media enriched with fetal bovine serum (10% *v/v*). Following 24 h of stabilization, the medium was exchanged for a serum-free one (in case of MAPKs detection) and the cells were treated for 30 min and 24 hours with increasing concentrations of SP600125 in the presence or absence of TCDD (final concentration 5 nM), SB203580 or U0126 (in case of MAPKs detection) or for 24 h with dexamethasone (DEX; 100 nM final concentration), 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM final concentration), TCDD+DEX and with DMSO as vehicle for control. Following the treatments, total protein extracts were prepared as follows: Cells were washed twice with 1 ml of ice-cold phosphate-buffered saline (PBS) and scraped into 1 ml of PBS. The suspension was centrifuged (1500 g/ 5 min/ 4°C) and the pellet was resuspended in 120 µl of ice-cold lysis buffer (10 mM Hepes pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM DTT; 0.1% *v/v* NP-40; anti-protease cocktail, 0.2% *v/v* sodium dodecyl sulfate). The mixture was incubated for 20 min on ice and then centrifuged (12,000 g/ 10 min/ 4°C). Supernatant was collected and the protein content in extracts was determined by the biscinchoninic acid method (Pierce, Rockford, IL.) [159]. The extracts were analyzed on SDS-PAGE gels (8%) which were run on a BIORAD (La Jolla, CA, USA) apparatus according to the general procedure. Protein transfer onto nitrocellulose membrane was carried out, the membrane was stained with Ponceau S red for control of transfer and then saturated with 8% non-fat dried milk for 2 hours. Blots were probed with primary antibodies against: aryl hydrocarbon receptor (Ah Receptor (H-211), rabbit polyclonal, dilution 1/500), human CYP1A1 (CYP1A1 (G-18) goat

polyclonal, dilution 1/500), p-c-Jun Ser 63/73 (goat polyclonal, dilution 1/500), b-actin (Actin (I-19) goat polyclonal, dilution 1/2000) all purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA); p38-P (Phospho-p38, Thr180/Tyr182, rabbit polyclonal), ERK-P (Phospho-p44/42, Thr202/Tyr204, rabbit polyclonal), JNK-P (Phospho-SAPK/JNK, Thr183/Tyr185, rabbit polyclonal) all purchased from Cell Signalling Technology, USA. Chemiluminescence detection using horseradish peroxidase conjugated secondary antibodies and an Amersham (GE Healthcare) ECL kit was performed.

4.4.3. Gene reporter assay

pDRE-luc plasmid containing two inverted repeats of the XRE (xenobiotic responsive element) of mouse *cyp1a1* upstream of the thymidine kinase promoter and luciferase reporter gene [160, 161] was kindly provided by Dr. L. Poellinger (Karolinska Institute, Stockholm, Sweden). p1A1-luc plasmid containing 5´-flanking region (-1566 to +73) of human CYP1A1 gene subcloned into the *Kpn*I–*Hin*dIII double-digested pGL3-Basic vector (Promega, Madison, WI) upstream of the firefly luciferase reporter gene [162] was a generous gift from Dr. Robert Barouki (INSERM U490, Paris, France). For gene reporter assays, HepG2 cells were transiently transfected by lipofection (FuGENE 6) or AMAXA solution V with 200 ng/well of pDRE-luc or p1A1-luc plasmid in 24-well plates. Following 16 h of stabilization, the cells were treated for 24 h with increasing concentrations of SP600125, SB203580 and U0126 in the presence or absence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; 5 nM final concentration). After the treatments, cells were lysed and luciferase activity was measured on a luminometer Berthold (Germany) and standardized per milligram of protein.

4.4.4. Statistical analyses

One-way ANOVA followed by Dunnett's multiple comparison *post hoc* test was used for statistical analysis of differences between two groups using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Two-way ANOVA with interaction

was used to compare induction profiles of CYP1A1 and CYP1A2 mRNAs in RT-PCR experiments. Data are plotted as means ± standard deviation (SD). EC_{50} (xenobiotic concentration required to achieve half-maximum promoter activation) and IC_{50} (the concentration that is required for 50% inhibition) values were determined according to Hill's equation by nonlinear regression analysis using GraphPad Prism Software from at least seven-point curves performed in triplicate (concerned only RNA data published in Biochemical Pharmacology 75).

4.4.5. Metabolism of SP600125 and SB203580

Metabolism of SP600125

SP600125 (100 μM final concentration) or vehicle (DMSO; 0.1%, *v/v*, final concentration) were incubated for 2 h with primary cultures of human hepatocytes, cultured HepG2 cells or pooled human liver microsomes. For human liver microsomes, reaction mixture contained in 200 ml: 100 pmol CYP, NADPH-generating system (3.76 mM isocitrate, 1.04 mUnits of isocitrate dehydrogenase, 0.485 mM $NADP^+$, 5 mM $MgSO_4$), 100 μM SP600125 in 50 mM Tris/KCl buffer, pH 7.4. The reaction was stopped by addition of 20 ml of 70% $HClO_4$. Metabolites were extracted to dichloromethane, vortexed for 15 s and the organic extract was subsequently evaporated under gentle stream of nitrogen. Subsequently, 100 ml of the mobile phase was added to the sample. Similarly, 1ml of culture media was deproteined by 100 ml of $HClO_4$ prior to the extraction with dichloromethane. As a positive control, SP600125 was added to the blank mixture/media post-denaturation to exclude possible non-enzymatic transformation of SP600125. The extracts were subjected to high performance liquid chromatography (HPLC) analyses.

HPLC analyses

Separations were done using a HPLC method based on chromatography on the Merck RP-18e LiChrospher 100 column (Darmstadt, Germany) with a mobile phase consisting of acetonitrile/water/acetic acid mixture (40:59.9:0.1, v/v). A Shimadzu Class VP system (Tokyo, Japan) was used with detection at 200 nm and a flow rate of 1ml/min.

Two minor metabolites of SP600125 were found in 3.9 and 4.7 min (M1 and M2). These metabolites were isolated by HPLC (fractionation, post-column collection) and subjected to mass spectrometry (MS) analyses.

μLC/MS^2 analyses

A standard of SP600125 and fractionated metabolites M1 and M2 were analyzed by μLC/MS^2. Micro-liquid chromatograph CapLC XE was hyphenated with Q-TOF Premier mass spectrometer (Waters, Milford, USA). Microcolumn Gemini C-18, particle size of the stationary phase 5 μm (Phenomenex, USA, column dimensions: 150mm x 300 μm i.d.) was used at a flow rate 5 μl/min. A binary gradient elution was performed. Mobile phase A was 5.7 mmol/l solution of acetic acid in water with 5% acetonitrile, mobile phase B was pure acetonitrile; gradient profile was as follows: 0–5 min 0–10% B, 5–25 min 10–50% B, 25– 40 min 50–80% B, 40–45 min 80 100% B. Evaporated samples were simply dissolved in mobile phase A (150 μl) and centrifuged prior to injection. Injection volume was 1 μl, sample was injected using an autosampler.

Optimized parameters of electrospray were: capillary voltage +2.5 kV, sampling cone 40 V, source temperature 50°C, desolvation temperature 200°C, cone gas flow 20 l/h and desolvation gas flow 300 l/h. Data were obtained in a single V mode. Data were collected in cyclic repeated scan events covering MS and MS/MS data (collision induced dissociation of parent ion in collision cell, previously isolated in first quadrupole of mass analyzer) during chromatographic run. MS spectra used for interpretation were averages of scans over chromatographic peaks (baseline subtraction was used to filter impurities of mobile phase). Retention times of SP600125, M1 and M2 were 26.7, 20.8 and 22.6 min, respectively. Solution of leucine-enkephaline (20 μg/l) in a mixture of water:methanol (1:1) was used for lock mass correction during exact mass measurement. Flow rate of the solution into the reference ESI probe of ion source was 2 μl/min.

Metabolism of SB203580

SB203580 (50 μM final concentration) or vehicle (DMSO; 0.1% *v/v* final concentration) were incubated for 30 min and 45 min with cultured HepG2 cells or

pooled human liver microsomes. For human liver microsomes, reaction mixture contained in 200 μl: 100 pmol CYP (cytochrome P450), NADPH-generating system (3.76 mM isocitrate, 1.04 mU of isocitrate dehydrogenase, 0.485 mM $NADP^+$, 5 mM $MgSO_4$) and 50 μM SB203580 in 50 μM Tris/KCl buffer, pH 7.4. The reaction was stopped by addition of two volumes of methanol. Mixture was centrifuged and supernatants were used for μLC/MS^2 analyses. Similarly, 1 ml of culture media was deproteined by methanol prior to the μLC/MS^2 analyses. As a positive control, SB203580 was added to the blank mixture/media post denaturation to exclude possible nonenzymatic transformation of SB203580.

μLC/MS^2 analyses

A standard of SB203580 and related metabolized samples were analyzed by μLC/MS^2. Micro-liquid chromatograph CapLC XE was hyphenated with Q-TOF Premier mass spectrometer (Waters, Milford, USA). Microcolumn Gemini C-18, particle size of the stationary phase 5 μm (Phenomenex, USA, column dimensions: 150 mm×300 μm I.D.) was used at a flow rate 5 μl/min.

A binary gradient elution was performed. Mobile phase A was prepared as 10 mmol/l solution of phosphoric acid in water adjusted to pH=7.4 with ammonia. After the pH adjustment 5% (*v/v*) of acetonitrile was added to the solution. Mobile phase B was pure acetonitrile. Gradient profile was as follows: 0–5min 10% B, 5–25 min 10–50 min, 25–40 min 90% B, 40–50 min 90–95% B. After each run an equilibration to the initial mobile phase composition (10% B) was performed for 10 min.

A standard of SB203580 was dissolved in mobile phase A and final concentration was adjusted to 10^{-4} mol/l. Samples of reaction mixture after metabolization and related positive control were centrifuged prior to analysis. Injection volume was 0.3 or 1 μl. Samples were injected using an autosampler.

