UNIVERSITY OF ALBERTA

IDENTIFYING ARYL HYDROCARBON RECEPTOR MODULATORS FROM A NATURAL SOURCE

By

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This work is dedicated to my family

ABSTRACT

Dioxins are widespread environmental contaminants that have been linked to a variety of deleterious effects on human health including increased cancer rates via aryl hydrocarbon receptor (AhR)-dependent mechanism. AhR is a transcription factor that regulates the expression of the carcinogen-activating enzyme, cytochrome P450 1A1 (CYP1A1). Activation of AhR and its regulated gene, CYP1A1, have been correlated with the incidence of several cancers. Therefore, the use of AhR antagonists has been proposed as a promising chemopreventative approach. Nonetheless, most of the currently used AhR antagonists are not specific to AhR and some of them act as partial agonists. Therefore, the search for new AhR antagonists is still in progress. The specific objectives of the present work were to identify new AhR modulators from a natural source. In this regard, first we demonstrated that *Peganum harmala*, a common traditional plant in Middle East, and North Africa, significantly inhibited the dioxin-mediated induction of CYP1A1 at mRNA, protein and activity levels using human and mouse hepatoma cells. The role of AhR was confirmed using AhR-dependent luciferase assay and electrophoretic mobility shift assay. Additionally, we identified two β-carboline alkaloids (harmine and harmaline) as the active constituents of the plant extract. Second, we demonstrated that harman, a common β-carboline in several foods and drinks and the parent structure of harmine, significantly induced CYP1A1 mainly through an AhR-dependent mechanism. Third, the active constituents of *Peganum harmala* extract, harmine and harmaline, and their metabolites, harmol and harmalol, significantly

decreased the dioxin-mediated induction of CYP1A1 at mRNA, protein and activity levels via transcriptional (through AhR) and post-translational (through ubiquitin-proteasomal pathway as well as a direct inhibitory effect on CYP1A1 enzyme). Additionally, we demonstrated that harmine, harmol, and harmalol can act as direct antagonists for AhR, whereas harmalol affected AhR activation without a direct interfering with AhR binding to its ligands. Finally, we confirmed the effect of harmine and harmaline on dioxin-mediated induction of Cyp1a1 *in vivo* using the responsive C57BL/6 mouse strain. In conclusion, our data clearly demonstrate the promising effects of *Peganum harmala*, harmine, harmol, harmaline, and harmalol to prevent the toxicity and carcinogenicity of several AhR ligands.

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LIST OF ABBREVIATIONS

3MC	3-Methylcholanthrene
7ER	7-Ethoxyresorufin
Act-D	Actinomycin D
AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
ALDH3A1	Aldehyde dehydrogenase 3A1
ANOVA	Analysis of variance
ARE	Antioxidant responsive element
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATCC	American type culture collection
ATP	Adenosine triphosphate
B[a]P	Benzo[a]pyrene
B[a]PDE	Benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide
bHLH	Basic-helix-loop-helix
*β*NF	*β*-naphthoflavone
Brg-1	Brahma-related gene 1
CAR	Constitutive androstane receptor
CARM1	Cofactor-associated arginine [R] methyltransferase 1
CBP	CREB-binding protein
CH223191	2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide
CHX	Cycloheximide

COX2	Cyclooxygenase-2
CREB	cAMP-response element-binding protein
CYP	Cytochrome P450
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
EMSA	Electrophoretic mobility shift assay
EROD	7-Ethoxyresorufin O-deethylase
FICZ	6-Formylindolo[3,2-b]carbazole
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GSTA1/2	Glutathione transferase A1/2
HAH	Halogenated aromatic hydrocarbon
HAP	Hydroxyapatite
HC	Herbal components
HDAC	Histone deacetylase
HSP90	Heat shock protein 90
i.p.	Intraperitoneal
IA	Intrinsic activity
IARC	International agency of research on cancer
JNK	c-JUN N-terminal kinase
LC-ESI-MS	Liquid chromatographic-electrospray ionization-mass spectrometry

LD_{50}	Median lethal dose
MG-132	Carbobenzoxy-l-leucyl-l-leucyl-leucinal
miR	microRNA
MMP	Matrix metalloproteinase
MNF	3′-Methoxy-4′-nitroflavone
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NES	Nuclear export sequences
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization sequences
NQO1	NAD(P)H:quinine oxidoreductase 1
Nrf2	Nuclear factor erythroid 2-related factor 2
PAH	Polycyclic aromatic hydrocarbon
PAS	Per-ARNT-Sim
PBS	Phosphate-buffered-saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PXR	Pregnane X receptor
Res	Resveratrol
RLU	Relative light unit
RT	Reverse transcription
SAhRM	Selective AhR modulator
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptor

SR1	StemRegenin 1
SRC1	Steroid receptor coactivator 1
$t_{1/2}$	Half-life
TCDD	2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-Tetrachlorodibenzofuran
TMF	6,2′,4′-Trimethoxyflavone
UGT1A6	Uridine diphosphate glucuronosyltransferase 1A6
XAP2	Hepatitis B virus X-associated protein 2
XMEs	Xenobiotic metabolizing enzymes
XRE	Xenobiotic responsive element

CHAPTER 1- INTRODUCTION

1.1. Aryl Hydrocarbon Receptor

1.1.1. Background and Historical Perspective

Fifty years ago, the early studies on the aryl hydrocarbon receptor (AhR) started when polycyclic aromatic hydrocarbons (PAHs) such as 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD) and 3-methylcholanthrene (3MC), induced drug-metabolizing enzymes in rat livers (Conney and Burns 1959, Conney 1982, Kato 1961, Remmer 1959). In 1962, Omura and Sato identified cytochrome P450 (CYP) as a microsomal component that gave a unique absorption spectrum with a peak at 450 nm when incubated with CO and NADPH (Omura and Sato 1962). Later, Cooper *et al.* discovered that the identified CYP is drug-metabolizing enzyme (Cooper et al 1965).

In 1976, Poland and his coworkers identified a novel cytoplasmic protein that bound to TCDD with high affinity and called this protein AhR (Poland et al 1976). Furthermore, they correlated AhR activation by TCDD to the induction of the drug-metabolizing CYP. In the 1980s, using a molecular cloning technology, the cDNA clone of 3MC-inducible P450s was isolated (Sogawa et al 1984). Additionally, the regulatory sequences responsible for 3MC-mediated induction of CYP1A1 were identified using a reporter gene technique, and this sequence was designated as xenobiotic responsive element (XRE) (Fujisawa-Sehara et al 1987).

In 1995, AhR deficient mice were first generated by a homologous recombination technique (Fernandez-Salguero et al 1995). The use of AhR-deficient mice has clearly demonstrated that AhR mediates several

pharmacological and toxicological effects of TCDD such as teratogenesis, immune suppression, tumor promotion, liver damage as well as the induction of several drug-metabolizing enzymes (Gonzalez and Fernandez-Salguero 1998, Mimura et al 1997).

1.1.2. Characterization of the AhR

The human *AhR* gene is localized on chromosome 7. It is about 50 kb long and encodes a 96 KDa protein (Bennett et al 1996, Micka et al 1997). AhR is a ligand-dependent transcription factor that belongs to the basic-helix-loop-helix (bHLH)/Per-ARNT-Sim (PAS) family of transcription factors (Beischlag et al 2008, Whitlock 1999). The bHLH/PAS family of proteins are essential regulatory proteins involved in maintenance of homeostasis, circadian rhythm and normal development (Whitlock 1999). The PAS is a region of amino acid sequences and designated PAS because their homologies were originally noted between *Drosophila* circadian rhythm protein period (Per), the mammalian AhR nuclear translocator (ARNT), and *Drosophila* neurogenic protein single-minded (Sim) (Whitlock 1999).

The process of AhR activation has been studied extensively and it revealed the involvement of AhR heterodimerization with another member of the bHLH/PAS family called AhR nuclear translocator (ARNT) (Denison et al 2002, Mimura and Fujii-Kuriyama 2003, Whitlock 1999). The structures of AhR and ARNT have similar features. Both of them contain bHLH domain near the N-terminal followed by two 51-amino acid PAS A-B repeats and C-terminal

glutamine-rich region (Q-rich region) (Hoffman et al 1991, Nambu et al 1991) (Fig. 1.1.). The bHLH region of the AhR functions in dimerization with ARNT, DNA binding, heat shock protein 90 (HSP 90) interaction and possesses sequences responsible for AhR nuclear localization (NLS) and nuclear export (NES) (Denison et al 2002). Next to the bHLH region, the PAS domain is localized and consists of two structural repeats (PAS A-B). PAS A functions in AhR dimerization with ARNT, whereas PAS B functions in AhR ligand and HSP90 binding. The C-terminal region of AhR protein consists of a glutamine-rich region (Q-rich region) that is responsible for the transactivation of AhR (Fig. 1.1.) (Denison et al 2002, Kumar et al 2001, Mimura and Fujii-Kuriyama 2003). Although AhR and ARNT have a similar structure, the PAS regions of AhR and ARNT possess different functions. For example, ARNT cannot bind to ligand or HSP90 as AhR. Furthermore, the transactivation of ARNT is simpler than AhR, as ARNT transactivation is constitutive and expressed by the intact protein (Hankinson 2005, Whitlock 1999).

Recently, the ability of AhR protein to bind to different AhR ligands, such as TCDD, 3MC, and β-naphthoflavone, has been studied using several docking models. It has been demonstrated that three amino acids play a key role in ligand-binding ability of AhR, namely, Phenylalanine 318 (Phe318), Isoleucine 319 (Ile319), and Histidine 320 (His320). Moreover, mutations of these amino acids can affect the activity of AhR protein and its binding ability to different AhR ligands (Goryo et al 2007).

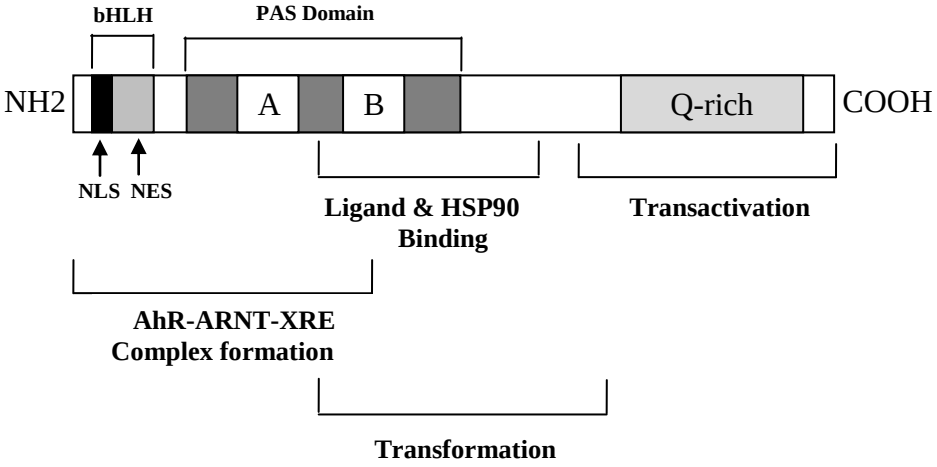


Fig. 1.1. Structural and functional domains of AhR. The N-terminal region of the AhR consists of the bHLH region that functions in dimerization with ARNT, DNA binding, HSP 90 interaction and possesses sequences responsible for AhR nuclear localization (NLS) and nuclear export (NES). Next to the bHLH region, the PAS domain is localized and consists of two structural repeats (PAS A-B). PAS A functions in AhR dimerization with ARNT, whereas PAS B functions in AhR ligand and HSP90 binding. The C-terminal region of AhR protein consists of a glutamine-rich region (Q-rich region) that responsible for the transactivation of AhR (Denison et al 2002).

1.1.3. Species, Strain, Tissue and Cellular Expression of AhR

AhR is an ubiquitous protein that has been identified for most, if not all, investigated mammalian and a number of nonmammalian vertebrate species (Bradshaw et al 2002). The effects of AhR agonists vary between different species and strains of the same species. For example, the median lethal dose (LD_{50}) of TCDD varies over 5000-fold among different species of animals as it ranges from 1 µg/kg for guinea pig, the most sensitive animal, to more than 5000 µg/kg for hamster, the most resistant animal (Poland and Knutson 1982). The main reason behind the different responses of AhR ligands between species is the species-specific biochemical and physiological characters including the variation of binding affinities of AhR between species (Denison et al 2011). In this context, several studies demonstrated that human AhR contains a point mutation in its ligand-binding domain that decreases its binding ability to TCDD by around 10-fold compared to the responsive C57BL/6 mouse AhR (Harper et al 1988, Ramadoss and Perdew 2004). Moreover, recent microarray gene expression analysis has revealed dramatic species-specific AhR-related differences between TCDD-treated hepatocytes obtained from the wild type C57BL/6 mice and the transgenic C57BL/6 mice in which the AhR has been selectively replaced by humanized AhR (Flaveny et al 2010).

An example of the different response of AhR agonists between strains of the same species is exemplified by 2 different mouse strains. C57BL/6 mice were found to be the high-responsive strain of mice to TCDD when compared to DBA/2 mice that are considered as a low-responsive strain. The difference in

response between the two strains to TCDD was attributed to a polymorphism of the AhR protein of DBA/2 mice that affected its binding ability to TCDD (Ema et al 1994).

Notably, the presence of AhR protein is different among tissues and cell types during the developmental stages with a remarkably higher level in placenta. However, in adults, AhR has been detected in several human tissues and cell types in culture including lung, liver, kidney, tonsils and B lymphocytes, whereas lower levels are expressed in hearts (Bradshaw et al 2002, Landers and Bunce 1991, Mehrabi et al 2002).

1.2. AhR-Regulated Genes

The AhR battery of genes includes four phase I and four phase II xenobiotic metabolizing enzymes (XMEs). The AhR-regulated genes that belong to phase I XMEs include *CYP1A1*, *CYP1A2*, *CYP1B1*, and *CYP2S1*, whereas the AhR-regulated genes of phase II XMEs include NAD(P)H:quinine oxidoreductase 1 (*NQO1*), glutathione transferase A1/2 (*GSTA1/2*), uridine diphosphate glucuronosyltransferase 1A6 (*UGT1A6*), and aldehyde dehydrogenase 3A1 (*ALDH3A1*) (Kohle and Bock 2006, Saarikoski et al 2005). Phase I XMEs are group of metabolic enzymes that generate highly reactive electrophiles. The detoxification of these electrophiles is usually carried out by phase II XMEs that conjugate the formed electrophile such as UGT1A6 or convert them to nucleophiles such as NQO1 enzyme (Kohle and Bock 2006).

1.2.1. The Phase I AhR-Regulated Genes

Phase I XMEs consist mainly of the CYP superfamily embedded in the membrane of the endoplasmic reticulum. CYP is composed of polypeptide membrane-bound haemoproteins that possess a key role in the oxidative metabolism of several exogenous and endogenous substances (Nebert and Russell 2002). CYP is approximately 45-55 kDa and contains haemoprotein that functions as an oxygen and substrate binding site. CYP enzymes are found in almost all human tissues; however, the highest abundance is found in liver > adrenal > small intestine >brain > kidney > lung and testis (Pelkonen et al 2008).

The CYP superfamily consists of approximately 7,000 identified CYPs in all living organisms including animals, plants, bacteria and fungi. Humans possess 57 CYP enzymes and their corresponding functions are almost identified. Out of these enzymes only 15 individual CYP enzymes in families 1, 2, and 3 metabolize different drugs and xenobiotics under the regulation of AhR, constitutive androstane receptor (CAR) or pregnane X receptor (PXR), respectively (Guengerich et al 2005, Lewis 2003). The other CYPs are responsible for the metabolism of endogenous substrates such as sterols and vitamins. One important aspects of CYP is its differential expression in several organs and tissues. It has been demonstrated that the differential expression of individual CYPs in certain tissues and cells of an organ can affect the final physiological function of that organ (Seliskar and Rozman 2007).