Electrospray ionization was used in all analyses. Optimized parameters of ion source (Z-spray) were: capillary voltage +2.8 kV, sampling cone 50 V, source temperature 120°C, desolvation temperature 150°C, cone gas flow 31 l/h and desolvation gas flow 400 l/h. Data were obtained in a single V mode. Data were collected in cyclic repeated scan events covering MS and MS/MS data (collision induced dissociation of

parent ion in collision cell, previously isolated in first quadrupole of mass analyzer) during the chromatographic run. MS spectra used for interpretation were averages of scans over chromatographic peaks (baseline subtraction was used to filter impurities of mobile phase). Solution of leucine-enkephaline (20 μg/l) in a mixture of water:methanol (1:1) was used for lock mass correction during exact mass measurement. Flow rate of the solution into the reference ESI probe of ion source was 3 μl/min.

5. RESULTS

5.1. EFFECTS OF MODEL ACTIVATORS AND INHIBITORS ON MAPKS ACTIVATION

5.1.1. Effects of MAPKs activators – epidermal growth factor, sorbitol and anisomycin on mitogen-activated protein kinases activation

In initial series of experiments, we examined the effects of MAPKs activators on the activation of ERK, p38 and JNK kinases in primary human hepatocytes and HepG2 cells (a cancer cell line derived from human liver cells, as the alternative to human hepatocytes) using sorbitol (SOR) to activate JNK by osmotic shock [163], anisomycin (ANI) to activate p38 by genotoxic shock [164] and epidermal growth factor (EGF) to activate ERK via EGF-receptor [165]. The HepG2 cells were treated with 0.4 M sorbitol, 5 μM anisomycin and 75 ng/ml EGF for 30 min, 6 h, 24 h and activation of ERK-P(Thr202/Tyr204), JNK-P(Thr183/Tyr185) and p38-P(Thr180/Tyr182) was monitored by western blot in total cellular extracts. We also tested the effect of TCDD on MAPKs activation. The HepG2 cells were treated with 5 nM TCDD for 30 min.

ERK was activated by EGF, anisomycin and slightly by sorbitol. TCDD did not activate ERK in HepG2 cells (Fig. 6). p38 was activated by anisomycin and very slightly by sorbitol but TCDD and EGF did not activate p38 in HepG2 cells (Fig. 6). Anisomycin and sorbitol activated JNK but TCDD and EGF did not cause JNK activation in HepG2 cells (Fig. 6).

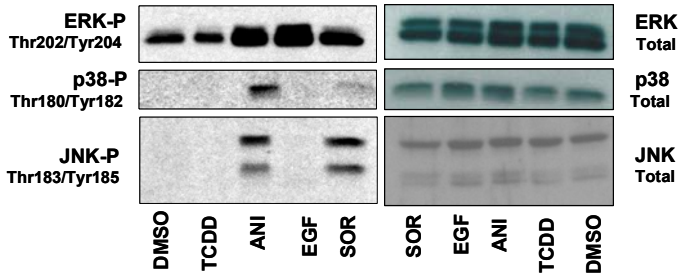


Fig. 6: The effects of MAPKs activators on the activation of ERK, p38 and JNK kinases activation in HepG2 cells. Cells were treated with vehicle (0.1% *v/v* DMSO), TCDD (5 nM), anisomycin (ANI) (5 μM), EGF (75 ng/ml) and sorbitol (SOR) (0.4 M) for 30 min. Total proteins were isolated and western blot analyses were performed. The membranes were probed with anti ERK-P(Thr202/Tyr204), JNK-P(Thr183/Tyr185) and p38-P(Thr180/Tyr182) antibodies.

In human hepatocytes, ERK was activated by EGF but not by sorbitol and anisomycin. The effect of EGF was more apparent after 30 min than after 6 h and 24 h (Fig. 7). JNK was activated by anisomycin and sorbitol and slightly by EGF in 30 min experiment. Very slight activation of JNK mediated by sorbitol was observed in 24 h experiment (Fig. 7). Similarly, p38 was activated by anisomycin, soritol and EGF. The most potent activator was anisomycin. Activation vanished after 6 h and 24 h of the treatment (Fig. 7).

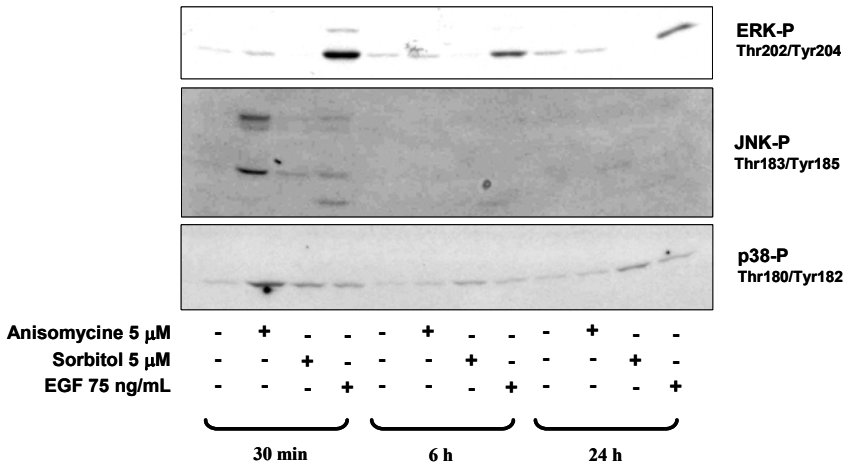


Fig. 7: Effects of model activators on MAPKs activity in primary cultures of human hepatocytes. Primary cultures of human hepatocytes (FT278) were treated with vehicle (0.1% *v/v*; DMSO), sorbitol (0.4 M), anisomycin (5 µM) and epidermal growth factor (75 ng/ml; EGF) for 30 min, 6 h and 24 h. Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185) and anti p38-P(Thr180/Tyr182) antibodies.

5.1.2. Effects of MAPKs inhibitors SP600125, SB203580 and U0126 on mitogen-activated protein kinases activation

Next, we examined the effects of SB203580 (a pharmacological inhibitor of p38 MAPK) together with U0126 (a pharmacological inhibitor of ERK) and SP600125 (a pharmacological inhibitor of JNK) on MAPKs pathways in human hepatoma cells HepG2. The HepG2 cells were treated with vehicle (0.1% *v/v*; DMSO), SB203580 (0.1-20 μM), SP600125 (10 μM), U0126 (10 μM) and with model activators - anisomycin (5 μM), sorbitol (0.4 M), epidermal growth factor (75 ng/ml; EGF) for 30 min. SB203580 dose-dependently activated ERK but not p38 and JNK. The maximal activation of ERK was attained for concentration of SB203580 ranging between 1 μM and 10 μM (Fig. 8). The effects of SB203580 on ERK were abolished by U0126, an inhibitor of the up-stream MEK kinase that phosphorylates ERK (Fig. 8). U0126 inhibited activation of ERK mediated by EGF and 10 μM SB203580 in HepG2 cells (Fig. 8). SP600125 inhibited very slightly activation of JNK mediated by sorbitol in HepG2 cells (Fig. 8).

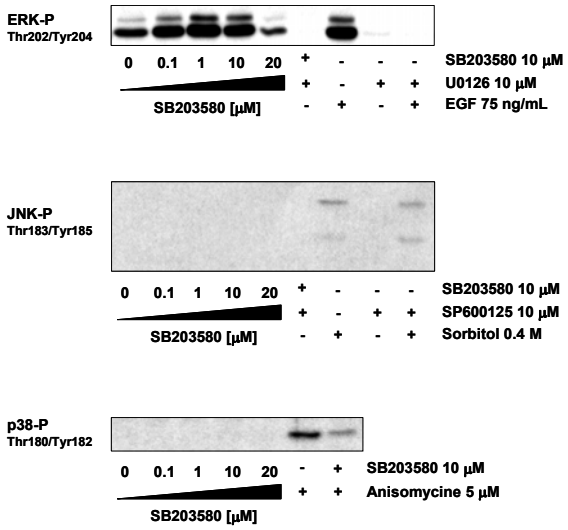


Fig. 8: Effects of SB203580 on MAPKs activity in HepG2 cells. Cells were treated with vehicle (0.1% *v/v*; DMSO), SB203580 (0.1 μM, 1 μM, 10 μM, 20 μM), anisomycin (5 μM), sorbitol (0.4 M), epidermal growth factor (75 ng/ml; EGF), specific inhibitor of ERK-MEK (10 μM; U0126) and inhibitor of JNK (10 μM; SP600125) for 30 min. Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185) and anti p38-P(Thr180/ Tyr182) antibodies.

We also treated primary cultures of human hepatocytes with MAPKs inhibitors for 30 min. For control, cells were treated with model activators, inhibitors or their combination for the respective MAPK kinase. We observed that SB203580 dose-dependently activated ERK and JNK MAPK kinases in primary cultures of human hepatocytes and the levels of ERK-P(Thr202/Tyr204), JNK-P(Thr183/Tyr185) were increased by SB203580 (Fig. 9, 10). This activation was inhibited by ERK and JNK inhibitors U0126 and SP600125, respectively. The activation of ERK by SB203580 occurred rapidly (30 min) (Fig. 9). The basal level of p38-P(Thr180/Tyr182) and the activation of p38 by anisomycin were diminished by SB203580 (Fig. 9). The effects of SB203580 on ERK were abolished by U0126 (Fig. 9). Activation of ERK by SB203580 occured after 30 min (Fig. 9, 10) and after 24 h were not so apparent (Fig. 11). Whereas the activation of JNK was more apparent after 24 h (Fig. 11). p38 was not activated by SB203580 in human hepatocytes (Fig. 10, 11). U0126 and SP600125 had similar effects in human hepatocytes as were observed in HepG2 cells (Fig. 8, 9).

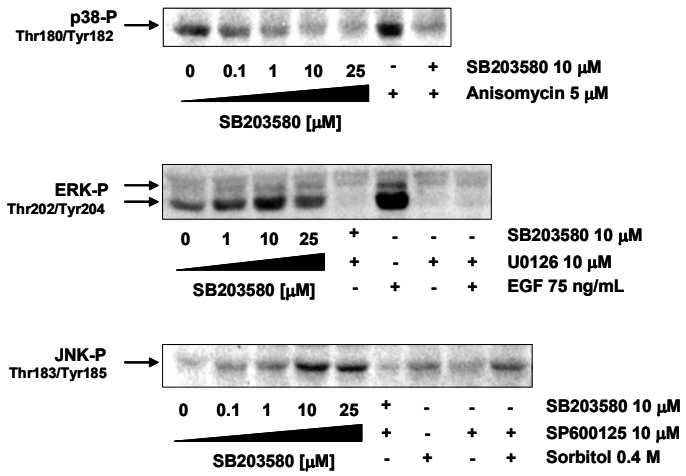


Fig. 9: Effects of SB203580, SP600125 and U0126 on MAPKs activity in primary cultures of human hepatocytes. Primary cultures of human hepatocytes (LH21) were challenged for 30 min with vehicle (0.1% *v/v*; DMSO), anisomycin (5 µM), sorbitol (0.4 M), epidermal growth factor (75 ng/ml; EGF), specific inhibitor of ERK-MEK (10 µM; U0126) and inhibitor of JNK (10 µM; SP600125) and/or SB203580 (0.1 µM; 1 µM; 10 µM; 25 µM). Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185), anti p38-P(Thr180/Tyr182) and anti c-Jun-P(Ser63/73) antibodies.

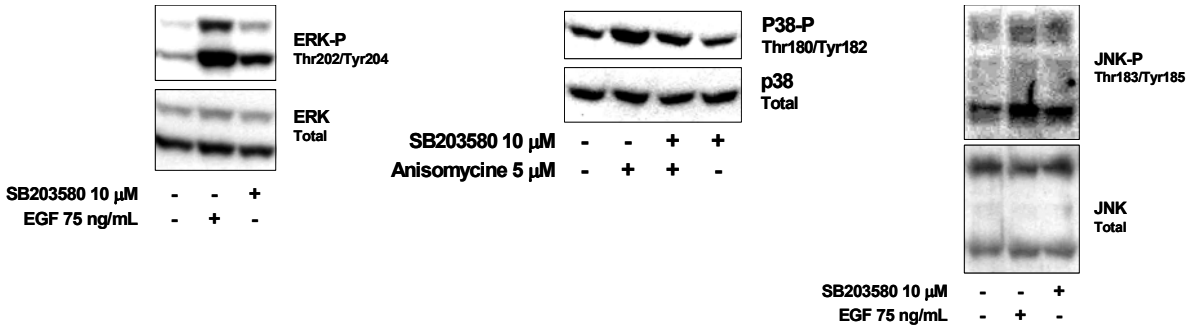


Fig. 10: Effects of SB203580 on MAPKs activity in primary cultures of human hepatocytes. Primary cultures of human hepatocytes (FT279) were challenged for 30 min with vehicle (0.1% *v/v*; DMSO), anisomycin (5 µM), epidermal growth factor (75 ng/ml; EGF), and SB203580 (10 µM). Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185), anti p38-P(Thr180/Tyr182) antibodies.

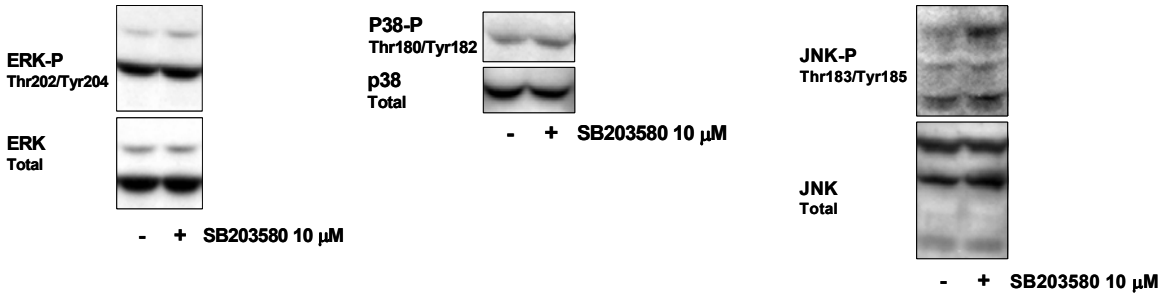


Fig. 11: Effects of SB203580 on MAPKs activity in primary cultures of human hepatocytes. Primary cultures of human hepatocytes (FT280) were challenged for 24 h with vehicle (0.1% *v/v*; DMSO), and SB203580 (10 µM). Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185), anti p38-P(Thr180/Tyr182) and anti c-Jun-P(Ser63/73) antibodies.

We showed that SB203580 exerts differential effects on JNK in normal cells (primary human hepatocytes) and in HepG2 cancer cell line derived from human liver cells (Fig. 8, 9). Since we observed differential activation of ERK and JNK by SB203580 in primary human hepatocytes and human hepatoma cells HepG2, we examined the effects of SB203580 in other unrelated cell lines. We used human promyelocytic leukemia cells HL-60, human primary osteogenic sarcoma SaOS-2 and immortalized nontumorigenic human keratinocytes cells HaCaT. Anisomycin activated p38 MAPK in all cell lines tested. This effect was abolished by SB203580 (Fig. 12). EGF activated ERK in SaOS-2 and HaCaT cells but not in HL-60 cells. Consistent with the effects in HepG2 cells, SB203580 activated ERK in SaOS-2, HL-60 and slightly in HaCaT cells (Fig. 12). In contrast, SB203580 did not activate JNK in either cell line used, (Fig. 12). Sorbitol was a potent activator of JNK in four cancer cell lines (Fig. 8, 12), whereas it stimulated JNK in human hepatocytes only moderately (Fig. 9). These data confirm the cell-type specificity of SB203580 action.

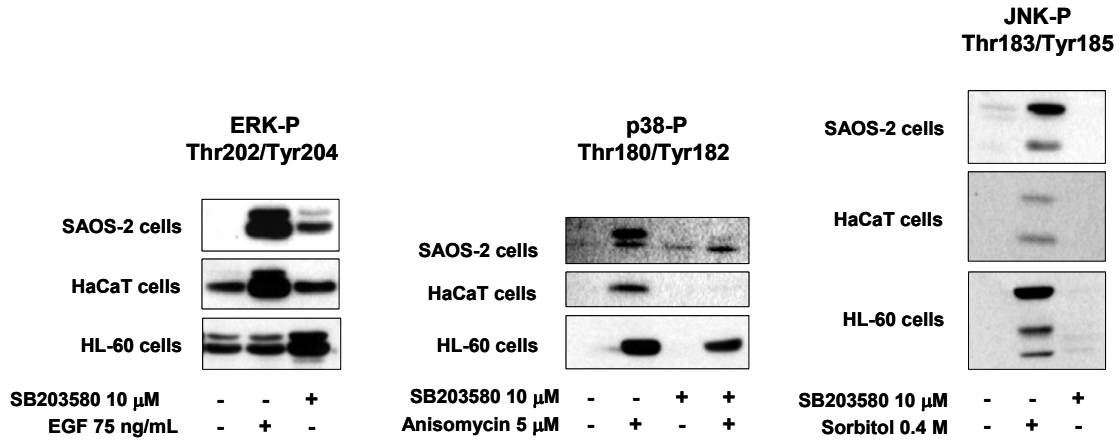


Fig. 12: The effects of SB203580 on MAPKs activity in HL-60, SaOS-2 and HaCaT cell lines. Cells were treated with vehicle (0.1% *v/v*; DMSO), SB203580 (10 μM), anisomycin (5 μM), sorbitol (0.4 M) and epidermal growth factor (75 ng/ml; EGF) for 30 min. Total proteins extracts were isolated, and after western blotting analysis the membranes were probed with anti ERK-P(Thr202/Tyr204), anti JNK-P(Thr183/Tyr185) and anti p38-P(Thr180/Tyr182) antibodies.

5.2. EFFECTS OF MAPKS INHIBITORS ON THE EXPRESSION OF CYP1A mRNA AND PROTEIN

5.2.1. Effects of SP600125, SB203580 and U0126 on the CYP1A protein expression in human hepatocytes

In next series of experiments, we examinated the effects of MAPKs inhibitors (SP600125, SB203580 and U0126) on CYP1A protein level in human hepatocytes. SP600125 decreased TCDD- inducible expression of CYP1A proteins and basal induction of CYP1A was unchanged in treatment with 10 μM SP600125 and slight decrease of CYP1A protein was observed in treatment with 100 μM (Fig. 13). U0126 induced expression of CYP1A proteins and SB203580 caused only slight induction of CYP1A proteins in comparison with negative control (DMSO). However, all inductions were smaller in comparison with positive control (TCDD) (Fig. 14).

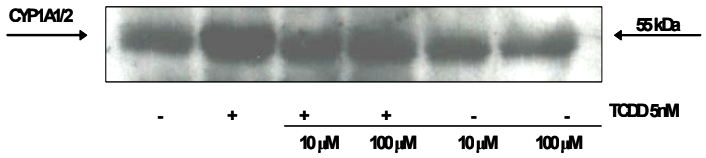


Fig. 13: Effects of SP600125 on expression and TCDD-inducible expression of CYP1A proteins in human hepatocytes. Representative western blotting analyses. Human hepatocytes were treated with vehicle (DMSO, 0.1% *v/v*), SP600125 (10, 100 μM) in the presence or absence of TCDD (5 nM) for 24 h.

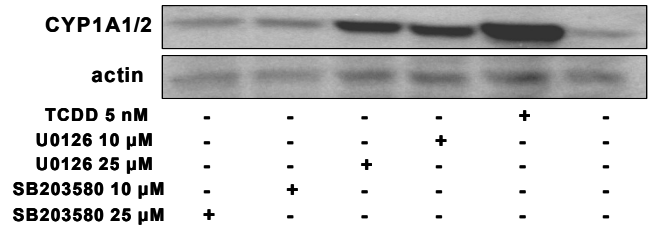


Fig. 14: Effects of U0126 and SB203580 on expression of CYP1A1 and CYP1A2 proteins in human hepatocytes. Representative western blotting analyses. Human hepatocytes were treated with vehicle (DMSO, 0.1% *v/v*), 5 nM TCDD, U0126 and SB203580 (10, 25 μM) for 24 h.

5.2.2. Effects of SP600125, SB203580 and U0126 on the CYP1A protein expression in HepG2 cells

We observed a somewhat different results in experiments with HepG2 cells in comparison with results obtained in human hepatocytes. The level of CYP1A protein was unchanged in treatments with SP600125 in comparison with DMSO (negative control). U0126 slightly induced expression of CYP1A protein and SB203580 caused the most potent induction of CYP1A protein in comparison to other inhititors (Fig. 15).