According to the amino acid sequences, different CYP enzymes are grouped in several families and subfamilies. CYP members of the same family possess more than 40% amino acid sequence similarity (Nelson 2006, Sim and Ingelman-Sundberg 2006). Inside the same family, members of CYP are further classified into subfamilies and the members belonging to the same subfamily have more than 55% amino acid sequence similarity. The unifying nomenclature of CYP designates a family by an Arabic number followed by a capital letter for subfamily and finally an Arabic number representing the individual CYP enzymes. It is important to note that the italicized font is usually used to refer to the encoding gene of certain CYP isoforms (for example, *CYP1A1*), while lower

case letters are used to refer to mouse enzymes (for example, Cyp1a1) (Nelson 2006, Sim and Ingelman-Sundberg 2006).

1.2.1.1. CYP1A1

Among all AhR-regulated genes, CYP1A1 is considered the best-characterized and a paradigm of AhR-induced enzymes. CYP1A1 induction was discovered from the observation that PAHs induce their own metabolism (Whitlock 1999). CYP1A1 is an extra-hepatic enzyme; however, it is highly inducible in liver and extra-hepatic tissues (Ma and Lu 2007). Notably, *CYP1A1* encoding gene has a cluster of XRE in the enhancer region of human and rodent CYP1A1 that explains its induction by several AhR agonists (Ueda et al 2006).

Humans are exposed to several environmental pollutants such as PAHs from a wide range of sources including tobacco smoke, automobile exhausts and smoked and cooked foods. Most of these compounds are inert and require metabolic activation to exert their carcinogenic effects. In this context it was previously demonstrated that CYP1A1 not only is induced by PAHs but also participates, as one of the major contributors, in metabolic activation of PAHs to their ultimate carcinogenic forms (Guengerich and Shimada 1991, Shimada and Fujii-Kuriyama 2004). Therefore, the induction level of CYP1A1 can be considered as a biomarker for exposure to several environmental pollutants such as PAHs (Williams et al 2000).

In agreement with this postulation, the role of CYP1A1 in metabolic activation has been previously confirmed. For example, the mutagenicity of 3MC

was found to be dependent on CYP1A1 enzyme in the V79 Chinese hamster cell line (Ellard et al 1991). Moreover, several studies demonstrated the correlation between the induction of CYP1A1 and the development of different human cancers such as lung, colon and rectal cancers (Oyama et al 2007, Shah et al 2009, Slattery et al 2004).

It is worth mentioning that the human *CYP1A1* gene is located on chromosome 15 and it has several mutations corresponding to 18 allelic variants (http://www.cypalleles.ki.se/cyp1a1.htm) (Androutsopoulos et al 2009, Murray et al 2001). The two most common polymorphisms of the *CYP1A1* gene are a point mutation that involves a thymine to cytosine substitution at the MspI site in the 3'-untranslated region (MspI polymorphism) and the second is a single nucleotide polymorphism of *CYP1A1* gene with adenine to guanine substitution in exon 7 at codon 462 that results in a substitution of isoleucine with valine (Ile462Val; exon 7 polymorphism) (Androutsopoulos et al 2009, Zhan et al 2011). Both polymorphisms are accompanied by induced catalytic activity of CYP1A1 and increased metabolic activation of benzo[a]pyrene (B[a]P) and the formation of DNA-adducts (Bartsch et al 2000, Lodovici et al 2004).

Using several meta-analyses, these 2 polymorphisms of the *CYP1A1* gene have been correlated with the increased incidence of several human cancers such as lung (exon 7 or MspI polymorphisms), cervical (exon 7 or MspI polymorphisms), ovarian (exon 7 polymorphism), esophageal (exon 7 polymorphism) , and breast (exon 7 polymorphism) (Hiyama et al 2007, Huang et

al 2012, Sergentanis and Economopoulos 2010, Sergentanis et al 2012, Zhan et al 2011).

CYP1A1 participates in the process of metabolic activation of several PAHs such as B[a]P. The process of activation of B[a]P starts with the combined action of CYP1A1 and CYP1B1 that result in the formation of an epoxide. Subsequently, the formed epoxide is subjected to hydrolysis by epoxide hydrolase enzyme to a dihydrodiol intermediate, which is further metabolized by CYP1A1 and CYP1B1 to the ultimate reactive species, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (B[a]PDE) (Fig.1.2.) (Singh et al 2006). B[a]PDE can be found as two diastereoisomers, namely, *anti*-B[a]PDE and *syn*-B[a]PDE and each compound of these diastereoisomers can be resolved into (+)- and (-)-enantiomers. Notably, both enantiomers can covalently react with the N^2 exocyclic amino group of guanine by cis- or trans-addition via the C10 position and to a lesser extent the N^6 and N^4 exocyclic amino groups of adenine and cytosine, respectively, forming DNA adducts. It has been shown that the isomer with the 7*R*,8*S*,9*S*,10*R*-configuration, (+)-*anti*-B[a]PDE, is the ultimate carcinogenic metabolite of B[*a*]P that can bind to DNA and induces mutations, DNA breakage, and cancer (Fig.1.2.) (Singh et al 2006).

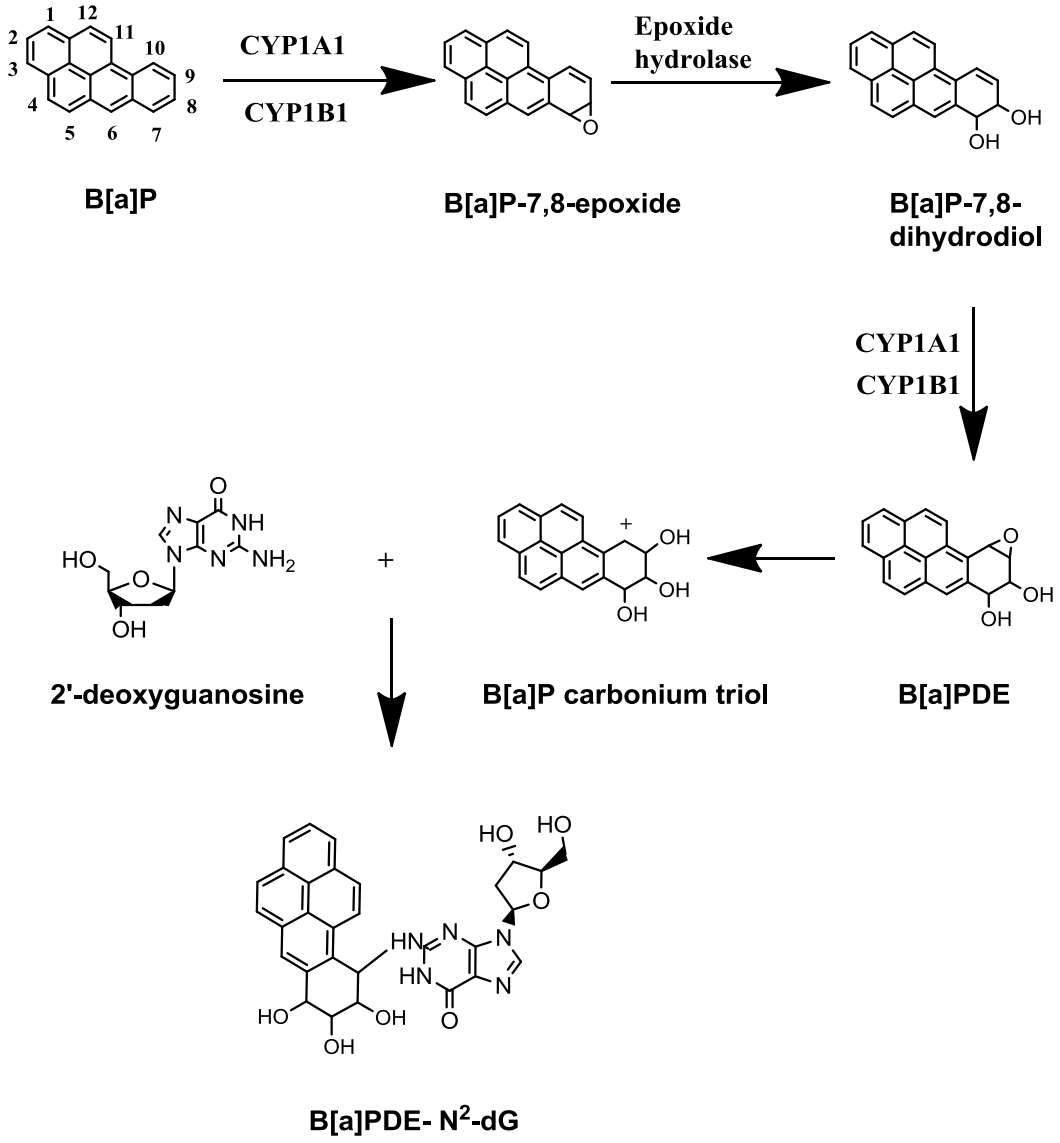


Fig.1.2. Role of CYP1A1 in B[a]P metabolic activation. CYP1A1 and CYP1B1 oxidize B[a]P that results in the formation of an epoxide. Subsequently, the formed epoxide is subjected to hydrolysis by epoxide hydrolase enzyme to a dihydrodiol intermediate, which is further metabolized by CYP1A1 and CYP1B1 to the ultimate reactive species, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (B[a]PDE) that can covalently react with the N^2 exocyclic amino group of guanine, forming DNA adducts (Singh et al 2006).

To the contrary, new postulation has been introduced demonstrating the beneficial role of Cyp1a1 in detoxification of oral B[a]P using *Cyp1a1* knockout mice (Uno et al 2004). This protective property of Cyp1a1 was found to be dependent on several factors such as the tissue in question, and the route of administration (Nebert et al 2004). However, the role of Cyp1a1 in metabolic activation of B[a]P is mainly a first step in the detoxification process by introducing a polar group that can be further conjugated by phase II XMEs to be eliminated out of the body. Therefore, if these reactive metabolites can be conjugated by phase II XMEs, the body will eliminate the PAHs. Conversely, if the balance between the activation (by CYP1A1) and detoxification (by phase II XMEs such as GST) is disturbed, several harmful effects of the reactive metabolites will appear, including mutations and cancer development. In agreement with this hypothesis, the effect of decreased activity of GST enzyme and increased activity of CYP1A1 due to polymorphism can be considered as a major risk factor for the formation of B[a]P-DNA adducts in human leukocytes of healthy smokers (Lodovici et al 2004).

1.2.1.2. Other Phase I AhR-Regulated Genes

In addition to CYP1A1, CYP1A2 and CYP1B1 are important phase I enzymes involved in the metabolic activation of several procarcinogens including PAHs. CYP1A2 is constitutively expressed in human liver and is further induced by AhR agonists such as TCDD (Kohle and Bock 2007). It is involved in the metabolic activation of aromatic amines and the metabolism of important drugs

such as phenacetin, theophylline, caffeine, imipramine, and propranolol (Brosen 1995, Cheng et al 2006). Moreover, CYP1A2 has been correlated with different cancers such as human tobacco-related cancers (Smith et al 1996).

On the other hand, the expression of CYP1B1 is mainly in extra-hepatic organs and is highly induced by PAHs and TCDD through an AhR-dependent mechanism (Shimada and Fujii-Kuriyama 2004). CYP1B1 is responsible for the metabolic activation of several PAHs to their reactive intermediates (Shimada and Fujii-Kuriyama 2004). In this context, several lines of evidence demonstrated the correlation between CYP1B1 and breast cancer development (Ragin et al 2010, Spink et al 1998). One of the postulated mechanisms of CYP1B1 involvement in breast cancer development is the ability of CYP1B1 to metabolize 17β-estradiol to 4-hydroxylated products (Spink et al 1998). These metabolites are recognized as potent carcinogens with high affinity to estrogen receptors that can cause breast cancer in women (Liehr and Ricci 1996, Spink et al 1998).

Despite the fact that TCDD induces mainly the CYP1 family, CYP2S1 mRNA has been induced by TCDD treatments in human and mouse cells through an AhR-dependent mechanism (Rivera et al 2002). The expression of CYP2S1 has been found in several tissues such as lung, stomach and intestine; however, lower levels were found in liver (Rylander et al 2001). The TCDD-mediated induction of CYP2S1 has been correlated with the presence of XRE in its promoter region, confirming the role of AhR in CYP2S1 regulation (Rivera et al 2002). Moreover, it has been demonstrated that CYP2S1 is involved in the process of metabolic activation of B[a]P (Bui et al 2009b).

1.2.2. The Phase II AhR-Regulated Genes

Phase II XMEs involve several enzymes catalyzing detoxification and conjugation reactions of the reactive and carcinogenic metabolites. Among these enzymes NQO1, GSTA1/2, UGT1A6, and ALDH3A1 are regulated by AhR. Moreover, it has been demonstrated that another transcriptional factor called nuclear factor erythroid 2-related factor 2 (Nrf2) participates in the regulation of most of these enzymes through another responsive element called the antioxidant responsive element (ARE) (Ma et al 2004, Nioi and Hayes 2004).

NQO1 is a homo-dimer flavoprotein with multiple cytoprotective functions. It has long been considered as an important chemopreventative enzyme that catalyzes the reduction and detoxification of various exogenous and endogenous quinines (Dinkova-Kostova and Talalay 2010, Nioi and Hayes 2004). It is regulated by both AhR and Nrf2. Most importantly, NQO1 activity has been correlated with the protection against several cancers in both animals and humans (Iskander and Jaiswal 2005, Smith et al 2002).

Additionally, GST enzymes play an essential role in carcinogen detoxification and there is an inverse relationship between the activity of GST enzymes and tumor incidence in humans (McIlwain et al 2006, Peters et al 1993). GST enzymes catalyze the conjugation reactions of several reactive intermediates with glutathione (GSH) and the subsequent excretion of these metabolites out of the body (Lamb and Franklin 2002). GSTA1 and GSTA2 are 2 closely related GST enzymes (GSTA1/2*)* and both of them are induced by AhR agonists and are

attenuated in AhR agonists-treated Nrf2-null mice, suggesting regulation by both transcription factors (Lu et al 2011).

Furthermore, UGT1A6 catalyzes conjugation of serotonin and several phenolic compounds such as paracetamol and planar metabolites of PAHs (Bock and Kohle 2005). Similarly, UGT1A6 of human and murine origin are regulated by AhR and Nrf2 (Kohle and Bock 2007).

In addition, ALDH3A1 is a cytosolic enzyme that catalyzes the oxidation of toxic endogenous and exogenous aliphatic and aromatic aldehydes to ketones and carboxylic acids (Nebert et al 2000, Uchida et al 1999). For example, it is involved in the oxidation of 4-hydroxy-2-nonenal, an end product of intracellular lipid peroxidation that induces H_2O_2 production (Uchida et al 1999). Beside AhR regulation, ALD3A1 has been found to be regulated by several other transcriptional factors such as Nrf2 and nuclear factor kappa B (NF-κB) (Vasiliou et al 1999).

1.3. AhR Activation

1.3.1. Ligand-Dependent Activation

AhR is found inactive in the cytoplasm in combination with two HSP90, the co-chaperone p23 and a 43-KDa protein termed hepatitis B virus X-associated protein 2 (XAP2) (Denison et al 2002). HSP90 is bound to the bHLH and the PAS-B domain of the AhR and its main function is to maintain AhR in a high affinity ligand binding conformation and to prevent its DNA-binding ability (Dolwick et al 1993). The HSP90-associated co-chaperone, p23, acts as a stimulatory factor of AhR activity (Beischlag et al 2008). p23 is necessary for the interaction between HSP90 and AhR and it prevents the spontaneous formation of AhR-ARNT heterodimer in absence of ligand (Kazlauskas et al 1999). Additionally, p23 protects the receptor from being degraded and facilitates the nuclear uptake of the AhR following ligand treatment (Cox and Miller 2002). On the other hand, XAP2 is essential for attenuating the nuclear export of the unliganded AhR to the nucleus (Kazlauskas et al 2000).

Activation of the AhR usually starts with binding to its ligand, leading to a conformational change that exposes its N-terminal nuclear localization sequence and the subsequent translocation of the liganded AhR complex to the nucleus (Denison et al 2011). In the nucleus, AhR heterodimerizes with a structurally related nuclear protein called ARNT. This binding results in the displacement of HSP90 and other subunits from the AhR leading to more interactions between AhR and ARNT, and activation/transformation of ligand-AhR-ARNT complex into high affinity DNA-binding form (Denison et al 2011).