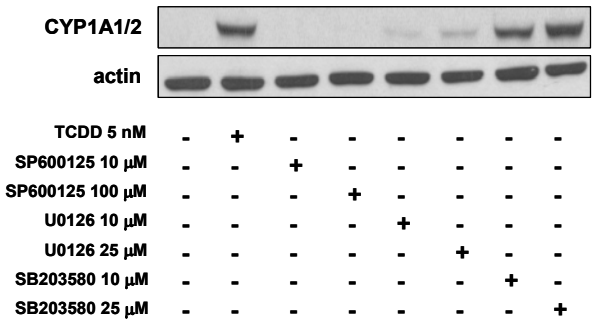


Fig. 15: Effects of SP600125, U0126 and SB203580 on expression of CYP1A proteins in HepG2 cells. Representative western blotting analyses. HepG2 cells were treated with vehicle (DMSO, 0.1% *v/v*), 5 nM TCDD, SP600125 (10, 100 μM), U0126 and SB203580 (10, 25 μM) for 24 h.

5.2.3. Effects of SP600125, SB203580 and U0126 on the expression and TCDD-inducible expression of CYP1A mRNAs in HepG2 cells and human hepatocytes

We tested whether MAPKs inhibitors are capable to induce CYP1A1 mRNA in the cancer cell line HepG2. Cells were treated with vehicle (0.1% *v/v* DMSO) (negative control), 100 μM SP600125 and 5 nM TCDD (positive control) in the presence or absence of 100 μM resveratrol (RVT) and levels of CYP1A1 mRNA were analysed by qRT-PCR. Both SP600125 and TCDD induced CYP1A1 mRNA in HepG2 cells but the induction mediated by SP600125 was smaller in comparison with TCDD. In addition, we used RVT (an antagonist of AhR [60]) to test whether the effects of SP600125 were AhR-dependent. RVT abolished these inductions (Fig. 16). These data confirm that SP600125

induced CYP1A1 genes via AhR in HepG2. We also observed a slight induction of CYP1A1 mRNA, which was mediated by RVT (Fig. 16). In next experiments we tested effects of U0126 and SB203580 on the expression of CYP1A1 mRNA in HepG2 cells. Cells were treated with 0.1% (*v/v)* DMSO (as negative control), 5 nM TCDD (as positive control), increasing concentrations of U0126 or SB203580. We observed a slight induction of CYP1A1 mRNA mediated by U0126 in a dose-dependent manner. SB203580 caused also a slight induction of CYP1A1 mRNA but the maximal induction was mediated by 10 μM SB203580 (Fig. 17).

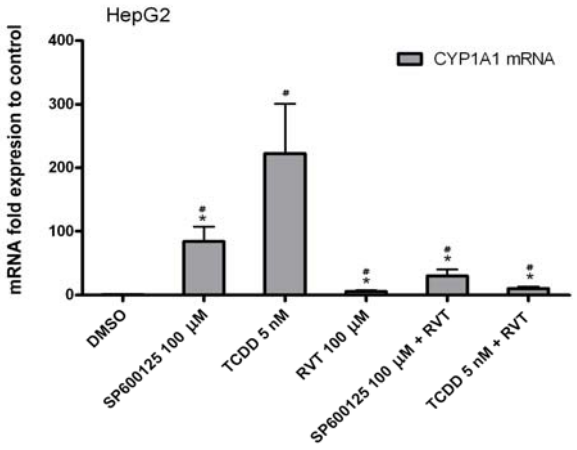


Fig. 16: Effects of SP600125 on the expression of CYP1A1 mRNA in HepG2 cells. Human hepatoma cells were treated with vehicle (0.1% *v/v* DMSO), SP600125 (100 μM) and TCDD (5 nM) in the presence or absence of AhR antagonist resveratrol (RVT; 100 μM) for 24 h. Representative RT-PCR analyses of CYP1A1 mRNA are shown. The data are mean ± S.D. from duplicate measurements and are expressed as fold induction over DMSO-treated cells. The data were normalized to GAPDH mRNA levels. #Significantly different from DMSO-treated cells ($p < 0.05$); *significantly different from TCDD-treated cells ($p < 0.05$).

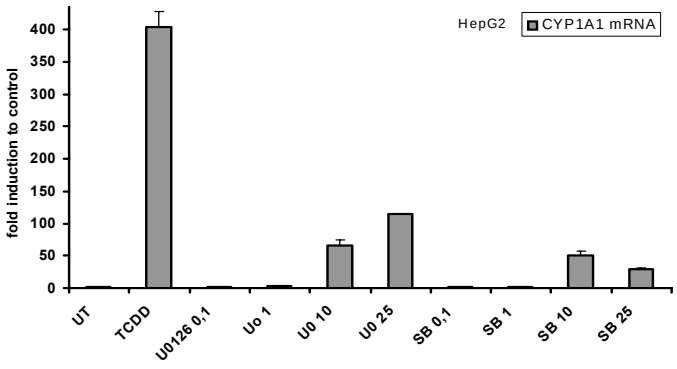


Fig. 17: Effects of U0126 and SB203580 on the expression of CYP1A1 mRNA in HepG2 cells. Human hepatoma cells were treated with vehicle (0.1% *v/v* DMSO), U0126 and SB203580 (0.1, 1, 10 and 25 μM) and TCDD (5 nM) for 24 h. Representative RT-PCR analyses of CYP1A1 mRNA are shown. The data are mean ± S.D. from duplicate measurements and are expressed as fold induction over DMSO-treated cells. The data were normalized to GAPDH mRNA levels.

We also tested the effects of SP600125, SB203580 and U0126 on AhR-dependent expression of CYP1A1 or CYP1A2 mRNAs in primary cultures of human hepatocytes. For this purpose, human hepatocytes were treated with vehicle (0.1% *v/v* DMSO), increasing concentrations of SP600125 (0.1 - 100 μM), SB203580 or U0126 (1- 25 μM), 5 nM TCDD and 100 μM RVT and the levels of CYP1A1 and CYP1A2 mRNAs were analyzed using qRT-PCR. SP600125 caused a dose-dependent induction of CYP1A1 and CYP1A2 mRNAs (Fig. 18). Maximal induction of CYP1A1 mRNA was observed after treatment with $1x10^{-4}$ M SP600125. But this induction was smaller compared to that reached by a typical AhR agonist TCDD. This finding was in accordance with results obtained in HepG2 cells (Fig 16). RVT inhibited CYP1A1 and CYP1A2 mRNAs induction, which were mediated both by SP600125 and TCDD (Fig. 18).

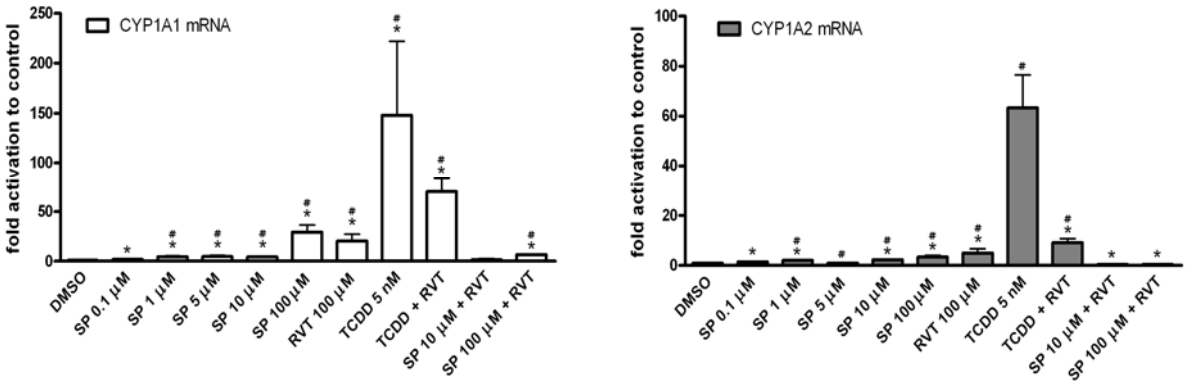


Fig. 18: Effects of SP600125 on the expression of CYP1A mRNAs in human hepatocytes. Human hepatocytes were treated with increasing concentrations of SP600125 (0.1–100 μM), TCDD (5 nM) and AhR antagonist resveratrol (RVT; 100 μM). As a control, hepatocytes were treated with vehicle (DMSO, 0.1% *v/v*). Representative RT-PCR analyses of CYP1A1 and CYP1A2 mRNAs are shown. The data are mean ± S.D. from duplicate measurements and are expressed as fold induction over DMSO-treated cells. The data were normalized to GAPDH mRNA levels. #Significantly different from DMSO-treated cells ($p < 0.05$); *significantly different from TCDD-treated cells ($p < 0.05$).

Since our findings indicated that SP600125 could be a partial agonist we tested the effects of SP600125 on TCDD-inducible expression of CYP1A mRNAs in human hepatocytes. Human hepatocytes were treated with increasing concentrations of SP600125 in the presence of TCDD and various levels of CYP1A mRNAs. SP600125 caused a dose-dependent inhibition of CYP1A mRNAs induction that were mediated by a full agonist of AhR - TCDD (Fig. 20).

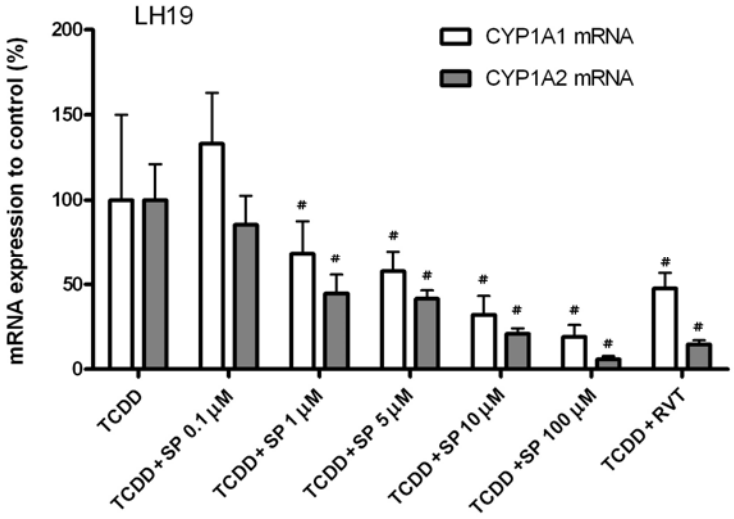


Fig. 19: Effects of SP600125 on TCDD-inducible expression of CYP1A mRNAs in human hepatocytes. Representative RT-PCR analyses of CYP1A1 and CYP1A2 mRNAs are shown. Human hepatocytes were treated with vehicle (DMSO, 0.1% *v/v*), AhR antagonist resveratrol (RVT, 100 µM), increasing concentrations of SP600125 (0.1–100 µM) in the presence of TCDD (5 nM) for 24 h. The data are mean ± S.D. from duplicate measurements and are expressed as % of induction attained by 5 nM TCDD (100% induction). Expression of tested genes was normalized to the mRNA levels of GAPDH housekeeping gene. #Significantly different from TCDD-treated cells ($p < 0.05$).

5.3. EXAMINATION OF SP600125, SB203580 AND U0126 EFFECTS ON ACTIVATION OF p1A1-LUC AND pDRE-LUC REPORTER PLASMIDS IN HEPG2 CELLS

Next we examined the effects of MAPKs inhibitors on AhR using gene reporter assays. For this purpose, HepG2 cells were transiently transfected with pDRE-luc plasmid containing two inverted repeats of the dioxin responsive element (DRE) or with p1A1-luc plasmid containing 5´-flanking region of human CYP1A1 gene. Thereafter, cells were treated with increasing concentrations of SP600125, SB203580 and U126 and SP600125 in the presence of TCDD. Both reporters are competent for monitoring AhR transcriptional activity. pDRE-luc contains only specific binding sites for AhR while p1A1-luc should be closer to physiological situation with respect to AhR-CYP1A signalling, since it contains the 5´-flanking region of *CYP1A1* gene from -1566 to +73.

SP600125 and U0126 activated pDRE-luc and p1A1-luc reporter plasmids in a dose-dependent manner (Fig. 20, 22) while SB203580 did not cause activation of both plasmids (Fig. 21). SP600125 transactivated pDRE-luc and p1A1-luc reporter plasmids with EC_{50} 0.005 μM and 1.89 μM, respectively (Fig. 19) and U0126 transactivated these plasmids with EC_{50} 10.805 μM and 0.717 μM, respectively. TCDD-dependent activation of pDRE-luc and p1A1-luc was inhibited by SP600125 with IC_{50} values of 1.54 and 2.63 μM, respectively (Fig. 20).

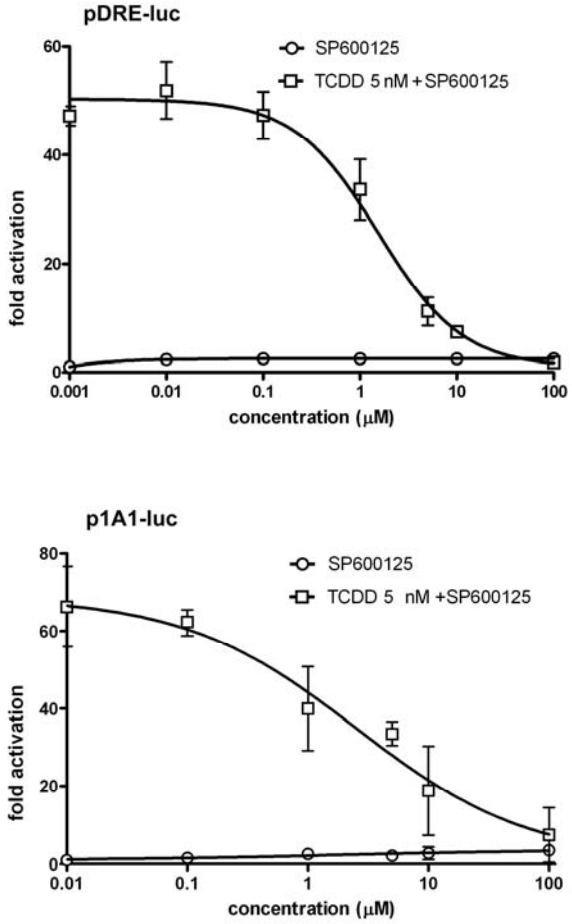


Fig. 20: Effects of SP600125 on activation of pDRE-luc and p1A1-luc reporter plasmids in HepG2 cells. HepG2 cells were transiently transfected by lipofection (FuGENE 6) with 200 ng/per well of pDRE-luc or p1A1-luc plasmid. Following 16 h of stabilization, the cells were treated with increasing concentrations of SP600125 in the presence or absence TCDD (5 nM) for 24 h. After the treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. The data are mean ± S.D. from triplicate measurements and are expressed as fold induction over DMSO-treated cells.

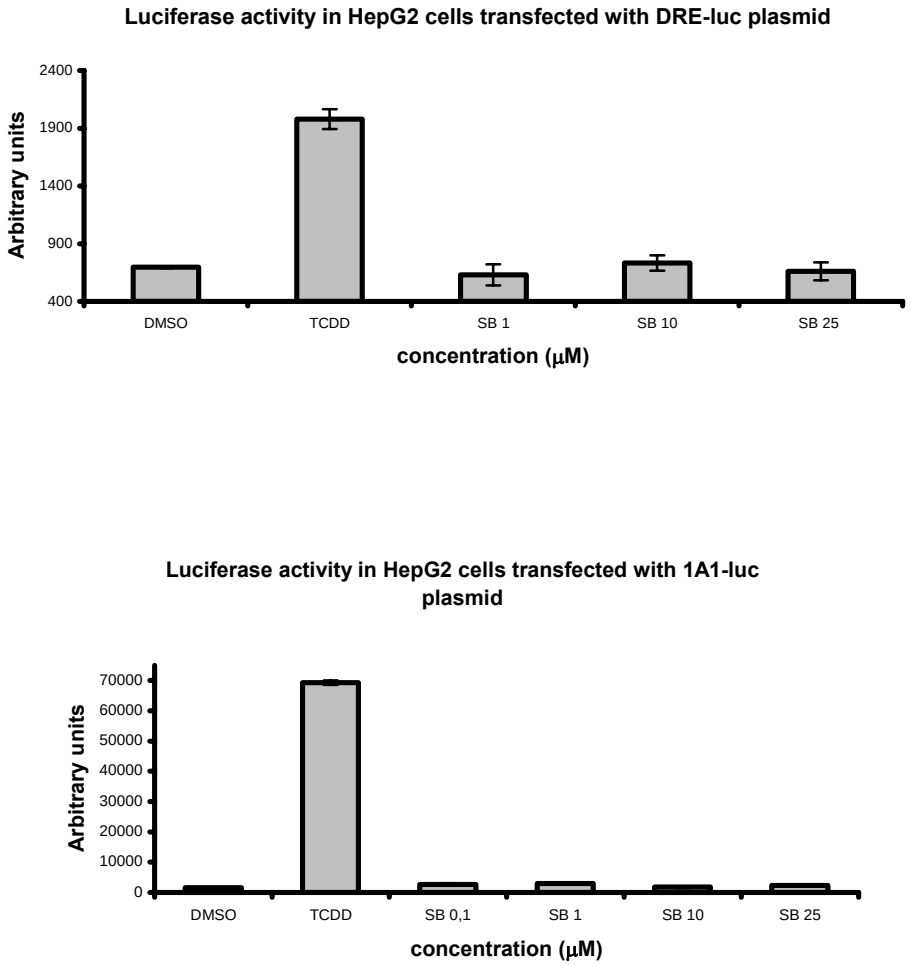
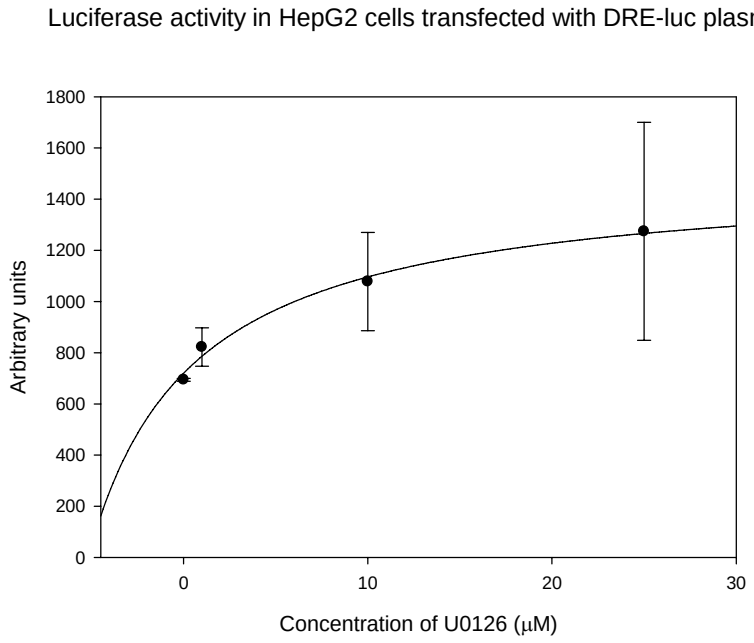


Fig. 21: Effects of SB203580 on activation of pDRE-luc and p1A1-luc reporter plasmids in HepG2 cells. HepG2 cells were transiently transfected by AMAXA cell line nucleofector solution V with 200 ng/per well of pDRE-luc or p1A1-luc plasmid using the Nucleofector II device. Following 24 h of stabilization, the cells were treated with 0.1% (*v/v*) DMSO, TCDD (5 nM) and increasing concentrations of SB203580 for 24 h. After the treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. The data are mean ± S.D. from duplicate.