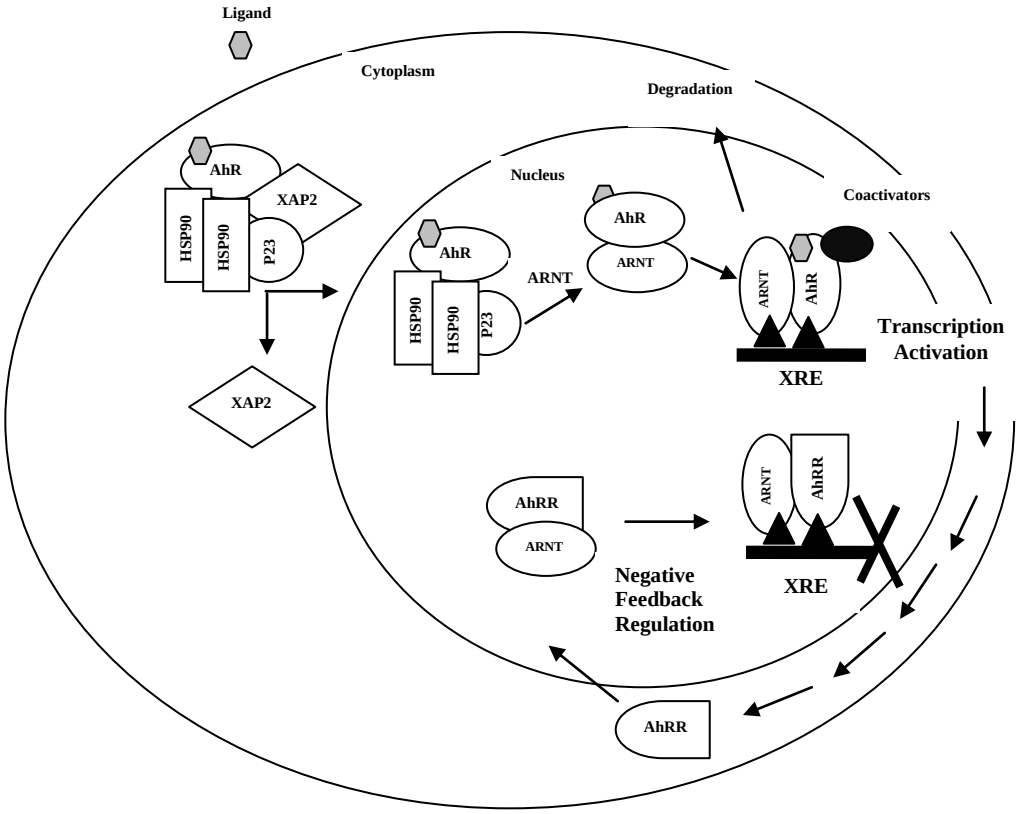


Fig. 1.3. Molecular mechanism of AhR-dependent gene regulation. AhR is found inactive in the cytoplasm in combination with two HSP90, the co-chaperone p23 and a XAP2. Upon ligand binding, AhR gets activated and the liganded AhR complex is translocated to the nucleus where it heterodimerizes with a structurally related nuclear protein called ARNT. The activation/transformation of ligand-AhR-ARNT complex leads to binding to its specific DNA sequence called XRE upstream of the AhR-regulated genes, including CYP1A1. A negative feedback regulation of the AhR pathway is carried out through the AhRR. AhRR can exert the negative feedback regulation on the AhR pathway by competing with AhR for ARNT and formation of inactive AhRR-ARNT transcriptional complexes on XRE. After transcription, AhR is exported out of the nucleus to the cytosol where it degraded by the ubiquitin-proteasomal pathway (Denison et al 2011).

The formed complex then binds to its specific DNA sequence called XRE upstream of the AhR-regulated genes, including CYP1A1, leading to DNA bending, coactivator recruitment, chromatin and nucleosome disruption, increased promoter accessibility to transcription factors, and increased rates of gene transcription (Denison and Nagy 2003, Denison et al 2011) (Fig. 1.3).

The identification of XRE and the cloning of AhR and ARNT participated in the identification of several novel AhR-regulated genes. AhR-regulated genes contain not only the phase I and II XMEs, but also several other genes that can explain the role of AhR and its activation in several physiological and toxicological effects of AhR ligands. Examples of these genes include Slug; a member of the snail/slug family of transcriptional repressors critical for cell adhesion and migration, matrix metalloproteinase-1 (MMP-1), MMP-9, the proto-oncogene Vav3, Cyclooxygenase-2 (COX2), AhR repressor (AhRR), and the metastasis marker Nedd9 (Baba et al 2001, Bui et al 2009a, Carvajal-Gonzalez et al 2009, Degner et al 2007, Ikuta and Kawajiri 2006, Villano et al 2006).

Termination of the transcription activation process of AhR-regulated genes starts with the dissociation of ligand-AhR-ARNT complex from XRE, nuclear export of AhR into the cytosol by its NES that leads to ubiquitination and proteasomal degradation of AhR (Denison et al 2011, Pollenz 2002).

Several lines of evidence demonstrated that the toxicity of numerous AhR ligands such as TCDD depends mainly on the binding and activation of AhR (Denison et al 2011). In agreement with this hypothesis, several studies using transgenic mice in which AhR or a selective function of AhR or ARNT has been

disrupted showed the requirement of AhR signaling pathway for TCDD to exert its toxic effects (Bunger et al 2003, Bunger et al 2008, Gonzalez and Fernandez-Salguero 1998).

1.3.2. Exogenous Ligand-Independent Activation

An increasing number of reports indicated that AhR, in the absence of any exogenous ligand, is trafficking between nucleus and cytosol. These data suggest that AhR can be activated in a ligand-independent manner (Richter et al 2001). Several postulations have been reported to explain this observation. For example, there are several endogenous compounds that can act as AhR activators. In this context, arachidonic acid metabolites have been suggested to be elaborated and induce CYP1A1 when cells were exposed to hydrodynamic shearing stress (Mufti and Shuler 1996). Moreover, UV and visible light exposure lead to formation of tryptophan metabolites that can activate AhR and induce CYP1A1 (Helferich and Denison 1991, Ma 2011). Another postulation has been introduced to explain the ligand-independent activation of AhR suggesting that AhR can be affected by several other signaling pathways. In agreement with this hypothesis, it was previously reported that protein kinase C (PKC) is involved in the activation of AhR in a relatively different way than 3MC-mediated activation (Delescluse et al 2000).

On the other hand, some chemical compounds showed an induction of AhR-regulated genes in an AhR ligand-independent manner. An example of these chemicals is omeprazole that induced CYP1A1 in rat and human hepatocytes;

however, it did not show any binding ability to AhR (Backlund and Ingelman-Sundberg 2004). In this regard, other mechanisms were reported to explain the effect of omeprazole, including its effect on protein tyrosine kinases which differs from the effect of the high affinity AhR ligands such as TCDD (Backlund and Ingelman-Sundberg 2005, Delescluse et al 2000).

1.3.3. AhR Ligands

AhR is a promiscuous receptor that can bind and be activated or inhibited by a wide variety of structurally different compounds. AhR ligands possess significant species-specific differences in ligand-binding specificity. The great variation in structures of AhR ligands can be attributed, at least in part, to the differential binding abilities of several classes of ligands within AhR ligand-binding pocket (Denison et al 2011, Stejskalova et al 2011).

It is worth noting that AhR ligands can be distinguished into several types according to their intrinsic activity (IA), a term that describes a maximal effect of a ligand relative to another highly potent ligand. AhR ligands can be described as *full agonists* that possess the maximum IA (IA = 1), *partial agonists* that possess a moderate IA (1 > IA > 0) or *antagonists* that do not possess any IA (IA = 0) (Carlier et al 2002, Leff 1995, Stejskalova et al 2011). Most AhR ligands are partial agonists that possess a similar affinity to AhR as full agonists. However, the IA of partial agonists is lower than that of full agonists. Moreover, partial agonists can compete with full agonists for AhR and decrease the effect of full agonists, thus behaving as antagonists in the presence of full agonists (Stejskalova

et al 2011). According to their structural specificity, AhR ligands can be classified into classical and non-classical AhR ligands (Kazmin et al 2006, Ozers et al 2005).

1.3.3.1. Classical AhR Ligands

The classical AhR ligands include environmental toxic and persistent carcinogens such as PAHs and halogenated aromatic hydrocarbons (HAHs). These compounds are AhR agonists that share some common characters and features of being aromatic, hydrophobic, planar or co-planar and would be between 12-14 Å (length), less than 12 Å (width) and not more than 5 Å deep and can fit in the AhR ligand-binding pocket (Nguyen and Bradfield 2008). According to their mode of action, classical AhR ligands can be sub-classified into 2 groups. The first group contains agents that are metabolized post-AhR binding and cause DNA adducts (e.g. B[a]P). The other group contains agents that induce persistent P450 activation that increase the likelihood of other pro-carcinogens being activated; an indirect contribution to carcinogenesis (e.g. TCDD) (Bradshaw et al 2002).

1.3.3.1.1. Genotoxic AhR Ligands

The compounds of this category involve several PAHs that formed during thermal decomposition of organic compounds. These compounds usually stimulate their own metabolism through binding and activation of AhR and its regulated genes including CYP1A1. Thereafter, they can be converted to

dihydrodiols by epoxide hydrolase enzyme. The formed intermediates can be further oxidized by CYP1A1 to form the ultimate carcinogenic forms of these compounds that are capable of binding DNA and forming several DNA adducts (Fig. 1.2.) (Singh et al 2006). The most common example of the genotoxic AhR ligands is B[a]P that is usually found as an environmental pollutant in cigarette smoke and automobile exhaust fumes and 3MC that is produced by burning organic compounds (Fig. 1.4.) (Shimada 2006, Wang et al 2008).

1.3.3.1.2. Non-Genotoxic AhR Ligands

This class is represented by several HAHs such as polychlorinated biphenyls and halogenated dioxins and furans. The polychlorinated biphenyls are used widely as capacitors and transformer oils, hydraulic fluids, lubricating oils and plasticizers. Halogenated dioxins and furans are formed in several industrial processes such as bleaching of wood pulp, combustion and as a by-product in production of phenoxyherbicides (Stejskalova et al 2011). Due to the lipophilic characteristics of these compounds as well as their resistance to metabolism, HAHs are widely distributed in the environment and their residues have been identified in air, water, aquatic and marine sediments, fish, human adipose tissue, serum and milk (Safe 1993a).

Structural-activity relationships and structural-binding relationships have been carried out for several HAHs. These studies revealed that the most active compounds in the competitive ligand binding assay are those possessing chlorine substituents in the lateral positions (2,3,7 and 8 for polychlorinated dibenzo-p-

dioxins (TCDD) and polychlorinated dibenzofurans) (Fig.1.4.). The addition of extra chlorine substituent in a non-lateral position or the removal of any of the lateral chlorine substituents resulted in a remarkable decrease of the AhR binding affinities of these compounds (Safe 1993b). TCDD and its related compounds are the most potent AhR activators. It has been previously reported that dioxin induces persistent expression of high levels of CYP1A1 that lead to increased metabolism of exogenous and endogenous chemicals, generation of reactive oxygen species and induction of oxidative stress. TCDD produces several toxicological adverse effects such as teratogenicity, tumor promotion, endocrine disruption, wasting syndrome, reproductive toxicity, and immune-, hepato-, cardio- and dermal-toxicity (Denison et al 2011).

On the other hand, β-naphthoflavone (βNF) is an exception to this group of compounds that does not contain any halogen substituent (Fig. 1.4.). βNF is a synthetic derivative of the naturally occurring flavonoids and it does not produce mutagenicity or genotoxicity by itself (Guengerich and Liebler 1985). Moreover, it has been demonstrated that βNF can potentially induce CYP1A enzymes through an AhR-dependent mechanism. Additionally, it has been reported that βNF can exert liver tumor-promoting activity through enhancement of oxidative stress responses in rats (Dewa et al 2008, Kuwata et al 2011).

1.3.3.2. Non-Classical AhR Ligands

A tremendous amount of research suggests that AhR can be activated by several novel chemicals that possess different structural and physiochemical

properties than classical AhR ligands. These compounds have striking structural differences from the classical AhR ligands regarding planarity, hydrophobicity and aromaticity. Most of these compounds are weak inducers or AhR ligands when compared to TCDD (Denison and Heath-Pagliuso 1998, Stejskalova et al 2011).

An example of a non-classical AhR ligand is primaquine, an 8-aminoquinoline derivative that is usually used as antimalarial drug (Fig. 1.4.). Primaquine induces CYP1A1 at mRNA and activity levels in human hepatoma HepG2 cells (Bapiro et al 2002). Although the direct binding of primaquine to AhR is a matter of debate, it has been demonstrated that primaquine induces dissociation of AhR and binding to XRE in rat hepatoma H4IIE cells (Backlund and Ingelman-Sundberg 2004, Fontaine et al 1999, Werlinder et al 2001). Several other synthetic compounds have been proven to induce CYP1A1 activity, including omperazole (Backlund and Ingelman-Sundberg 2004), sulindac (Ciolino et al 2006), tranilast (Prud'homme et al 2010), leflunomide (O'Donnell et al 2010) and several kinase inhibitors such as SP600125 [a c-JUN N-terminal kinase (JNK) inhibitor] which has been demonstrated to be an AhR partial agonist (Dvorak et al 2008a).

On the other hand, several lines of evidence demonstrated that AhR can be induced in absence of exogenous ligands. This observation was augmented by the identification of several endogenous ligands for AhR that are synthesized inside the bodies of higher organisms. Heme metabolites such as bilirubin, biliverdin and hemin; arachidonic acid metabolites such as lipoxin A4; tryptophan

derivatives such as indoleacetic acid, kynurenic acid, xanthurenic acid, and 6-formylindolo[3,2-b]carbazole (FICZ) are examples of endogenous AhR ligands (Nguyen and Bradfield 2008, Schaldach et al 1999). Most of these endogenous ligands show their effect as AhR agonists at relatively higher non-physiologically relevant concentrations except for kynurenic acid and xanthurenic acid. These tryptophan derivatives are direct ligand/agonists for AhR and can activate AhR in physiologically attainable concentrations (DiNatale et al 2010). Although the main pathway that generates these compounds is the indoleamine-2,3-dioxygenase pathway that is important in maintaining T cell functions, the exact physiologic role of these compounds needs further investigation (Omiecinski et al 2011).

1.3.3.3. AhR Antagonists

AhR antagonists are a group of compounds that are capable of binding to AhR and prevent its activation (Henry et al 1999). Given that several AhR ligands possess numerous toxic and carcinogenic effects, it has been proposed that the use of AhR antagonists may be one of the good approaches to prevent and/or to treat AhR ligand-mediated toxicity and carcinogenicity (Puppala et al 2008). In agreement with this hypothesis, pre-treatment with several AhR antagonists, such as 3′-methoxy-4′-nitroflavone (MNF), were proven to inhibit genotoxicity in mice treated with B[a]P (Dertinger et al 2001). Interestingly, MNF has species-specific effects as a partial agonist/antagonist for AhR. MNF showed a full agonist effect for AhR in guinea pig cells, whereas it was a partial agonist/antagonist of TCDD-

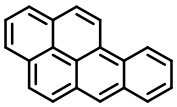
induced AhR in mouse cells (Henry and Gasiewicz 2008, Murray et al 2010, Zhou and Gasiewicz 2003). The species-specific effect of MNF was attributed to a single amino acid difference of the ligand binding domain of the two species (Henry and Gasiewicz 2008).

In addition to MNF, another synthetic flavone called 6,2′,4′-trimethoxy-flavone (TMF) (Fig. 1.4) has been introduced as a promising AhR antagonist in human and murine hepatoma cells. Unlike MNF, TMF showed no species-specificity with regard to AhR antagonism. Moreover, TMF did not show any AhR agonistic effect in short term exposure; however, a modest increase in AhR-dependent gene expression after extended exposure has been detected (Murray et al 2010).