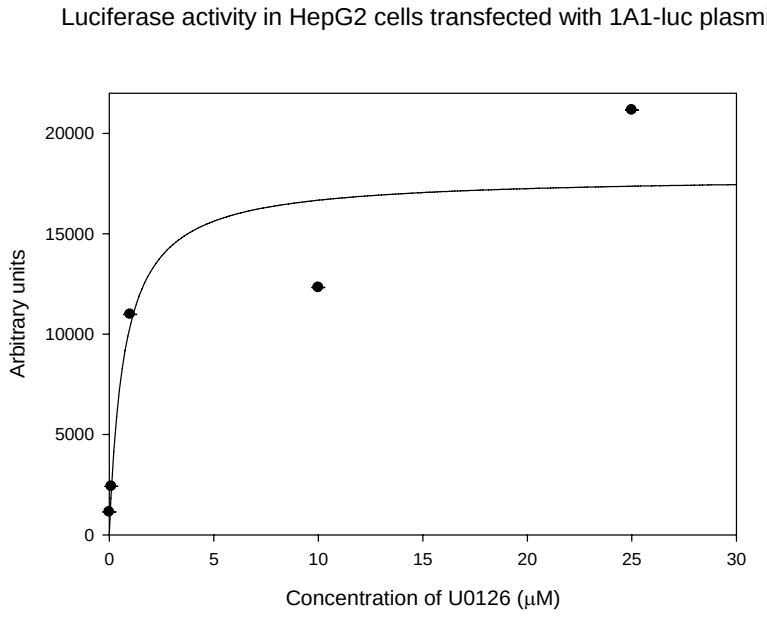


Fig. 22: Effects of U0126 on activation of pDRE-luc and p1A1-luc reporter plasmids in HepG2 cells. HepG2 cells were transiently transfected by AMAXA cell line nucleofector solution V with 200 ng/per well of pDRE-luc or p1A1-luc plasmid using the Nucleofector II device. Following 24 h of stabilization, the cells were treated with 0.1% (*v/v*) DMSO, TCDD (5 nM) and increasing concentrations of U0126 for 24 h. After the treatments, cells were lysed and luciferase activity was measured and standardized per milligram of protein. The data are mean ± S.D. from duplicate.

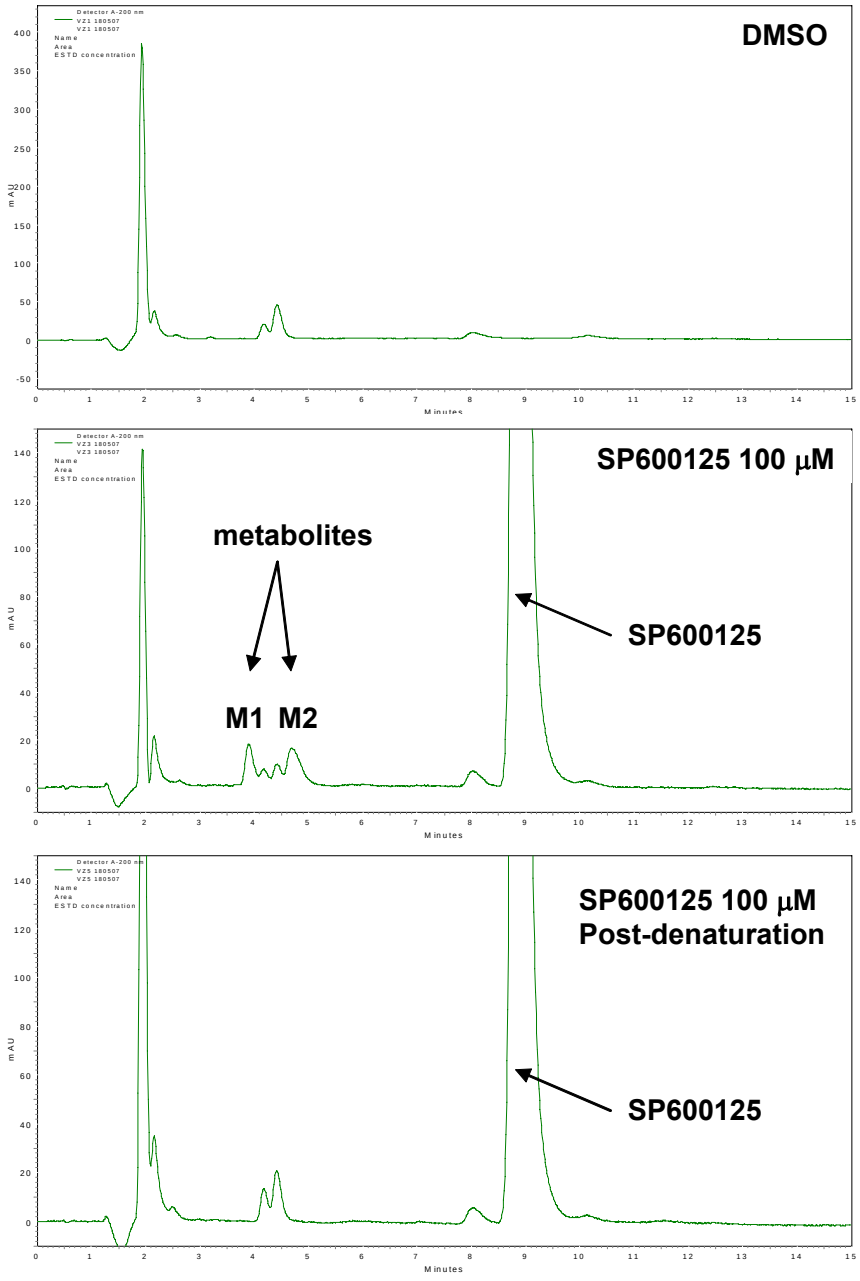
5.4. SP600125 AND SB203580 METABOLISM IN HUMAN HEPATOCYTES AND IN HEPG2 CELLS

To test whether the activation of human AhR is caused by metabolites of SP600125 a metabolite study was perfomed. SP600125 was incubated (100 μM final concentration) for 2 h with primary cultures of human hepatocytes, cultured HepG2 cells or pooled human liver microsomes. Two minor metabolites (M1 = 3.9 min; M2 = 4.7 min) of SP600125 were detected using HPLC/UV analyses in human hepatocytes and human liver microsomes, but not in HepG2 cells (Fig. 23 a)) – data from microsomes are shown only). These data imply that biotransformation is not responsible for the effects of SP600125 on AhR signalling.

M1 and M2 metabolites were isolated by HPLC fractionation (post-column collection) and the obtained fractions were analyzed by micro-liquid chromatography/mass spectrometry (μLC/MS^2). Electrospray ionization (ESI) of SP600125 provided a quasi-molecular ion $[M+H]^+$ with m/z 221 in the first order mass spectra (MS). Ion m/z 237 dominated in the related spectra of both metabolites M1 and M2 after their chromatographic separation (Fig. 23 b), spectra on the left). The mass difference $\Delta m/z$ = 16 between maternal compound SP600125 and metabolites (M1 and/or M2) suggests incorporation of oxygen atom into the molecule. Taking in account the polyaromatic structure of SP600125, the formation of two isomeric monohydroxylated metabolites is plausible. MS/MS experiment was performed to elucidate the structure of metabolites. Characteristic elimination of carbon monooxide and/or nitrogen molecule (both $\Delta m/z$ = 28) leading to ions of *m/z* 193 and *m/z* 165 (dominant fragment ion) was observed in MS/MS spectra of SP600126 (Fig. 23 b), spectra on the right). The same fragmentation pattern (i.e. elimination CO and/or N_2) was observed in MS/MS spectra of M1 and M2 (fragment ions with *m/z* 209 and *m/z* 181) suggesting that studied metabolites have the same structural base as the original compound. In addition to it, ion *m/z* 153 occurs in collision spectra which could be explained by elimination of carbon monooxide from hydroxylated aromatic ring. Finally, abundant fragment ion *m/z* 127 can be formed by the cleavage of acetylene ($\Delta m/z$ = 26) from ion *m/z* 153. Exact mass measurement of parent ions of both metabolites supports the identification of monohydroxylated

derivatives. Their calculated mass was *m/z* 237.0664. Measured value was *m/z* 237.0662 for M1 (Δ -0.8 ppm) and *m/z* 237.0670 for M2 (Δ +2.5 ppm), respectively. In sum, mass spectrometry unambiguously identified metabolites M1 and M2 as monohydroxylated derivatives of SP600125, however, the position of hydroxy group in the molecule has not been determined.

(a)



(b)

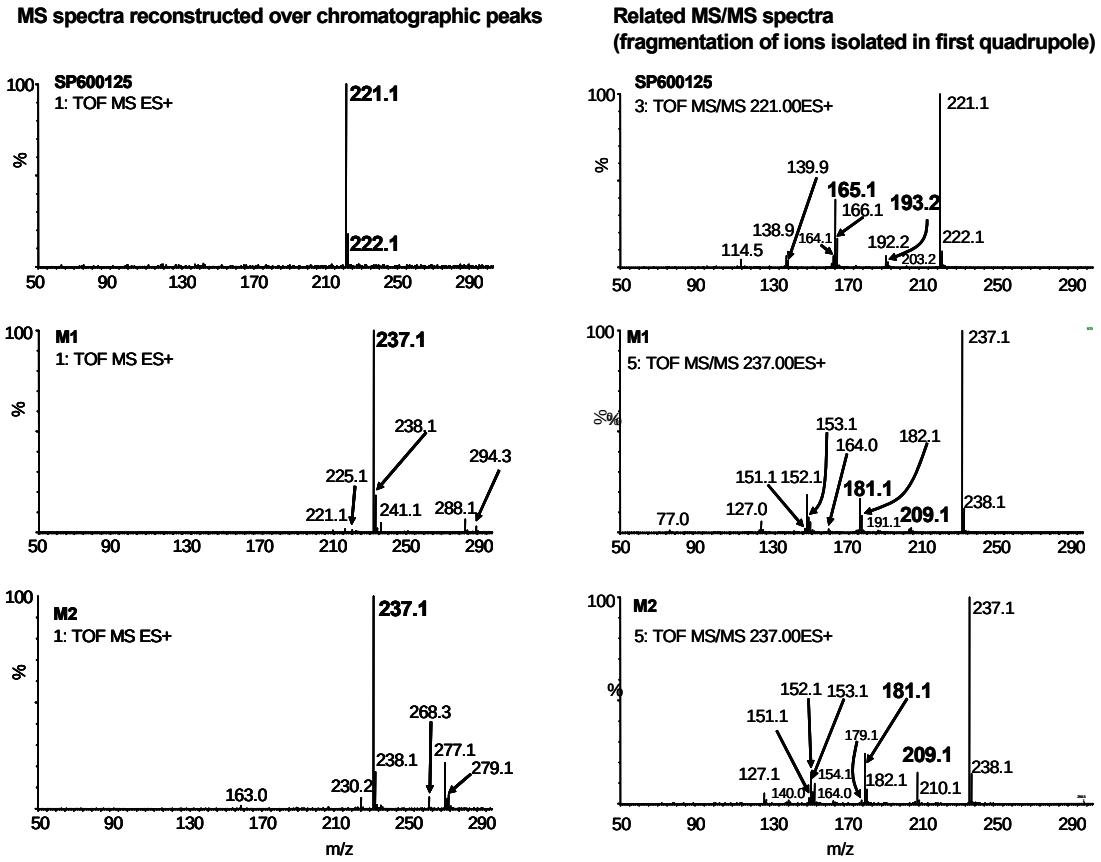
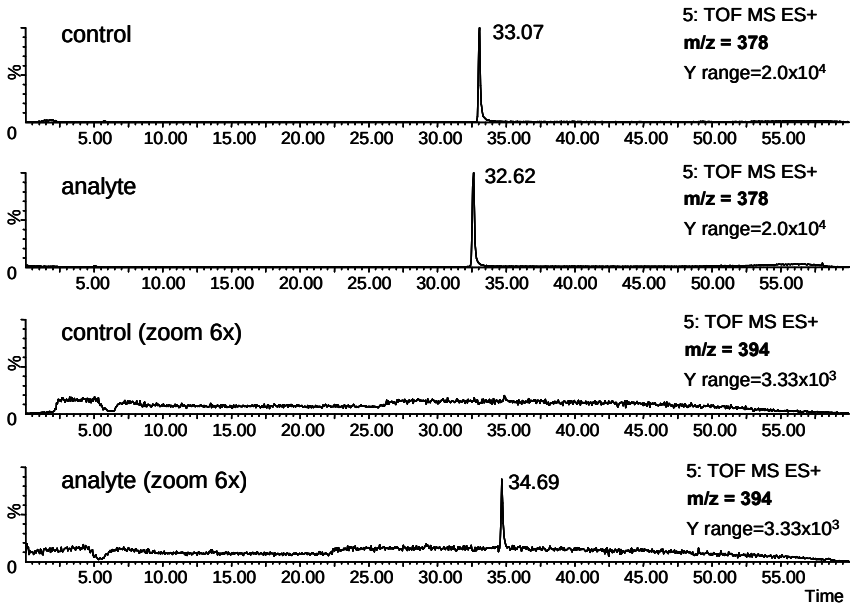


Fig. 23: Metabolism of SP600125 in human liver microsomes. SP600125 (100 μM final concentration) or vehicle (DMSO; 0.1% v/v, final concentration) were incubated for 2 h with human liver microsomes. The mixture was deproteined by $HClO_4$, as described in Methods section. As a positive control, SP600125 was added to the blank mixture/media post-denaturation. Following centrifugation (13,000 rpm/3 min), supernatants were subjected to HPLC analyses. **(Panel a)** Representative HPLC chromatograms (UV detection at 200 nm): upper panel - negative control (Blank; DMSO); middle panel—SP600125 and its metabolites M1 and M2; lower panel positive control (Blank; SP600125 post-denaturation). **(Panel b)** Metabolites M1 and M2 shown in Panel a) were isolated by fractionation (post-column collection). SP600125, M1 and M2 were subjected to μLC/MS^2 analyses. Representative ESI-MS and related MS/MS spectra of SP600125 and metabolites M1 and M2 are shown.

We also investigated metabolism of SB203580 in human hepatocytes and HepG2 cells. SB203580 was incubated (50 μM final concentration) for 30 min and 45 min with cultured HepG2 cells or pooled human liver microsomes. Using micro-liquid chromatography/mass spectrometry (μLC/MS) analyses, one minor metabolite of SB203580 in human liver microsomes but not in HepG2 cells was detected. Fig. 24 a) shows the reconstructed chromatograms of metabolized sample and positive control. The upper two traces show reconstructed chromatograms of quasimolecular ion of SB203580 ($[M+H]^+$, *m/z*=378). The peak of the compound was found in both analyses suggesting that a considerable amount of SB203580 was not metabolized. The bottom traces show the reconstructed chromatograms of a potential metabolite having one more oxygen in the molecule with respect to the original molecule ($[M+H+16]^+$, *m/z*=394). A distinct peak can be observed in the metabolized sample. In the control sample the peak is missing. Hydroxylation, *N*- and *S*-oxidation could be considered regarding this metabolite. Fig. 24 b) shows the fragmentation pattern of SB203580 and its metabolite. Cleavage of methyl radical (363=378−15) and CH_3SO group (315=378−63) was observed in the collision spectra of SB203580 (after chromatographic separation and isolation of parent ion in first quadrupole). In the corresponding collision spectra of metabolite the related cleavage of methyl radical (379=394−15) was not observed at all and the main fragment ion had m/z=315 (315=394−79). The loss of 79 corresponds to the cleavage of CH_3SO_2 group. This behavior clearly indicates that the metabolite is a product of *S*-oxidation (methanesulfinyl group is oxidized to methanesulfonyl one). The identity of SB203580 and the metabolite was further supported by exact mass measurement (*m/z*=378.1083; mass error 1.9 ppm and *m/z*=394.1029; mass error 0.8 ppm, respectively). The peak area of metabolite M progressively increased with the time of incubation. In addition, this peak did not appear in the incubation buffer containing SB203580 or in microsomes with SB203580 added post-denaturation (data not shown). Taken together, the formation of metabolite M is due to enzymatic transformation of SB203580 and not to chemical conversion.

(a)



(b)

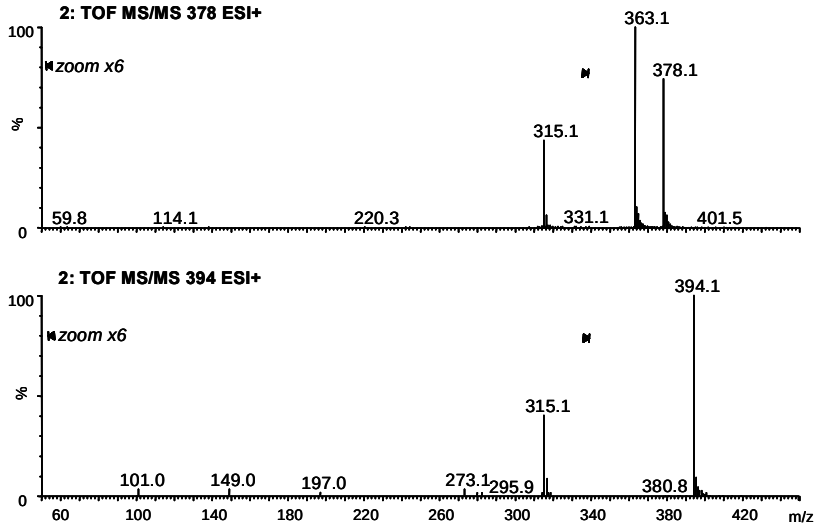


Fig. 24: Metabolism of SB203580 in human liver microsomes. SB203580 (50 µM final concentration) or vehicle (DMSO; 0.1% *v/v* final concentration) were incubated for 30 min and 45 min with human liver microsomes. The mixture was deproteined by methanol. As a positive control, SB203580 was added to the blank mixture/media post-denaturation. Following centrifugation (13000 g/3 min), supernatants were subjected to µLC/MS analyses. **Panel a**: µLC/MS^2 analysis of metabolism of SB203580. Reconstructed chromatograms for original compound (*m/z*=378) and metabolite M (*m/z*=394). **Panel b**: MS/MS spectra of SB203580 and its metabolite M. Spectra averaged over chromatographic peaks, parent ions were isolated in the first quadrupole and subsequently fragmented in the collision cell of mass spectrometer.

6. DISCUSSION

Discovery of chemical MAPKs inhibitors such as SP600125 (inhibitor for JNK) [80], SB203580 (inhibitor for p38) [79] and U0126 (inhibitor for ERK) [105] was an important moment in the MAPKs research since these kinases are involved in inflammation, immune and oncological diseases, renal failure or pathophysiological processes in the heart (heart failure, ischemia etc.) and some of them are appropriate therapeutic targets [165-167]. For instance, p38 inhibitors could be use in inhibition of production of pro-inflammatory cytokines, MEK 1/2 and Raf-1 inhibitors could be used for treatment of cancer and inhibitors for all three studied types of MAPKs (JNK, ERK and p38) could be use in treatment of renal diseases. Moreover, MEK inhibitors are currently undergoing clinical trials [168]. Their disadvantage is interaction with other cellular targets. For example, interaction between U0126 and aryl hydrocarbon receptor [14], SP600125 and aryl hydrocarbon receptor [13, 57], SB203580 and c-Jun *N*-terminal kinase [15, 169] was described. Thus we analyzed these three chemical inhibitors to confirm and find out interactions with other cellular targets (AhR and MAPKs). The goal of this thesis was to point out interactions among AhR and MAPKs inhibitors and to point out differences among cancer cell lines and primary cells and relevance of studies on primary cells.

The interference of JNK specific inhibitor SP600125, p38 specific inhibitor SB203580 and U0126, an inhibitor of the up-stream MEK kinase, with the AhR signalling pathway were studied. Our studies were carried out particularly on primary cultures of human hepatocytes that are quiescent non-transformed cells and on HepG2 cells that is a cancer cell line derived from human hepatocytes.

One part of experiments was focused on selected MAPKs activators. MAPKs activation by model activators (epidermal growth factor, sorbitol and anisomycin) in human hepatocytes was only transient and vanished within 24 h (Fig. 7). In addition, we observed that one activator activated more than one MAPK both in HepG2 cells and human hepatocytes (Fig. 6, 7) [15]. These findings imply that these activators do not act specifically. Moreover, TCDD was demonstrated to activate ERK in Hepa-1 [115], murine macrophages RAW [118], human macrophages [119] and in human promonocytic leukemia cell line THP-1 [120] or JNK in CV-1 cells and mouse embyonic fibroblasts

[115] but we did not observed activation of neither ERK, JNK nor p38 in HepG2 cells (Fig. 6). Thus, these results indicate that activation of MAPKs by TCDD could be cell type-dependent.