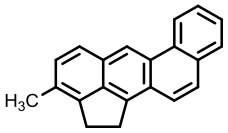
In addition to synthetic flavonoids as antagonists of AhR, a new purine derivative called StemRegenin 1 (SR1) showed AhR antagonistic activity. Similar to MNF, the effect of SR1 is a species-specific AhR antagonism. SR1 completely inhibited TCDD-mediated induction of AhR-regulated gene expression in human cells, weak inhibition in mouse cells, and no effect on rat AhR-dependent activation of signal transduction (Boitano et al 2010).

A novel compound, 2-methyl-2*H*-pyrazole-3-carboxylic acid (2-methyl-4-*o*-tolylazo-phenyl)-amide (CH223191) (Fig. 1.4.), has been identified as an AhR antagonist. CH223191 efficiently inhibited TCDD-mediated activation of AhR, blocked the binding of TCDD to AhR and prevented the TCDD-mediated nuclear translocation of AhR and subsequently inhibited the transcription of AhR-regulated genes (Kim et al 2006).

Classical genotoxic AhR agonists

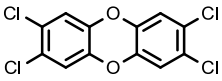


Benzo[a]pyrene (B[a]P)

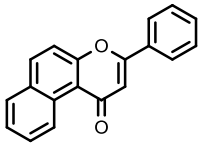


3-Methylcholanthrene (3MC)

Classical non-genotoxic AhR agonists

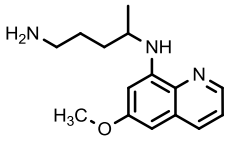


2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)

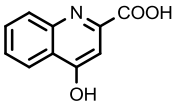


***β*-naphthoflavone (*β*NF)**

Non-Classical AhR agonists

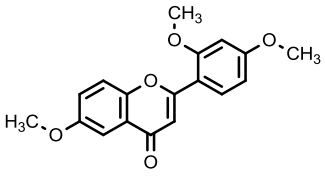


Primaquine

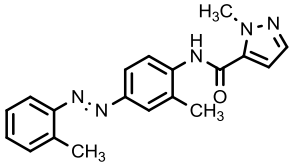


Kynurenic acid

AhR antagonists



6,2',4'-Trimethoxyflavone (TMF)



CH223191

Fig. 1.4. Representative structures of several classes of AhR ligands

Unlike MNF and SR1, the effect of CH223191 was not species-specific as it inhibited TCDD-mediated activation of AhR in mouse, rat, human, and guinea pig cell lines. However, CH223191 showed a unique characteristic of being a ligand-selective antagonist. CH223191 was found to preferentially inhibit the ability of some classes of AhR agonists such as TCDD and other HAHs, whereas it did not show similar effects using non-HAHs such as PAHs and βNF (Zhao et al 2010). The effect was attributed to the differences of binding between HAHs and non-HAHs within the AhR ligand binding pocket. Moreover, it has been postulated that CH223191 acts as a selective AhR modulator (SAhRM). CH223191 can bind to AhR outside the ligand binding pocket and act as allosteric modulator that affects AhR structure, leading to preferentially inhibiting some ligands, but not others (Zhao et al 2010).

1.3.4. Regulatory Proteins of AhR Activation/Deactivation

AhR shares a common characteristic with other transcriptional factors of recruiting protein complexes consisting of common pools of coactivator/corepressor proteins that are described as coregulators or master regulators of gene transcription (Glass and Rosenfeld 2000, McKenna and O'Malley 2002). These coregulators modulate the intrinsic functions of transcriptional factors through regulation of wide variety of functions including chromatin remodeling, RNA elongation and processing, translation, DNA break formation, and transcriptional termination (Ju et al 2006, O'Malley 2007).

Upon ligand binding, AhR gets activated and translocates to the nucleus where it binds to the XRE sequence in the enhancer region of CYP1A1 that causes nucleosomal disruption and recruitment of coactivators and other transcriptional factors by the transactivation domain of the AhR, leading to transcription of *CYP1A1* gene (Fig. 1.3.) (Hankinson 2005). Several coactivators such as the following have been recognized to affect *Cyp1a1* gene transcription: coactivators with histone acetyltransferase activity [e.g. steroid receptor coactivator 1 (SRC1), CREB-binding protein (CBP), and p300], coactivators with histone methyltransferase activity [e.g. cofactor-associated arginine [R] methyltransferase 1 (CARM1)], and coactivators with ATPase-dependent histone modifiers [e.g. Brahma-related gene 1 (Brg-1)] (Hankinson 2005, Kumar and Perdew 1999).

On the other hand, several coregulators are capable of suppressing the transcription of AhR-regulated genes. For example, it has been shown that interaction of the AhR/ARNT complex with silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) significantly reduces the activation and binding of AhR to XRE and represses the expression of AhR-regulated genes (Nguyen et al 1999). Moreover, several histone deacetylases (HDAC) were reported to suppress the transcription activation of AhR-regulated genes by deacetylating histones and stabilizing chromatin such as HDAC1, HDAC4 and HDAC5 (Oshima et al 2007, Suzuki and Nohara 2007).

The differential recruitment of coactivators/corepressors has been postulated as one of the main factors of species- and tissue-specific responses of

different AhR ligands. In this context, the differences in gene expression response to TCDD between human hepatoma HepG2 cells and murine hepatoma Hepa 1c1c7 cells was attributed to the different profile of coactivators and corepressors in both cells (Suzuki and Nohara 2007). Moreover, it has been demonstrated that TCDD shows a higher induction of Cyp1a1 and AhR-dependent luciferase activity in human embryonic kidney HEK293 cells than its effect in murine hepatoma Hepa 1c1c7 cells. The effect was correlated with the recruitment of several coactivators in HEK293, such as CARM1, that was not detected in Hepa 1c1c7 cells (Zhang et al 2008).

In addition to corepressors, another negative regulatory mechanism for AhR has been reported involving the AhRR. AhRR is a bHLH-PAS protein that inhibits both constitutively-active and xenobiotic-induced AhR transcription activity in multiple species (Hahn et al 2009). AhRR has a similar structural to AhR; however, it cannot bind AhR ligands (Mimura et al 1999). AhRR is induced by AhR agonists and acts as a negative feedback regulator of AhR activation through 2 main pathways. First, AhRR can quench AhR-dependent up-regulation of transcription by competing with AhR for binding to XRE found in the AhR-target genes (Fig. 1.3.). Second, AhRR may transrepress AhR through protein-protein interaction but independent of direct DNA binding (Hahn et al 2009, Harper et al 2006). The AhRR regulatory mechanism can give an explanation of some cell- and tissue-specific effects of AhR agonists. For example, human cervical carcinoma HeLa cells were found to be relatively resistant to several AhR

agonists, such as TCDD and 3MC, that was attributed to the higher level of AhRR expression in these cells (Tsuchiya et al 2003).

1.4. Mechanisms Participating in Regulation of AhR-Regulated Genes

There are several mechanisms introduced to determine the effect of several compounds on AhR-regulated genes including transcriptional, post-transcriptional, and post-translational mechanisms.

1.4.1. Transcriptional Mechanisms

In general, the activation of AhR and the subsequent induction of its regulated genes are controlled mainly by transcriptional mechanisms. These mechanisms start with ligand binding to AhR, translocation to the nucleus, activation and transformation of AhR, binding to its specific DNA sequence and starting transcription process of the AhR-regulated genes. All these steps can be examined; for example, AhR binding can be examined using competitive ligand binding assays, where a radiolabelled TCDD is incubated with cytosolic AhR in the presence and absence of the test compound. This assay tests the ability of the compound to compete with radiolabelled TCDD for AhR (Denison et al 1986). Moreover, AhR transformation and binding to its specific DNA sequence can be tested using an electrophoretic mobility shift assay (EMSA). In this assay, the specific DNA sequence of AhR, XRE, is post-labelled using radiolabelled ATP. If AhR gets activated, it will bind to its radiolabelled XRE and the formed complex can be resolved using a polyacrylamide gel (Denison et al 2002).

Another method to test AhR activation and binding to XRE is the use of an AhR-dependent luciferase assay. In this assay, XRE-driven firefly luciferase reporter gene plasmid is transfected inside the cells. If AhR is activated, it will

bind to its XRE that is found upstream of AhR-regulated genes along with the luciferase DNA. Therefore, the luciferase activity can be used to confirm the transcriptional activation (Jeuken et al 2003, Jiang et al 2008). Finally, to determine the effect of a test compound on a specific gene expression, real-time Polymerase Chain Reaction (real-time PCR) can be used with specific primers for the needed gene (Nishimura et al 2002).

1.4.2. Post-Transcriptional Mechanisms

The steady-state level of mRNA is a result of mRNA synthesis and mRNA degradation. Therefore, an increase in the level of mRNA can be attributed either to an increase of mRNA synthesis or a decrease of mRNA degradation. The degradation process of mRNA is mediated mainly by exonucleases that catalyze mRNA. The post-transcriptional modifications can be assessed using the half-life ($t_{1/2}$) of a certain mRNA as a measure of its stability (Lekas et al 2000). Actinomycin D (Act-D) is a known transcriptional inhibitor that has been used extensively to stop the synthesis of the *de novo* mRNA and therefore, the decay curve of a certain gene can be plotted against time and the $t_{1/2}$ can be calculated (Elbekai and El-Kadi 2007). Several studies have demonstrated the $t_{1/2}$ of several CYPs including CYP1A1, CYP1A2 and CYP1B1 in human hepatoma HepG2 cells after treatment with TCDD. The decay curves demonstrated that CYP1A2 and CYP1B1 possess longer half-lives (> 24 h), whereas CYP1A1 possesses a shorter $t_{1/2}$ (2.4-4.4 h) (Lekas et al 2000, Suzuki and Nohara 2007). Moreover, it has been demonstrated that the stability of CYP1A1 mRNA is higher in human

HepG2 cells than murine Hepa 1c1c7 cells (Suzuki and Nohara 2007). Several compounds can affect CYP1A1 through post-transcriptional mechanisms. For example, the natural compound resveratrol (Res) and the adrenal steroidal hormone dehydroepiandrosterone decreased the stability of CYP1A1 mRNA in human breast cancer T47D cells (Lee and Safe 2001). Recently, a microRNA has been identified in the 3'-untranslated region of CYP1A1. This microRNA (miR-892a) has been recognized as a negative regulator of CYP1A1 that can repress the expression of CYP1A1 post-transcriptionally (Choi et al 2012).

1.4.3. Post-Translational Mechanisms

Post-translational modification can be defined as any difference between a functional protein and the linear polypeptide sequence encoded between the initiation and the termination codons of its structural gene (Han and Martinage 1992). The post-translational modifications of CYP enzymes can be classified into covalent and noncovalent modifications. The noncovalent modifications involve the incorporation of cofactors such as heme, and protein folding. Moreover, it involves several substrate and inhibitor interactions that affect the final activation of CYP enzymes (Hlavica and Lewis 2001). The covalent modifications of CYP enzymes involve the cleavage of a single peptide and the alterations of the amino acid residues including phosphorylation, nitration, glycosylation, methylation, sulfation, and acetylation (Aguiar et al 2005).

One of the common approaches to test the effect of a substance on the post-translational modification of CYP1A1 is to co-incubate the test substance with the translational inhibitor cycloheximide (CHX) that inhibits new protein synthesis. Decay curves can be plotted against time and protein $t_{1/2}$ can be calculated (Elbekai and El-Kadi 2007).

1.4.3.1. Phosphorylation-Dependent Mechanism

Phosphorylation of protein is a reversible process that involves transfer of phosphate from adenosine triphosphate (ATP) to a specific protein by protein kinases and phosphatases (Han and Martinage 1992). Several studies suggested that AhR undergoes changes through phosphorylation. For example, treatment of Hepa 1c1c7 cells with c-AMP resulted in activation and translocation of AhR to the nucleus in a different way than that observed with TCDD (Oesch-Bartlomowicz et al 2005). Moreover, phosphatase inhibitors, such as ortho-vanadate, reduced the CYP1A1/2-dependent formation of mutagenic metabolites of aromatic amines and aromatic amides in rat liver hepatocytes and rat liver homogenates (Oesch-Bartlomowicz et al 1997).

1.4.3.2. 26S Proteasome-Dependent Mechanism

The 26S proteasome is a multi-enzyme protein complex that possesses an essential role in the bulk of protein degradation inside the cells. The complex has critical functions in several vital processes inside the cell such as cell cycle, differentiation and apoptosis (Weissman 2001). The process of protein

degradation starts with labelling the designated protein with a 76 amino polypeptide called ubiquitin. Several enzymes are involved in the following steps until a protein is conjugated with polyubiquitin subunits. Thereafter, the polyubiquitinated protein is recognized by the 26S proteasome, unfolded, the ubiquitin subunits are cleaved and the target protein is degraded into short peptides (Weissman 2001).

Several lines of evidence demonstrated that the ubiquitin-proteasomal pathway plays a key role in CYP1A regulation. For example, the ubiquitin-proteasomal pathway has been demonstrated to be the main pathway involved in degradation of AhR after ligand activation (Davarinos and Pollenz 1999, Pollenz 2007). Additionally, the TCDD-mediated induction of AhR-dependent luciferase activity was induced by the proteasomal inhibitor Carbobenzoxy-l-leucyl-l-leucyl-leucinal (MG-132) (Davarinos and Pollenz 1999). On the other hand, several CYPs, such as CYP2E1 and CYP3A4, are known to be a substrate for the ubiquitin-proteasomal pathway, leading to their degradation (Aguiar et al 2005, Roberts 1997).

1.5. Role of AhR in Toxicity and Carcinogenicity

Despite its physiological and developmental role, AhR has been correlated with the toxic and carcinogenic effects of several AhR agonists. For example, TCDD has been shown to produce a spectrum of AhR-dependent toxic effects that include tumorgenesis, teratogenicity, cardio-, hepato-, immune- and dermal-toxicity (Denison et al 2011, Kopf et al 2010). The main mechanism of TCDD toxicity has been found to be through persistent activation of AhR and its regulated genes (Denison et al 2011, Kopf et al 2010). In agreement with this hypothesis are the results of experiments using transgenic mice in which the activity of AhR has been compromised (Denison et al 2011). It has been demonstrated in these experiments that the toxicity of TCDD absolutely requires the activation of the AhR signaling pathway (Bunger et al 2003, Bunger et al 2008, Gonzalez and Fernandez-Salguero 1998).

It is important to note that the level of AhR expression has been correlated with malignancy (Belguise et al 2007). Moreover, the role of AhR has been implicated in all stages of cancer development starting from initiation to promotion and ending with progression (Dietrich and Kaina 2010). AhR possesses a key role in the initiation of chemical carcinogenesis through its ability to induce the expression of several CYPs that metabolically activate procarcinogens, such as PAHs, to their ultimate carcinogenic forms, leading to DNA binding and formation of DNA adducts (Omiecinski et al 2011). The fact that the carcinogenicity of PAHs is lost in AhR knockout mice demonstrated that

AhR is required for tumor initiation by PAHs in animals and most likely in humans (Dietrich and Kaina 2010, Nakatsuru et al 2004, Shimizu et al 2000).

On the other hand the nongenotoxic agonists of AhR, such as TCDD, are known carcinogens to animals and humans. For example, TCDD has been classified as a human carcinogen by the International Agency of Research on Cancer (IARC) since 1997. In 2009, the IARC confirmed that TCDD (or dioxin) is a human carcinogen that is correlated with the increased mortality from all types of human cancers combined (Warner et al 2011). The main mechanisms of the tumorigenic effect of TCDD involve induction of tumor promotion and progression through an AhR-dependent pathway (Dietrich and Kaina 2010).