We tested effects of SB203580 on MAPKs activation since we observed that this inhibitor caused activation of ERK (Fig. 8, 9, 10, 11). The activation of ERK occurred both in HepG2 cells and human hepatocytes. The most potent ERK activation was mediated by 10 μM SB203580. Higher concentration of SB203580 was not so effective. It was caused due to its toxicity (results from toxicity measurements are published in [15]). We also observed activation of JNK by SB203580 in primary human hepatocytes (Fig. 9, 10, 11). However, we did not observe JNK activation by SB203580 in human hepatoma cells (HepG2), human promyelocytic leukemia cells (HL-60), human primary osteogenic sarcoma (SaOS-2) and immortalized non-tumorigenic human keratinocytes cells (HaCat) (Fig. 8, 12) [15]. On the other hand, Muniyappa et al. (2008) showed JNK activation by SB203580 in human cell line using lung alveolar epithelial cells A549 [169]. All these finding are in accordance with Dhillon et al. (2007) who referred the different role of MAPKs among different cell lines [170]. These observations point out the importance to study cellular processes in normal primary cells. Whereas a major part of studies deal with cell lines that are proliferating and transformed, we used not only cell lines but also primary cultures of human hepatocytes, normal, non-transformed quiescent cells, as the unique model for our studies of MAPKs and AhR [13, 15]. We observed as well ERK activation by SB203580 in primary cultures of human hepatocytes, HepG2, HL-60, SaOS-2 and HaCaT human cell lines (Fig. 8, 9, 10, 11, 12) [15]. Since SB203580 is an inhibitor of p38 MAP kinase and one-way cross-talk between p38 and ERK was described in various cells such as mouse fibroblasts NIH3T3 [147], HepG2 cells [148], rat cardiac myoblasts H9c2 [149], T-lymphocytes [150], and rat cortical neuron cultures [151], our findings that ERK is activated by SB203580 support all these cross-talk observations. Since SB203580 activated JNK in human hepatocytes (Fig. 9) but not in HepG2 cells (Fig. 8), we considered a hypothesis that the different effects of SB203580 on JNK activation in HepG2 cell line and primary human hepatocytes could be due to its metabolic transformation. Metabolic analysis of SB203580 showed formation of a minor metabolite of SB203580 in human liver microsomes but no metabolite was found in HepG2 cells (Fig. 24). These findings imply that different effects of SB203580 in human hepatocytes and HepG2 cells could be at least partly mediated by its metabolic

transformation in human hepatocytes. It points out on differences among primary cell cultures and cell lines and different effects of one compound (SB203580 MAPK inhibitor) in a different type of cells.

Thereafter, the inhibitors of MAPKs were tested for our hypothesis to interact with AhR. We observed a slight induction of CYP1A proteins mediated by SB203580 in human hepatocytes (Fig. 14) and induction of CYP1A proteins in HepG2 cells (Fig. 15). The effects of SB203580 on the expression of CYP1A1 mRNA in HepG2 cells was as follows: 10 μM SB203580 was the most potent inducer of CYP1A1 mRNA in HepG2 cells whereas 25 μM SB203580 caused smaller induction of CYP1A1 mRNA in HepG2 cells (Fig. 17). These effects of SB203580 could be due to its toxicity (data are found in [15]). However, SB203580 did not induce basal reporter activity of p1A1-luc and pDRE-luc plasmids transfected in HepG2 cells (Fig. 21). Similar results were obtained by Shibazaki et al. [144] in Hepa-1 cells transfected with CYP1A1 plasmid. In one paper published by Shibazaki et al. [145] it was shown that TCDD-induced histone acetylation level was decreased by SB203580. These results indicate that SB203580 also interacts with the AhR signalling pathway similarly as SP600125. Taken together, it indicates that SB203580 interacts with AhR signalling pathway.

It was shown that U0126 activated AhR and induced both CYP1A1 mRNA and protein in HepG2 cells and primary rat hepatocytes [14]. We confimed results obtained by Andieux et al. [14] in HepG2 cells since we observed that CYP1A protein was slightly induced (Fig. 15). In accordance with these findings we observed that U0126 induced expression of CYP1A protein in human hepatocytes (Fig. 14). U0126 induced CYP1A1 mRNA in a dose-dependent manner in HepG2 cells (Fig. 17). Luciferase activity in HepG2 cells transfected with pDRE-luc and p1A1-luc plasmids was also increased in a dose-dependent manner after incubation with U0126 (Fig. 22). Andieux et al. [14] showed also increased CYP1A activity. All these findings imply that U0126 interacts with AhR in both cell lines and primary cells and that its use in the AhR studies is doubtful [171].

SP600125 was referred to as a ligand of AhR [57]. Joiakim et al. [57] showed that SP600125 inhibited induction of CYP1A1 mRNA mediated by TCDD in a dose-dependent manner in MCF10A human breast epithelial cell line. We obtained similar results with SP600125 as well as with U0126 on mRNA level both in human hepatocytes (Fig. 18, 19) and in HepG2 cells (Fig. 16). Joiakim et al. [57] showed that SP600125 is an

AhR ligand using ligand binding assay. However, a ligand could be more defined as: full agonist, partial agonist or antagonist. Results obtained by Joiakim et al. [57] and our results indicated that SP600125 could be a partial agonist. Evidence supporting this statement is as follows: SP600125 induced CYP1A mRNAs in human hepatocytes (Fig. 18) and CYP1A1 mRNA in HepG2 cells (Fig. 16) but the magnitude of the inductions did not correspond to the full activation of AhR mediated by TCDD. SP600125 inhibited CYP1A mRNAs TCDD-mediated induction in human hepatocytes at the same time (Fig. 19). Similar effects of SP600125 were observed in HepG2 cells (Fig. 16). All these effects of SP600125 probably occurred via AhR since resveratrol (AhR antagonist) abolished SP600125-mediated induction of CYP1A genes in human hepatocytes and HepG2 cells by SP600125 and TCDD (Fig. 16, 18). We observed a very slight induction of expression of CYP1A on protein level mediated by 10 μM SP600125 in human hepatocytes (Fig. 13) but not in HepG2 cells (Fig. 15). Protein analyses also showed SP600125 inhibition of CYP1A TCDD-mediated induction in human hepatocytes (Fig. 13) which is in accordance with observation on mRNA level (Fig. 18, 19). Another proof that SP600125 is a partial agonist of AhR was observed in experiments with pDRE-luc and p1A1-luc plasmids. SP600125 caused sub-maximal induction of basal reporter activity of p1A1-luc and pDRE-luc plasmids transfected in HepG2 cells but TCDD-induced reporter activity was inhibited by SP600125 (Fig. 20). Finally, we tested whether all SP600125 effects were due to its metabolic transformation. Analysis of SP600125 metabolism showed indeed that effects of SP600125 on the AhR pathway were not due to its metabolic transformation since similar results were observed in human hepatocytes and HepG2 cells and no metabolite of SP600125 was found in HepG2 cells (Fig. 23). In contrast, we observed the formation of two minor, monohydroxylated metabolites of SP600125 in human hepatocytes.

Taken together, it is important to know and consider interactions among various signalling pathways since every cellular tagret can be affected by other cellular target. It is also important to carry out studies on both models such as cancer cell lines and primary cells.

7. CONCLUSIONS

Results in this thesis confirm that selected chemical inhibitors of mitogen-activated protein kinases (that are reffered in literature as specific inhibitors) interact with other cellular targets (aryl hydrocarbon receptor and mitogen-activated protein kinases). These thesis also point out relevance of studies on primary cells.

- epidermal growth factor, sorbitol and anisomycin, the „classical“ model activators of extracellular regulated kinase, c-Jun *N*-terminal kinase and p38 mitogen-activated protein kinase, respectively did not act specifically, since one activator caused activation of more than one mitogen-activated protein kinase both in HepG2 cells and human hepatocytes
- dioxin (TCDD, aryl hydrocarbon receptor agonist) did not activated studied mitogen-activated protein kinases in HepG2 cells
- 1,9–pyrazoloanthrone (SP600125) is a partial agonist of aryl hydrocarbon receptor and cellular effects of this inhibitor were not mediated due to its metabolic transformation

→ SP600125 interaction with aryl hydrocarbon receptor should be taken into account and its use in aryl hydrocarbon receptor studies is doubtful

- we confirm that 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene ethanolate (U0126) interacts with the aryl hydrocarbon receptor signalling pathway both in human hepatocytes and HepG2 cells

→ use of U0126 in the aryl hydrocarbon receptor studies is of uncertain outcome as well

- 4-(4-fluorophenyl)-2-(4- methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580) interacted with the aryl hydrocarbon receptor signalling pathway
- SB203580 activated extracellular regulated kinase and c-Jun *N*-terminal kinase in human hepatocytes but in HepG2 cells we observed only activation of extracellular regulated kinase

→ activation of extracellular regulated kinase and c-Jun *N*-terminal kinase by SB203580 inhibitor should be considered in future mitogen-activated protein kinases studies, use of SB203580 has its limits in the aryl hydrocarbon receptor studies as well

8. PUBLICATION AND CONFERENCE REPORTS

Publications:

1. Dvorak Z, Vrzal R, Henklova P, Jancova P, Anzenbacherova E, Maurel P, Svecova L, Pavek P, Ehrmann J, Havlik R, Bednar P, Lemr K, Ulrichova J: **JNK is a partial agonist of human aryl hydrocarbon receptor and induces CYP1A1 and CYP1A2 genes in primary human hepatocytes**. *Biochem Pharmacol 2008*, **75**: 580-588.
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6. Henklova P, Dvorak Z, Vrzal R, Jancova P, Anzenbacherova E, Maurel P, Svecova L, Pavek P, Ehrmann J, Havlik R, Bednar P, Lemr K, Ulrichova J: Human aryl hydrocarbon receptor is activated by c-Jun-N-Terminal kinase inhibitor SP600125. *Konference vědeckých prací studentů DSP 2008*. 3. - 4. 9. 2008, Olomouc, Česká republika.

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