The role of AhR in mediating tumor promotion and progression has been recently suggested through several findings. First, AhR regulates a battery of genes that participates in tumor promotion and progression of several tumor cell lines such as Slug, MMP-1, MMP-9, Vav3, COX2 and Nedd9 (Bui et al 2009a, Carvajal-Gonzalez et al 2009, Degner et al 2007, Ikuta and Kawajiri 2006, Villano et al 2006). Second, AhR ligands have been found to induce the expression of the tumor-promoting cytokine IL6 in tumor cell lines (DiNatale et al 2010). Finally, TCDD was found to induce tumor promotion, a reversible process in which actively proliferating pre-neoplastic cells accumulate, through inhibiting apoptosis. It has been demonstrated that apoptosis participates in the elimination of the damaged cells after the initiation stage of carcinogenesis and inhibition of apoptosis by TCDD supports tumor formation and promotion (Chopra and Schrenk 2011, Dietrich and Kaina 2010, Hattis et al 2009).

Regarding the role of AhR in tumor progression, a transition from a benign to a malignant tumor with invasion and metastatic potential, recent studies have demonstrated that AhR expression is higher in invasive than non-invasive tumor cells (Ishida et al 2010, Villano et al 2006, Yang et al 2005). Moreover, the level of AhR expression has been correlated with malignancy in lung tumors (Chang et al 2007). One of the postulated mechanisms of AhR ability to induce invasion of cells has been correlated with its role in the induction of several matrix metalloproteinase such as MMP-1 and MMP-9 (Ishida et al 2010). Additionally, fibroblasts deprived from AhR knockout mice possessed a decreased tumorigenic and migration ability in a xenograft model (Mulero-Navarro et al 2005). This effect was found to be due to the loss of the proto-oncogene Vav3 that is regulated by AhR (Carvajal-Gonzalez et al 2009). Finally, recent studies have demonstrated that AhR activation can induce some genes that affect cell-cell adhesion such as Slug. The induction of these genes by AhR agonists, such as TCDD, breaks down the cell-cell adhesion that leads to induction of tumor proliferation and progression (Dietrich and Kaina 2010).

Taken together, these data demonstrate that AhR plays an essential role in the production of the adverse effects of several AhR agonists. In addition, AhR has been extensively implicated in all stages of the process of carcinogenesis. Most importantly, these data suggest the use of AhR antagonists as promising agents for cancer prevention as well as treatment.

1.6. Herbs and Herbal Components (HC) as Potential Therapeutic Agents

Herbal plants have been used for health reasons for thousands of years and their use has been increased over the last decade (Ernst and Pittler 2002, Wachtel-Galor and Benzie 2011). It has been estimated that 70% of developing countries' and around 33% of developed countries' populations use herbal products (Chin et al 2006, Jiang and Hu 2009, Lennox and Henderson 2003, Olisa and Oyelola 2009). Moreover, natural compounds or natural product-derived drugs constitute approximately 28% and 24% of all drugs developed between 1981 and 2002, respectively (Chin et al 2006).

There is therapeutic and financial importance of herbal medicine. For example, herbal plants produce a number of very useful drugs that are difficult to produce by the commercial synthetic techniques such as alkaloids of opium and cardiotonic glycosides of digitalis. Moreover, herbal plants produce compounds that have been used as prototype model for synthetic drugs such as cocaine and its synthetic local anesthetics analogue procaine. On the other hand, drugs derived from herbal plants possess a key role in the pharmaceutical market. For instance, the annual market of taxol exceeds $1 billion dollar per year. Furthermore, plant-derived steroids account for about 15% ($22 billion) of the annual world pharmaceutical market (Ebadi 2007).

1.6.1. HC and Cancer

1.6.1.1. HC and Cancer Treatment

Herbal plants are one of the major sources of discovery of several drugs, especially for treatment of cancer (Newman et al 2000). It has been estimated that

more than 60% of currently used anti-cancer drugs have been derived from natural sources including plants (Cragg and Newman 2005, Newman et al 2003). There are several clinically used phytochemicals that can be categorized into four main classes of compounds: camptothecins, podophyllotoxins, taxanes, and vinca (or *Catharanthus*) alkaloids (Table 1.1) (van Der Heijden et al 2004). Furthermore, there are numerous phytochemicals in various phases of clinical trials. These include curcumin (extract from *Curcuma longa* Linn; head and neck, colorectal and pancreatic cancer), epigallocatechin gallate (EGCG; extract from green tea; breast, lung, bladder and prostate cancer), and soy isoflavones (breast and prostate cancer) (www.clinicaltrials.gov) (Aggarwal et al 2003, Bar-Sela et al 2010, Swami et al 2009).

Table 1.1. Anticancer drugs of plant origins

Phytochemicals	Source	Mechanism of action	Therapeutic use
Camptothecins (i) -Topotecan (Hycamtin) -Irinotecan HCl (Camptosar)	Barks of the Chinese *Camptotheca acuminata*	DNA Topoisomerase I inhibitors	- Metastatic ovarian cancers -Metastatic colorectal cancer
Podophyllotoxins (ii) -Etopside (VP-16) -Teniposide (VM-26)	Resin of *Podophyllum peltatum* & *Podophyllum hexandrum*	DNA Topoisomerase II inhibitors	-Lung cancer & lymphomas -Acute leukemia in children & brain tumors
Taxanes (iii) - Paclitaxel (Taxol) -Docetaxel (Taxotere)	Bark of yew tree *Taxus brevifolia* & leaves of *Taxus baccata*	Microtubule stabilizing agents	Ovarian, breast, colon, head, and lung cancers
Vinca alkaloids (iv) -Vinblastine (Velban) -Vincristine (Oncovin) -Vinorelbine (Navelbine)	-Leaves of *Catharanthus roseus*	Spindle poisons	-Testicular tumors & Hodgkin's lymphoma -Leukemia & Hodgkin's lymphoma -Breast & lung cancer

(i): (Pommier 2006)

(ii): (Belani et al 1994), (Xu et al 2009)

(iii): (Vaishampayan et al 1999), (Baker and Dorr 2001)

(iv): (Galano et al 2011), (Cragg and Newman 2005)

1.6.1.2. HC and Cancer Chemoprevention

Chemoprevention can be defined as the use of natural or synthetic compounds to allow suppression, retardation or inversion of carcinogenesis (Kelloff et al 1994). Several mechanisms are proposed to explain the chemopreventative effects of several phytochemicals. These include antioxidant, anti-inflammatory and immune enhancing effects and modification of phase I/II enzymes (Tsuda et al 2004). For example, carotenoids exert their chemopreventative effects through antioxidant, anti-inflammatory and immune enhancing mechanisms.

Moreover, emodin, an anthraquinone from *Cassia siamea,* exhibited strong anti-tumor promoting effect on mouse skin tumors induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Koyama et al 2002).

However, as most chemical carcinogens are inert and require metabolic activation to exert their carcinogenic effects, compounds that can affect xenobiotic metabolism are of great importance. There are several compounds known to inhibit phase I metabolism through AhR-dependent mechanism. In this context, Res, a trihydroxystilbene compound naturally occuring in red wine, was found to prevent genotoxicity in mice treated with B[a]P (Revel et al 2003). Another mechanism of chemoprevention is to induce phase II metabolizing enzymes, leading to conjugation and detoxification of several reactive metabolites. Several sulfur-containing phytochemicals such as sulforaphane and phenyethyl isothiocyanate (found in broccoli and other cruciferous vegetables) are potent inducers of phase II metabolizing enzymes through the Nrf2 signaling

pathway. These sulfur-containing compounds show great promise as chemopreventative agents (Conaway et al 2005, Laskin et al 2004).

1.6.2. AhR Modulators from Natural Sources

There are several AhR modulators from natural sources. For example, it has been demonstrated that several flavonoids can affect AhR activation/deactivation. Although a small number of flavonoids, such as daidzein and diosmin, are shown to act as weak AhR agonists, a huge number of dietary flavonoids exert AhR antagonistic activity such as apigenin, Res, quercetin, emodine, myricetin, kaempferol and chrysin (Ashida et al 2000, Ciolino et al 1998b, Ciolino and Yeh 1999, Doostdar et al 2000). Moreover, dietary flavonoids inhibited B[a]P-induced mutagenicity and carcinogenicity (Revel et al 2003, Schwarz and Roots 2003). Of particular note, flavonoids are found extensively in our foods and represent the most abundant class of dietary natural products (Androutsopoulos et al 2010). The AhR antagonistic effects of flavonoids were found to be exerted within the physiologically attainable concentrations (Ashida et al 2000). These data can justify why diets rich in vegetables and fruits are healthier than other types of diets. Furthermore, several AhR antagonists have been derived from flavonoid structures such as MNF and TMF (Murray et al 2010, Zhou and Gasiewicz 2003). Finally, it has been demonstrated that some flavonoids, such as kaempferol and Res, act as substrates and inhibitors of CYP1A1, leading to a decreased activity of the carcinogen-activating enzyme CYP1A1 (Ciolino and Yeh 1999, Silva et al 1997).

Several screening programs have been carried out to identify new AhR antagonists of herbal origin. Out of 41 kinds of extracts from vegetables and fruits, molokhia extract (*Corchorus olitorius* L.) (Egyptian spinach) showed the strongest inhibitory effect of TCDD-induced AhR transformation in murine and human hepatoma and human colon cell lines (Nishiumi et al 2006).

Furthermore, green tea is a major source of polyphenolic compounds that possess antitumor effects in several animal models. The most active ingredient of green tea is EGCG. It has been reported that EGCG is a potent AhR antagonist that alters AhR transcription and is responsible for most of AhR antagonistic effects of green tea extract (Palermo et al 2003). EGCG has been found to block AhR-dependent transcription indirectly by binding to HSP90, leading to stabilization of the AhR-HSP90-XAP2 complex that is unable to neither dimerize with ARNT nor bind to XRE (Yin et al 2009). In support of its AhR antagonistic effect, EGCG prevented 7,12-dimethylbenzo[a]anthracene-induced mammary tumors in rats (Belguise et al 2007).

1.6.3. *Peganum harmala*

Peganum harmala L. (Syrian rue, harmal, harmel) (Family Zygophyllaceae) is a perennial herbal plant native to North Africa, the Mediterranean Sea, the Middle East, Pakistan and India, and has been introduced to parts of the Southwest USA and a few areas in Australia and South Africa (Herraiz et al 2010). *Peganum harmala* has been used traditionally for psychoactive purposes since ancient times. Moreover, it possesses hypothermic,

hallucinogenic, aphrodisiac, stimulant, sedative, emmenagogue, abortifacient and emetic properties (Abdel-Fattah et al 1995, el Bahri and Chemli 1991). In addition, it has been used for the treatment of asthma, jaundice, diabetes, rheumatism, colic, hypertension, malaria, lumbago, microbial infections in poultry, and several tumors (Arshad et al 2008a, Astulla et al 2008, Farouk et al 2008, Lamchouri et al 1999, Tahraoui et al 2007).

Peganum harmala contains several *β*-carbolines such as harmine, harmaline, harmol, and harmalol. The presence of these alkaloids in the plants depends mainly on the part of the plant used. For example, seeds contain mainly harmine and harmaline, whereas roots contain mainly harmine and harmol (Herraiz et al 2010). Several lines of evidence demonstrated that *β*-carbolines are responsible for several pharmacological and toxicological effects of the plant extract including inhibition of MAO-A, antidepressant, antimalarial and antimicrobial effects (Arshad et al 2008a, Astulla et al 2008, Herraiz et al 2010).

1.6.4. *β*-Carbolines

β-Carbolines are a large group of natural and synthetic indole alkaloids that are widely distributed in nature, including various foods, plants, marine creatures, insects, mammalians, as well as human tissues (Cao et al 2007). *β*-Carbolines have been considered as an important group of compounds as they possess diverse pharmacological activities such as sedative, hypnotic, anxiolytic, anticonvulsant, antitumor, antithrombotic, antiparasitic, antimicrobial, as well as antiviral activities (Cao et al 2007).

Harmine, 7-methoxy-1-methyl-9H-pyrido[3,4-b]indole and harmol, 1-methyl-9H-pyrido[3,4-b]indole-7-ol (Fig.1.5.), are *β*-carboline compounds naturally found in several medicinal plants including *Peganum harmala* and *Banisteriopsis Caapi* (Malpighiaceae) (Herraiz et al 2010, Samoylenko et al 2010). Harmine possesses several pharmacological activities such as antiplatelet aggregating, antimicrobial, antioxidant and antiprotozoal activity (Arshad et al 2008b, Di Giorgio et al 2004, Im et al 2009, Moura et al 2007). Moreover, harmine is highly cytotoxic to several human tumor cell lines and showed promising antitumor effects in mice bearing tumor cells (Cao et al 2005).

Harmol, the metabolite of harmine, possesses limited or no neuropharmacological side effects (Cao et al 2007, Herraiz et al 2010). Moreover, harmol showed a promising effect as an anticancer agent through the induction of apoptosis in human lung carcinoma cells (Abe and Yamada 2009).

On the other hand, harmaline, 4,9-dihydro-7-methoxy-1-methyl-3H-pyrido[3,4-b]indole and harmalol, 1-methyl-4,9-dihydro-3H-pyrido[3,4-b]indole-7-ol (Fig.1.5.), are common dihydro-*β*-carboline compounds that are naturally found in several alcoholic beverages and medicinal plants including *Peganum harmala* (Herraiz et al 2010, Park et al 2010). Harmaline and harmalol possess antimicrobial, antioxidant and antiprotozoal activities (Arshad et al 2008b, Moura et al 2007). Similar to harmine, harmaline can interact with several enzymes and neurotransmittors including topoisomerase I and MAO-A (Herraiz et al 2010, Sobhani et al 2002).

It is important to note that the main structural nucleus of harmine and harmol compounds is another β-carboline compound called harman. Harman, 1-methyl-9H-pyrido[3,4-b]indole (Fig. 1.5.), has been identified in several traditional plants such as *Tribulus terrestris* (Zygophyllaceae), *Virola spp.* (Myristicaceae) and *Banisteriopsis spp.* (Malpighiaceae) (Boeira et al 2002, Pfau and Skog 2004). Harman is found in cigarette smoke and several foods and drinks such over-cooked foods and coffee (Herraiz 2004, Pfau and Skog 2004).

On the other hand, several studies demonstrated the mutagenic effects of harman (Boeira et al 2001, Boeira et al 2002, Madle et al 1981). Moreover, it was found that there is a correlation between the higher blood concentration of harman and the development of colon cancer in patients with essential tremors; however, the exact mechanism has not been fully identified (Louis et al 2008).

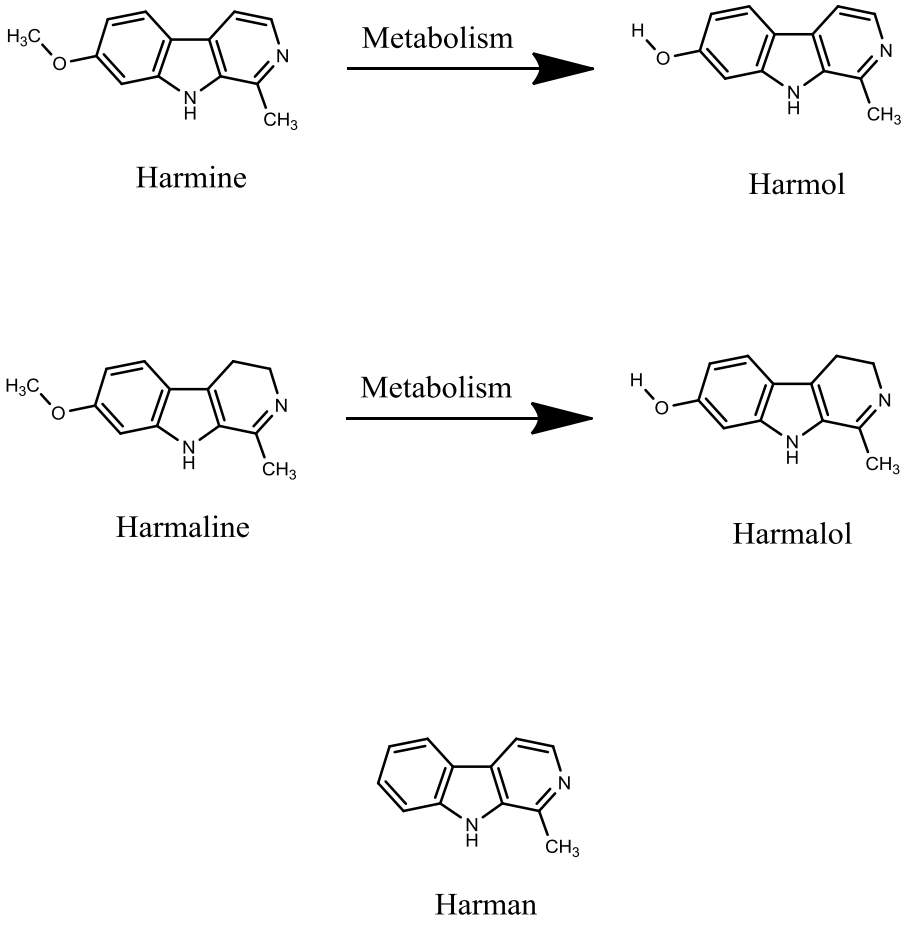


Fig. 1.5. Structures of the *β*-carbolines used in the current study

1.7. Rationale, Hypothesis, and Objectives

1.7.1. Rationale

AhR is a ubiquitous transcription factor that regulates several phase I and phase II metabolizing enzymes. AhR is activated by several structurally different compounds including PAHs (such as B[a]P) and HAHs (such as TCDD) (Denison et al 2002). It has been demonstrated that several AhR ligands cause toxic effects including cancer either directly through formation of genotoxic metabolites (such as B[a]P) or indirectly through induction of tumor promotion and progression (such as TCDD) (Denison et al 2011). Conversely, several cancers have been correlated with a high level of AhR activation (Belguise et al 2007).

AhR activation leads to increased transcription of several AhR-regulated genes including the carcinogen-activating enzyme CYP1A1 (Ma and Lu 2007). Most of chemical carcinogens are inert and require metabolic activation to exert mutagenic and carcinogenic effects. CYP1A1 has been identified as one of the main participating enzymes in the process of metabolic activation of several PAHs to their ultimate carcinogenic forms (Singh et al 2006). In agreement with this postulation, the mutagenicity of 3MC was found to be dependent on CYP1A1 enzyme in the V79 Chinese hamster lung cell line (Ellard et al 1991). Moreover, several studies demonstrated a correlation between the induction of CYP1A1 and the development of different human cancers such as lung, colon and rectal cancers (Oyama et al 2007, Shah et al 2009, Slattery et al 2004).

The fact that several AhR agonists possess toxic and carcinogenic properties suggests the use of AhR antagonists as promising chemopreventative

agents. Indeed, AhR antagonists such as Res and MNF prevented the genotoxic effects in B[a]P-treated mice. Nonetheless, the problem of the currently used AhR antagonists and their use as chemopreventative agents relies mainly on the fact that most of them, such as Res, are not specific to AhR (Signorelli and Ghidoni 2005) or like MNF they act as a partial agonist(Zhou and Gasiewicz 2003). Therefore, the search for new AhR antagonist is still in progress (Puppala et al 2008).

On the other hand, herbal plants have been used for centuries to treat several diseases including cancer. Several drugs, such as vincristine and vinblastine alkaloids, have been in clinical use for treatment of several types of cancers (Cragg and Newman 2005). Moreover, other herbal components, such as sulforaphane, showed great promise as chemopreventative agents (Conaway et al 2005). In addition several flavonoids, such as Res, act as AhR antagonists and prevented the genotoxicity induced by PAHs (Revel et al 2003).

1.7.2. Hypotheses

Based on the literature review we proposed the following hypotheses:

(1) *Peganum harmala* inhibits the carcinogen-activating enzyme CYP1A1 through an AhR-dependent mechanism.

(2) Harman induces the carcinogen-activating enzyme CYP1A1 through an AhR-dependent mechanism.

(3) The active constituents of *Peganum harmala* extract (harmine and harmaline and their metabolites, harmol and harmalol) inhibit CYP1A1 through transcriptional and post-transcriptional mechanisms.

(4) Harmine and harmaline inhibit the carcinogen-activating enzyme Cyp1a1 *in vivo* using C57BL/6 mice.

1.7.3. Objectives

The specific objectives of the studies described here were:

(1) To investigate the effect of *Peganum harmala* on AhR activation and regulation of CYP1A1 gene in human hepatoma HepG2 and murine hepatoma Hepa 1c1c7 cells and to identify the active constituents of the plant extract.

(2) To investigate the effect of harman, a widely distributed *β*-carboline, on CYP1A1 and the AhR-signaling pathway in human HepG2 cells and rat hepatoma H4IIE cells.

(3) To examine the effect of harmine and its metabolite, harmol, on AhR activation as well as CYP1A1 enzyme in human hepatoma HepG2 and murine Hepa 1c1c7 cells.

(4) To investigate the effect of harmaline and its metabolite, harmalol, on AhR activation as well as CYP1A1 in human hepatoma HepG2 cells.

(5) To confirm the obtained effects of harmine and harmaline *in vivo* using a responsive mouse strain (C57BL/6).

The importance and significance of the work derives from its great impact on the development of new drugs from natural products that might have anti-cancer effects. Lead compounds can be further utilized to synthesize different analogues of the active compounds. Furthermore, this research might help in improving treatment strategies of carcinogenicity and toxicity mediated by several AhR ligands. Eventually, this research might help in decreasing cancer mortality rate and hence diminish health care costs.

CHAPTER 2-

MATERIALS AND METHODS

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), CHX, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 7-ethoxyresorufin (7ER), fluorescamine, protease inhibitor cocktail, B[a]P, harmine hydrochloride (>98% pure), Res (99% pure), βNF, 3MC, and rabbit anti-goat IgG secondary antibody were purchased from Sigma-Aldrich (St. Louis, MO). TCDD (>99% pure) was obtained from Cambridge Isotope Laboratories (Woburn, MA). Harman, 98% pure, was obtained from Toronto Research Chemicals Inc. (North York, ON). Harmaline hydrochloride dihydrate (>90% pure) was supplied from ACROS Organics (Morris Plains, NJ). Harmol hydrochloride (99% pure), and harmalol hydrochloride (>98% pure) were obtained from MP Biomedicals (Solon, OH). Penicillin-streptomycin, L-glutamine, fetal bovine serum, TRIzol and Lipofectamine 2000 reagent were obtained from Invitrogen (Carlsbad, CA). Primary anti-mouse/human CYP1A1 antibody, primary goat anti-mouse/human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, primary rabbit anti-mouse actin antibody, goat anti-rabbit IgG peroxidase secondary antibody and goat anti-ARNT antibody were purchased from Santa Cruz (Santa Cruz, CA). Goat anti-mouse/human IgG peroxidase secondary antibody was obtained from R&D systems (Minneapolis, MN). Primary mouse anti-human aryl hydrocarbon receptor antibody was obtained from Abcam Inc. (Cambridge, MA). High-Capacity cDNA Reverse Transcription Kit, SYBR® Green PCR Master Mix, and 96-well optical reaction plates with optical adhesive films were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized

by Integrated DNA Technologies Inc. (San Diego, CA) according to previously published sequences (Table. 2.1.). MG-132 and Act-D were purchased from Calbiochem (San Diego, CA). Acrylamide, N',N'-bis-methylene-acrylamide, ammonium persulphate, glycine, nitrocellulose membranes (0.45 μm), β-mercaptoethanol and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence Western blotting detection reagents were obtained from GE Healthcare Life Sciences (Piscataway, NJ). [γ-^{32}P]-ATP was supplied by Perkin Elmer (Boston, MA). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and [^{3}H]TCDD (13 Ci/mmole) were obtained from Dr. Stephen Safe (Texas A&M University). Luciferase Reporter Assay System was obtained from Promega Corporation (Madison, WI). INTERFERin siRNA transfecting reagent was supplied by Polyplus Transfection (Illkirch, France). Reagents used for liquid chromatographic-electrospray ionization-mass spectrometry (LC-ESI-MS) were at HPLC grade. Water and Acetonitrile (HPLC grade) were obtained from EM Scientific (Gibbstawn, NJ). All other chemicals were purchased from Thermo Fisher Scientific (Toronto, ON, Canada).

2.2. Methods

2.2.1. Cell Model

To test the hypotheses raised in this study, several hepatoma cell lines were used, namely, human hepatoma HepG2, murine hepatoma Hepa 1c1c7, and rat hepatoma H4IIE cells.

HepG2 cells are one of the most widely used human hepatoma cells and are considered a potential useful model for several toxicological studies (Dehn et al 2004). HepG2 cells contain both Phase I (such as cytochrome P450–dependent monooxygenase enzymes, and a functional AhR) and Phase II metabolizing enzymes (such as glucuronic- and sulfate-conjugation enzymes) (Dvorak et al 2008b, Grant et al 1988, Rodriguez-Antona et al 2002, Westerink and Schoonen 2007). Therefore, HepG2 cells are capable of performing the metabolic and biotransformation reactions required for detoxification of several xenobiotics (Dehn et al 2004). Most importantly, HepG2 cells' response to dioxin is similar to that observed in primary human hepatocytes (Silkworth et al 2005). Moreover, they have been used extensively to study the regulation of the AhR signaling pathway as well as detecting new agonists and antagonists of AhR (Abdelrahim et al 2003, Backlund and Ingelman-Sundberg 2004, Beedanagari et al 2009, Ciolino and Yeh 1999, Dvorak et al 2008b, Zhang et al 2003).

On the other hand, due to the species-specificity of several AhR agonists and antagonists, two other hepatoma cells from different species have been used. Murine hepatoma Hepa 1c1c7 cells have been widely used to study mouse AhR and the regulation of the AhR signaling pathway (Hankinson 2005, Henry et al

1999). Furthermore, Hepa 1c1c7 cells have been derived from the high-affinity-AhR-expressing C57BL/6 mouse and have been utilized to detect the effect of several compounds on Cyp1a1 and murine AhR (Dere et al 2006, Nishiumi et al 2008). Additionally, rat hepatoma H4IIE cells have been derived from *Rattus norvegicus* and have been utilized to detect the toxic effects of several toxicants including dioxins (Whyte et al 2004). Furthermore, H4IIE cells have been used to detect the effect of several substances on CYP1A1 and the AhR signaling pathway (Hummerich et al 2004, Mueller et al 2010). Of particular note, HepG2, Hepa 1c1c7, and H4IIE cells have been used to detect the effect of species-specific differences in response to AhR ligands (Dere et al 2011, Mueller et al 2010, Suzuki and Nohara 2007, Zhou et al 2003).

2.2.2. Cell Culture and Treatments

Human hepatoma HepG2 (American Type Culture Collection (ATCC) number: HB-8065), murine hepatoma Hepa 1c1c7 (ATCC number: CRL-2026), and rat hepatoma H4IIE (ATCC number: CRL-1548) cell lines were purchased from ATCC (Manassas, VA). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/mL streptomycin. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified incubator.

Cells were treated in serum-free medium with various concentrations of *Peganum harmala* extract, *β*-carboline compounds or AhR agonists, namely, TCDD, *β*NF, 3MC, and B[a]P as specified under each experiment. The control

used in all experiments consists of cells treated with the same volume of vehicle. Harman, harmine, harmaline, harmol, harmalol, TCDD, Act-D, βNF, 3MC, B[a]P and Res were dissolved in dimethyl sulfoxide (DMSO) whereas CHX was dissolved in sterile distilled water. In all treatments, the DMSO concentration did not exceed 0.05% (v/v).

2.2.3. Plant Material

Peganum harmala L. fruiting tops were collected from Libya, identified by Dr. R. Treki (Faculty of Pharmacy, Al-Fateh University) and a voucher specimen was kept at University of Alberta Herbarium possessing number G1001.

2.2.4. Extraction and Isolation of Plant Extract

Peganum harmala L. fruiting tops containing seeds were obtained in the spring and dried at a temperature $\leq 40^{o}$C in the shade. The dried plant was ground and 13 g of the powdered plant were extracted with 200 mL methanol at room temperature overnight by maceration followed by 2 filtration steps through cotton plug and filter paper. The methanol was then evaporated under reduced pressure at 40^{o}C using a Rotavapor (Buchi-R114, Flawil, Switzerland), and then freeze dried using a benchTop K series freeze dryer (VirTis, NY). The lyophilized extract (9.2 % w/w) obtained was stored at -80^{o}C.

2.2.5. Characterization and Quantification of Active Components of the Extract

Harman, harmine and harmaline were characterized using a Waters Micromass ZQ 4000 spectrometer (Waters, Milford, MA). The qualitative analyses were carried out using the MH^+ ions at 182.9 (harman), 212.9 (harmine) and 214.9 (harmaline). The extract was then analyzed as above and MH^+ ion peaks for both harmine and harmaline were observed.

Harmine and harmaline were quantitatively analyzed in the plant extract using Micromass coupled liquid chromatography (Waters 2795). The mass spectrometer was operated in positive ionization mode (ES^+) with single-ion recording acquisition. The nebulizer gas was obtained from an in-house high-purity nitrogen source. The temperature of the source was set at 150°C, and the voltages of the capillary and the cone were 3.4 kV and 25 V, respectively. Desolvation gas flow was 550 L/min and cone gas flow was at 50 L/min. Desolvation temperature was maintained at 275°C. The samples (10 µL) were separated on a reversed phase C18 column (Agilent, 250 x 3.2 mm) using a linear gradient mobile phase system; 40% acetonitrile and 60% water with 0.2% ammonium hydroxide and 0.0032 mM ammonium formate at a flow rate of 0.25 mL/min. The mobile phase system started at 40% acetonitrile and increased linearly to 60% acetonitrile in 10 min. Standard curves were established for harmine and harmaline and the percentages of each of them in the plant extract were calculated using harman as an internal standard.

2.2.6. Animals

All experimental procedures involving animals were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. Male Hartley guinea pigs weighing 250–300 g and C57BL/6 mice weighing 20-25 g were obtained from Charles River Canada (St. Constant, QC, Canada). All animals were exposed to 12 h of light and 12 h of dark daily and given free access to food and water.

2.2.7. Determination of Cell Viability

The effect of *Peganum harmala* extract or *β*-carboline compounds (harmine, harmaline, harmol, harmalol, and harman) on cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT to formazan crystals as described previously (Mosmann 1983). Briefly, cells (10^4 cells per well) were seeded onto 96-well tissue culture plates until 70-80% confluency. Cells were incubated with increasing concentrations of *Peganum harmala* extract or *β*-carboline compounds in presence and absence of TCDD (1 nM) onto a 96-well cell culture plate at 37°C under a 5% CO_2 humidified incubator, the media were removed and 100 µL of serum-free media containing 1.2 mM of MTT dissolved in phosphate-buffered-saline (PBS), pH 7.4, was added to each well. After 2 h incubation in a CO_2 incubator at 37°C, the media were then decanted off by inverting the plate, and a 100 µL of isopropyl alcohol was added to each well, with shaking for 1 h to dissolve the formazan crystals. The color intensity of the blue formazan solution formed in each well

was measured at wavelength of 550 nm using BIO-TEK Instruments EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT). The percentage of cell viability was calculated relative to control wells designated as 100% viable cells.

2.2.8. RNA Extraction from Cell Cultures

Cells were pre-incubated with increasing concentrations of *Peganum harmala* extract, harmine, harmol, harmaline, or harmalol for 30 min before addition of TCDD (1 nM) for 6 h. Moreover, cells were incubated with either harman (25 μM) for different time points or increasing concentrations of harman (1-50 μM) for 6 h. Thereafter, the total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions (Invitrogen). Briefly, TRIzol reagent (600 μL) was added to each well of the six-well cell culture plates. Thereafter, cell lysate was collected into 1.5-mL Eppendorf tube and mixed with chloroform (120 μL), followed by centrifugation at 12,000 x *g* for 15 min at 4°C. The aqueous upper layer that contains RNA was isolated, transferred to a clean Eppendorf tube, and isopropyl alcohol (300 μL) was added to each tube and the tubes were stored at -20°C overnight to precipitation the RNA. The RNA was collected by centrifugation at 12,000 x *g* for 10 min at 4°C and the pellet was washed by 75% ethanol in diethyl pyrocarbonate (DEPC)-treated water. The resultant RNA pellet was dissolved in DEPC-treated water and quantified by measuring the absorbance at 260 nm.

2.2.9. Reverse Transcription (RT)

RNA quality was determined by measuring the 260/280 ratio and the first strand cDNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems®) according to the manufacturer's instructions. Briefly, 1.0 µg of total RNA from each sample was added to a mix of 2.0 µL of 10X reverse transcriptase buffer, 0.8 µL of 25X dNTP mix (100 mM), 2.0 µL of 10X reverse transcriptase random primers, 1.0 µL of MultiScribe reverse transcriptase, and 4.2 µL of nuclease-free water. The final reaction mix was kept at 25°C for 10 min, heated to 37°C for 120 min, heated for 85°C for 5 s, and finally cooled to 4°C (Zordoky et al 2008).

2.2.10. Quantification of mRNA Expression by Real-time Polymerase Chain Reaction (Real-time PCR)

Real-time PCR reactions were performed using an ABI 7500 system (Applied Biosystems®, Inc. Foster City, CA) as described previously (Zordoky et al 2008). The reaction mixture (25 µL) contained 0.075 µL of 25 µM forward primer and 0.075 µL of 25 µM reverse primer (75 nM final concentration of each primer), 12.5 µL of SYBR Green Universal Master Mix, 11.15 µL of nuclease-free water, and 1.25 µL of cDNA sample. The primers used in the current study were chosen from previously published studies (Anwar-Mohamed and El-Kadi 2009, Schmittgen and Zakrajsek 2000, Zordoky et al 2010) and were purchased from Integrated DNA Technologies (IDT, Coralville, IA) (Table 2.1). Assay controls were incorporated onto the same plate, namely, no-template controls to

test for the contamination of any assay reagents. The thermocycling program was initiated at 95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and anneal/extension at 60°C for 1 min. The PCR product was monitored by fluorescence of SYBR Green dye and the level of expression of each mRNA was normalized to simultaneously assayed reference gene expression. A melting curve (dissociation stage) was performed by the end of each test to ascertain the specificity of the primers and the purity of the final PCR product. The real-time PCR data were analyzed using the relative gene expression (ΔΔCt) method, as described in Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen 2001). Briefly, the data are presented as the fold change in gene expression normalized to the reference gene and were determined using the equation fold change = $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct (*target gene*) − Ct (*reference gene*) and Δ(ΔCt) = ΔCt (treated)− ΔCt (control).

Table 2.1. Primer sequences used for real-time PCR reactions

Gene	Forward Primer	Reverse Primer
Human *CYP1A1*	5′CGGCCCCGGCTCTCT3′	5′CGGAAGGTCTCCAGGATGAA3′
Human *β-Actin*	5′CTGGCACCCAGCACAATG3′	5′GCCGATCCACACGGAGTACT3′
Rat *CYP1A1*	5′CCAAACGAGTTCCGGCCT3′	5′TGCCCAAACCAAAGAGAATGA3′
Rat *β-Actin*	5′CCAGATCATGTTTGAGACCTTCA A3′	5′GTGGTACGACCAGAGGCATACA3′
Murine *Cyp1a1*	5′GGTTAACCATGACCGGGAACT3′	5′TGCCCAAACCAAAGAGAGTGA3′
Murine *β-Actin*	5′TATTGGCAACGAGCGGTTCC3′	5′GGCATAGAGGTCTTTACGGATGTC3′
Murine *18S*	5′GTAACCCGTTGAACCCCATT3′	5′CCATCCAATCGGTAGTAGCG3′

2.2.11. Protein Extraction and Western Blot Analysis

Western blot analysis was performed according to a previously described method (Gharavi and El-Kadi 2005). Cells were incubated with increasing concentrations of *Peganum harmala* extract or harmine, harmol, harmaline, and harmalol for 30 min before addition of TCDD (1 nM) for 24 h. Moreover, cells were incubated with increasing concentrations of harman for 24 h. Thereafter, the cells were washed once with cold PBS and collected by scraping in 100 µL of lysis buffer (50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5 µL/mL of protease inhibitor cocktail). Cell lysates were incubated on ice for 1 h with intermittent vortexing every 10 min. Thereafter, the supernatant containing cell lysate was separated from cellular debris by centrifugation at 12,000 x *g* for 10 min at 4°C and stored at -80°C freezer. The protein content of cell lysate was determined using the Lowry method (Lowry et al 1951). Proteins (50 µg for HepG2, and 25 µg for Hepa 1c1c7) from each treatment group were diluted with the same volume of 2 X loading buffer (0.1M Tris–HCl, pH 6.8, 4% SDS, 1.5% bromophenol blue, 20% glycerol, 5% *β*-mercaptoethanol), boiled and loaded onto 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The protein blots were blocked for 24 h at 4°C in blocking buffer [5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween 20 in Tris-buffered saline solution (0.15 M sodium chloride, 3 mM potassium chloride and 25 mM Tris base)]. Thereafter, the protein blots were incubated with primary CYP1A1 antibody for 2 h at 37°C, or primary goat GAPDH antibody for 24 h at 4°C. Finally, the

membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature, namely, goat IgG peroxidase secondary antibody for CYP1A1, and rabbit anti-goat IgG secondary antibody for GAPDH. The formed bands were visualized with the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The intensity of protein bands was quantified relative to the signals obtained for GAPDH protein using a densitometer (TBX, Tobias associates, Inc., PA).

2.2.12. Determination of CYP1A1 Enzymatic Activity

CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) activity was performed on intact, living cells using 7ER as a substrate. Cells (10^4 cells per well) were seeded onto 96-well microtiter cell culture plates until 70-80% confluency. Increasing concentrations of *Peganum harmala* extract, harmine, harmol, harmaline or harmalol were added to the cells for 30 min before addition of TCDD (1 nM) for 24 h. Moreover, cells were incubated with increasing concentrations of harman for 24 h. Thereafter, cells were washed with PBS and 200 μL of 7ER solution (2 μM) were added to each well. The amount of resorufin formed in each well at each time-point (every 5 min) was detected and the fluorescence measurement for each treatment per minute was determined by comparison with a standard curve of known concentrations (excitation, 545 nm; emission, 575 nm; Baxter 96-well fluorometer) (Kennedy et al 1993). The CYP1A1 enzymatic activity was normalized to cellular protein content using a modified fluorescence method (Lorenzen and Kennedy 1993).

2.2.13. Transfecting HepG2 cells with AhR siRNA

HepG2 cells were plated onto 24-well cell culture plates. Each well of cells was transfected with AhR siRNA (IDT, Coralville, IA) or Silencer Select Negative Control No. 2 siRNA (Applied Biosystems, Foster City, CA) at a concentration of 140 nM using INTERFERin reagent according to the manufacturer's instructions (Polyplus, Illkirch, France). AhR siRNA duplex was 5′-UAC UUC CAC CUC AGU UGG CTT-3′ and 5′-GCC AAC UGA GGU GGA AGU ATT-3′ (Abdelrahim et al 2003). Transfection efficiency was determined using Western blot analysis for AhR protein after 48 h. At 48 h post-transfection, medium was replaced with fresh medium containing harman (25 μM) for an additional 24 h to determine CYP1A1 protein and catalytic activity levels.

2.2.14. Transient Transfection and Luciferase Assay

Cells (10^5 cells per well) were plated onto six-well cell culture plates. Each well was transfected with 4 μg of XRE-driven luciferase reporter plasmid (pGudLuc 6.1. for HepG2 cells, and pGudLuc 1.1. for Hepa 1c1c7 cells). The plasmids were provided as a gift from Dr. Michael S. Denison (University of California, Davis, CA). The transfection procedure was carried out using Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen) and the luciferase assay was performed according to the manufacturer's instructions (Promega) as described previously (Elbekai and El-Kadi 2007). Thereafter, cells were incubated with *Peganum harmala* extract, harmine, harmol, harmaline or harmalol for 30 min before addition of TCDD (1

nM) for 24 h. Moreover, cells were incubated with increasing concentrations of harman for 24 h. The luciferase activity was determined according to manufacturer's instructions (Promega) and reported as relative light unit (RLU).

2.2.15. Electrophoretic Mobility Shift Assay (EMSA)

A guinea pig hepatic cytosol has been selected to study the transformation and activation of AhR using *in vitro* EMSA. Guinea pig hepatic cytosol was found to have the greatest degree of AhR transformation and activation, after treatment with AhR ligands, when compared with other hepatic cytosols obtained from different species including mouse and human (Bohonowych and Denison 2007, Soshilov and Denison 2012). A guinea pig was sacrificed and the liver was excised and placed in a cold HEDG solution (0.6% w/v Hepes, 0.037% w/v EDTA, 1mM dithiothreitol and 10% v/v glycerol). The tissue was homogenized using a Teflon homogenizer and the resultant homogenate was centrifuged at 9,000 x *g* for 20 min at 4°C. The supernatant was ultra-centrifuged at 100,000 x *g* for 60 min at 4°C. Hepatic cytosolic extracts (2 mg) were pre-incubated with *Peganum harmala* extract, harmine, harmol, harmaline or harmalol for 30 min before addition of TCDD (20 nM) for 2 h. Moreover, harman was incubated alone with hepatic cytosolic extracts for 2 h. The ability of the test compound to affect the transformation and DNA binding of the AhR was tested using a synthetic pair of oligonucleotides for the XRE binding site that involve the following sequence 5′-GAT CTG GCT CTT CTC ACG CAA CTC CG-3′ and 5′-GAT CCG GAG TTG CGT GAG AAG AGC CA-3′. These oligonucleotides were post-labelled

with [γ-^{32}P]ATP as described previously (Denison et al 2002). The products of this binding were separated on a 4% polyacrylamide gel. The specificity of binding was confirmed by competition experiments, cytosolic extracts were preincubated at room temperature for 20 min with a 100-fold molar excess of unlabeled XRE or 0.6 μg of anti-ARNT antibody (Santa Cruz Biotechnology, Inc.) before the addition of the labeled XRE. AhR-XRE complexes formed on the gel were visualized by autoradiography.

2.2.16. Competitive Ligand Binding Assay

A hydroxyapatite (HAP) assay was performed to determine the ligand binding ability of harmine, harmol, harmaline, and harmalol as described previously (Denison et al 1986). Briefly, untreated guinea pig and C57BL/6 mouse hepatic cytosols were diluted to 2 mg/mL in MEDG buffer (3-(*N*-morpholino) propanesulfonic acid (25 mM), pH 7.5, ethylenediaminetetraacetic acid (1 mM), dithiothreitol (1 mM), and glycerol (10%, v/v). Several aliquots of guinea pig or mice cytosols (200 μL) were incubated at room temperature for 1 h with [^{3}H]TCDD (2 nM) alone (total binding), [^{3}H]TCDD (2 nM), and TCDF (200 nM, 100-fold excess of competitor, nonspecific binding) or [^{3}H]TCDD (2 nM) in the presence of increasing concentrations of harmine, harmol, harmaline, and harmalol. All chemicals were dissolved in DMSO, in which DMSO content in reactions was adjusted to 2% (v/v) where necessary. Thereafter, HAP suspension (250 μL) was added to the different reaction mixtures and incubated for an additional 30 min with gentle vortexing every 10 min. The reactions were washed

three times with 1 mL of MEGT buffer (3-(*N*-morpholino) propanesulfonic acid (25 mM), pH 7.5, ethylenediaminetetraacetic acid (1 mM), glycerol (10%, v/v), and Tween 80 (0.5%, v/v). The HAP pellets were transferred to 4 mL scintillation vials, scintillation cocktail was added, and reactions were counted in a scintillation counter.

2.2.17. CYP1A1 mRNA Stability

The $t_{1/2}$ of CYP1A1 mRNA was analyzed by the Act-D-chase assay. To determine the effect of harman on the CYP1A1 mRNA $t_{1/2}$ at the constitutive level, cells were treated with Act-D (5 μg/mL), to inhibit further RNA synthesis, immediately before treatment with harman or TCDD (1 nM). Thereafter, total RNA was extracted at 0, 1, 3, 6, and 12 h post-treatment.

To determine the effect of *β*-carboline compounds (harmine, harmol, harmaline, harmalol, and harman) at the inducible level, cells were pre-treated with TCDD (1 nM) for 6 h. Thereafter, cells were washed and incubated with Act-D (5 μg/mL) immediately before treatment with the test compound. Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with the test compound. Real-time PCR reactions were performed as described in the materials and methods section (Zordoky et al 2008). The mRNA $t_{1/2}$ values were determined from semilog plots of mRNA amounts and expressed as percentage of treatment at t = 0, versus time.

2.2.18. CYP1A1 Protein Stability

The $t_{1/2}$ of CYP1A1 protein was analyzed by the CHX-chase assay. HepG2 cells were pretreated with TCDD (1 nM) for 24 h. Cells were then washed three times with serum free media and incubated with CHX (10 μg/mL), to inhibit further protein synthesis, immediately before treatment with *β*-carboline compounds (harmine, harmol, harmaline, harmalol, and harman). Cell homogenates were extracted after incubation with the *β*-carboline compounds at several time points. CYP1A1 protein was measured by Western blotting as described previously (Gharavi and El-Kadi 2005). The protein $t_{1/2}$ values were determined from semilog plots of integrated densities versus time.

2.2.19. Direct Inhibitory Study

To test the direct inhibitory effect of harmine, harmol, harmaline, and harmalol on CYP1A1 enzyme, a method similar to that described for the EROD assay was performed, with slight modifications. Briefly, HepG2 cells were incubated with TCDD (1nM) for 24 h. Thereafter, media were removed, cells were washed three times with PBS, and test compounds in assay buffer (Tris (0.05 M), NaCl (0.1 M), pH 7.8) were added to the cells for 15 min prior to the addition of 7ER (2 μM final concentration) as a substrate for EROD measurement and normalized for cellular protein content.

2.2.20. Mice Treatment and Tissues Isolation

Twenty-four male C57BL/6 mice were divided randomly into four groups (n = 6). The first group served as weight-matched controls and received the same volume of vehicle for the indicated time points. The second group were treated intraperitoneally (i.p.) with TCDD (15 μg/kg) dissolved in corn oil at 0 h. The third and fourth groups were treated either with harmine hydrochloride or harmaline hydrochloride (10 mg/kg, i.p.) dissolved in normal saline with sonication and heating for 30 minutes at 40°C and TCDD (15 μg/kg, i.p.). Harmine hydrochloride and harmaline hydrochloride were administered to mice at -4, 0, and +4 h, while TCDD was treated once at 0 h (Table 2.2.). All animals were sacrificed by cervical dislocation after +14 h from TCDD treatment. Liver and lung tissues were excised, divided in two separate parts; one smaller part was kept for total RNA isolation and the larger part was used for microsomal fraction isolation, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

2.2.21. RNA Isolation and cDNA Synthesis from Tissues

Total RNA was isolated from frozen livers and lungs using TRIzol reagent according to manufacturer's instructions. Briefly, TRIzol reagent (800 μL) was used to homogenize frozen tissues (approximately 0.25 g). The tissue homogenate was collected into a 1.5-mL Eppendorf tube and mixed with chloroform (160 μL), followed by centrifugation at 12,000 x *g* for 15 min at 4°C. The aqueous upper layer that contains RNA was isolated, transferred to a clean Eppendorf tube, and

isopropyl alcohol (400 µL) was added to each tube, and the tubes were stored at -20°C overnight to precipitate the RNA. The RNA was collected by centrifugation at 12,000 x *g* for 10 min at 4°C and the pellet was washed by 75% ethanol in DEPC-treated water. The resultant RNA pellet was dissolved in DEPC-treated water and quantified by measuring the absorbance at 260 nm. Reverse transcription and the first strand cDNA was synthesized using the High-Capacity cDNA reverse transcription kit (Applied Biosystems®) according to the manufacturer's instructions as described under section 2.2.9.

Table 2.2. Representation of different groups of C57BL/6 mice and the dose schedule for each group

	Time (h)			
Treatments groups	**-4**	**0**	**+4**	**+14**
Group 1	Saline	Saline + corn oil	Saline	Sacrifice
Group 2	Saline	Saline + T* (15 µg/kg)	Saline	Sacrifice
Group 3	Harmine (10 mg/kg)	Harmine (10 mg/kg) + T (15 µg/kg)	Harmine (10 mg/kg)	Sacrifice
Group 4	Harmaline (10 mg/kg)	Harmaline (10 mg/kg) + T (15 µg/kg)	Harmaline (10 mg/kg)	Sacrifice

T*: TCDD

2.2.22. Microsomal Incubation and Determination of Cyp1a1 Enzymatic Activity

Microsomes from liver (0.25 mg/mL) or lung (0.15 mg/mL) were suspended in incubation buffer containing 3 mM magnesium chloride hexahydrate dissolved in 0.5 M potassium phosphate buffer pH 7.4 at 37°C in a shaking water bath (100 rpm). 7-Ethoxyresorufin was used as a substrate and its final concentration was 2 µM. A pre-equilibration period of 5 min was performed before initiating the reaction with NADPH (1mM). After an incubation period of 3 min for liver microsomes and 30 min for lung microsomes, the reaction was stopped by adding 1 mL of ice-cold methanol. The reaction was carried out in duplicate using a reaction mixture without NADPH as a blank for each microsome sample. The amount of resorufin in the supernatant of each reaction mixture was determined using the Baxter 96-well fluorescence plate reader using excitation and emission wavelengths of 545 and 575 nm, respectively. Formation of resorufin was linear with incubation time and protein amount. The amount of resorufin was calculated using a standard curve of known resorufin concentrations and the final amount was calculated by subtracting the amount of resorufin formed in each blank from its corresponding reaction mixture. The final enzymatic activities were expressed as picomole of resorufin formed per minute and per milligram of microsomal proteins.

2.2.23. Statistical Analysis

All results are presented as mean ± S.E.M., and statistical differences between treatment groups were determined using one way ANOVA followed by the Student-Newman-Keuls post hoc test using SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

CHAPTER 3 - RESULTS

3.1. Effect of *Peganum harmala* Extract on Dioxin-Mediated Induction of CYP1A1

*Most of the results of this section have been published in "Planta Med. 2010; **76**:671-677.".*

3.1.1. Effect of *P. harmala* Extract on Cell Viability

To examine the effect of the *P. harmala* on cell viability, Hepa 1c1c7 cells were incubated with increasing concentrations of the plant extract for 24 h and the cell viability was determined using MTT assay. Our results showed that incubation of the cells with *P. harmala* extract for 24 h did not significantly affect the cell viability up to 100 µg/mL in the presence or absence of TCDD (1 nM) (Fig. 3.1A). Therefore, we have chosen 1, 10 and 50 µg/mL as safe concentrations for the following experiments.

3.1.2. Effect of *P. harmala* Extract on TCDD-Mediated Induction of Cyp1a1 mRNA Expression in Hepa 1c1c7 Cells

To investigate whether *P. harmala* extract affects the TCDD-mediated induction of Cyp1a1 mRNA, Hepa 1c1c7 cells were incubated with increasing concentrations of *P. harmala* extract in the presence of TCDD (1 nM) for 6 h. Our data showed that TCDD alone significantly increased the gene expression of *Cyp1a1* by 11-fold (Fig. 3.2.). On the other hand, *P. harmala* extract decreased the TCDD-mediated induction of *Cyp1a1* gene expression in a concentration-dependent manner by 5%, 12% and 48% with *P. harmala* concentration of 1, 10

and 50 μg/mL, respectively. The effect was only significant at the highest concentration tested, 50 μg/mL (Fig. 3.2.). The positive control, Res (25 μM) significantly decreased the induced Cyp1a1 mRNA by 19% (Fig. 3.2.).

3.1.3. Effect of *P. harmala* Extract on TCDD-Mediated Induction of Cyp1a1 Protein in Hepa 1c1c7 Cells

Western blot analysis was performed to examine whether the obtained inhibition on TCDD-mediated induction of Cyp1a1 mRNA level was translated to a relevant effect at the protein level. Figure 3.3. shows that *P. harmala* extract caused a concentration-dependent inhibition of the TCDD-mediated induction of Cyp1a1 protein by 9%, 17.5% and 32% when incubated for 24 h with *P. harmala* extract concentrations of 1, 10 and 50 μg/mL, respectively. Similar to the gene expression, the effect was only significant at the highest tested concentration, 50 μg/mL (Fig. 3.3.).

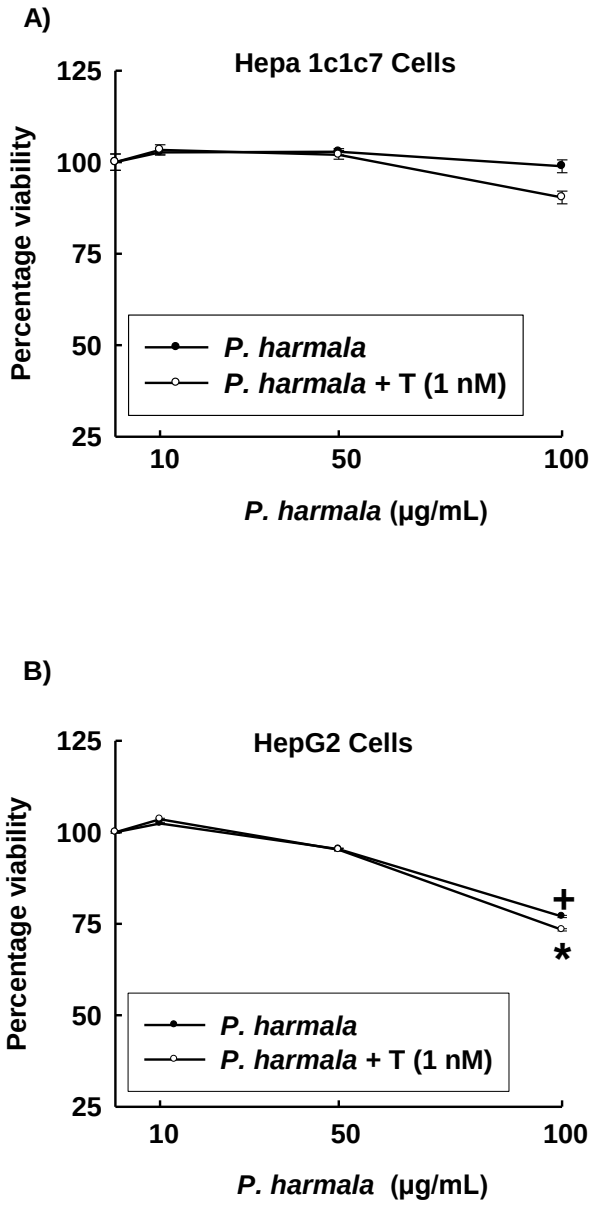


Fig. 3.1. Effect of *P. harmala* extract on murine hepatoma Hepa 1c1c7 (A) and human hepatoma HepG2 (B) cell viability. Viability was tested 24 h after treatment with different concentrations of *P. harmala* extract in the presence and absence of TCDD (T, 1 nM), using the MTT assay. Data were expressed as percentage of control, which was set at 100%, ± S.E.M. (n = 5). (+) $P<0.05$ compared with control, (*) $P<0.05$ compared with T.

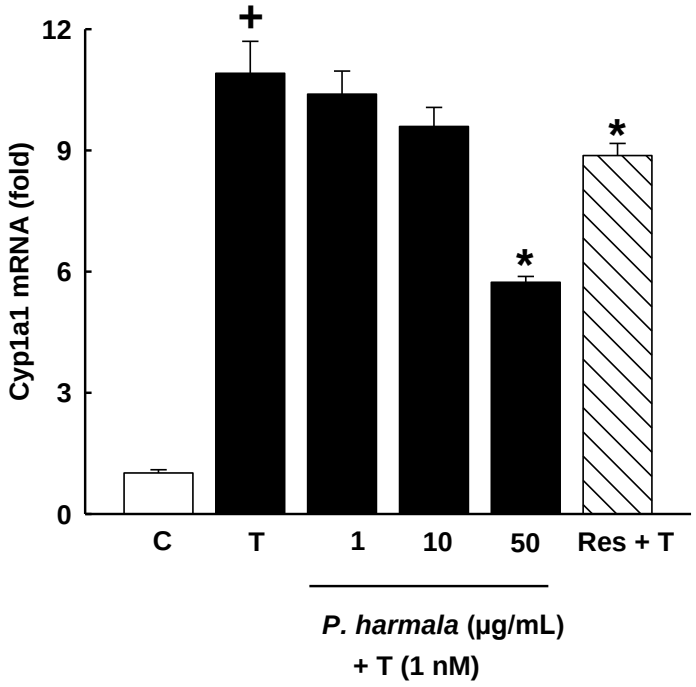


Fig. 3.2. Effect of *P. harmala* extract on TCDD-mediated induction of Cyp1a1 mRNA in Hepa 1c1c7 cells. Cells were incubated with increasing concentrations of *P. harmala* extract (1, 10 and 50 µg/mL) or Res (25 µM) in the presence of TCDD (T, 1 nM**)**. The amount of Cyp1a1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=5). (+) $P<0.05$ compared with control (C), (*) $P<0.05$ compared with T.

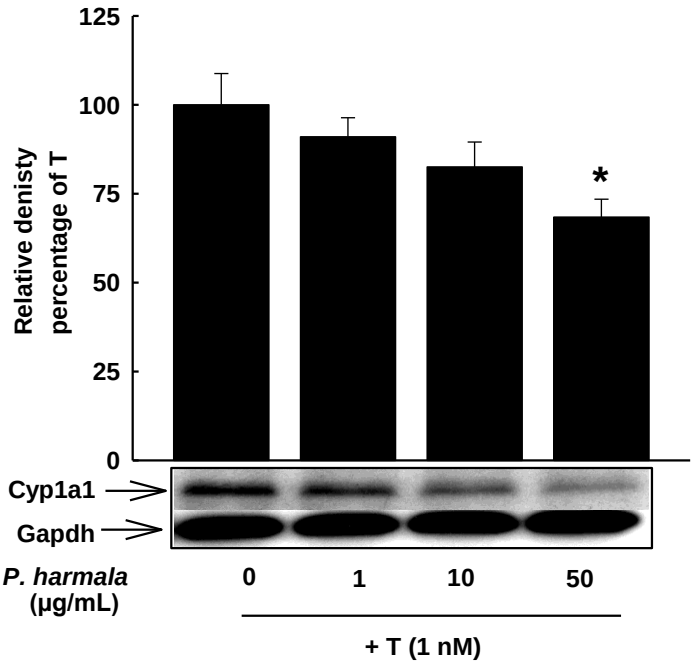


Fig. 3.3. Effect of *P. harmala* extract on TCDD-mediated induction of Cyp1a1 protein in Hepa 1c1c7 cells. Cells were treated with increasing concentrations of *P. harmala* extract (1, 10 and 50 µg/mL) in the presence of TCDD (T, 1 nM) for 24 h. Thereafter, cells were harvested and protein was extracted. Protein was separated on 12.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary Cyp1a1 antibody for 2 h at 4°C, followed by 1 h incubation with secondary antibody at room temperature. Cyp1a1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as a loading control. Graph represents the average optical density (± S.E.M.) of bands from three different experiments as a percentage of T. (*) $P<0.05$ compared with T.

3.1.4. Effect of *P. harmala* Extract on TCDD-Mediated Induction of Cyp1a1 Catalytic Activity in Hepa 1c1c7 Cells

To determine whether the effect of *P. harmala* on Cyp1a1 mRNA and protein is translated to Cyp1a1 catalytic activity, the cells were incubated with increasing concentrations of *P. harmala* extract (1, 10 and 50 μg/mL) in the presence of TCDD (1 nM) for 24 h. Our data showed that *P. harmal*a extract significantly decreased the TCDD-mediated induction of Cyp1a1 catalytic activity by 29%, 64%, and 70% with *P. harmala* extract concentrations of 1, 10, and 50 μg/mL, respectively (Fig. 3.4.). To examine whether or not this inhibitory effect is specific for TCDD, we tested the effect of *P. harmala* extract with different AhR ligands, namely, *β*NF, 3MC and B[a]P. Our results showed that all AhR ligands alone significantly increased Cyp1a1 activity. On the other hand, *P. harmala* extract significantly decreased the induced Cyp1a1 catalytic activity by all AhR ligands tested in a concentration-dependent manner (Fig. 3.4.). The positive control, Res (25 μM), significantly decreased the induced Cyp1a1 catalytic activity by all AhR ligands tested (Fig. 3.4.).

3.1.5. Transcriptional Inhibition of Cyp1a1 Induction by *P. harmala*

To determine the mechanism by which *P. harmala* extract inhibits Cyp1a1, we investigated the role of transcriptional mechanism. First, we examined the potential inhibitory effect of *P. harmala* extract on direct activation and transformation of cytosolic AhR by TCDD and subsequent DNA binding to XRE, the enhancer sequence of Cyp1a1, using EMSA. Figure 3.5. shows that

TCDD alone induced the activation and transformation of AhR and the formation of AhR-ARNT-XRE complex. On the other hand, *P. harmala* extract decreased TCDD-induced AhR-ARNT-XRE binding. The specificity of TCDD-induced AhR binding to XRE was confirmed by a competition assay using a 100-fold molar excess of unlabelled XRE (Fig. 3.5.).

Second, the obtained effect of *P. harmala* extract at the transcriptional level was confirmed by the XRE-dependent luciferase activity in Hepa 1c1c7 cells. The obtained results of luciferase activity were consistent with EMSA results. Figure 3.6. shows that TCDD alone significantly induced the luciferase activity by 4.6-fold compared to control. On the other hand, co-treatment with *P. harmala* extract and TCDD significantly decreased the luciferase activity compared to TCDD alone (Fig. 3.6.).

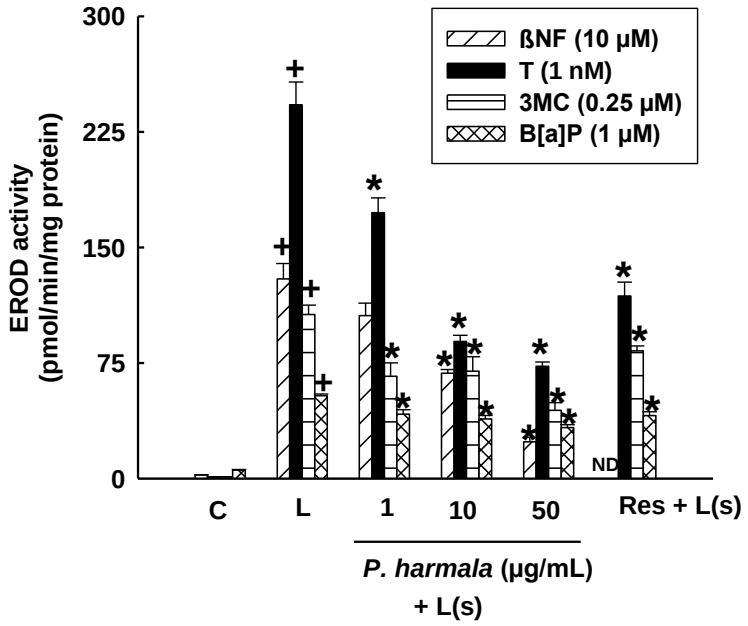


Fig. 3.4. Effect of *P. harmala* extract on induced Cyp1a1 activity by different AhR ligands in Hepa 1c1c7 cells. Cells were incubated with increasing concentrations of *P. harmala* extract (1, 10 and 50 µg/mL) or Res (25 µM) in the presence of TCDD (T, 1 nM), 3MC (0.25 µM), *β*NF (10 µM), or B[a]P (1µM) for 24 h. Cyp1a1 activity was determined using a Cyp1a1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P<0.05$ compared with control (C), (*) $P<0.05$ compared with AhR ligand (L), (ND): not detected.

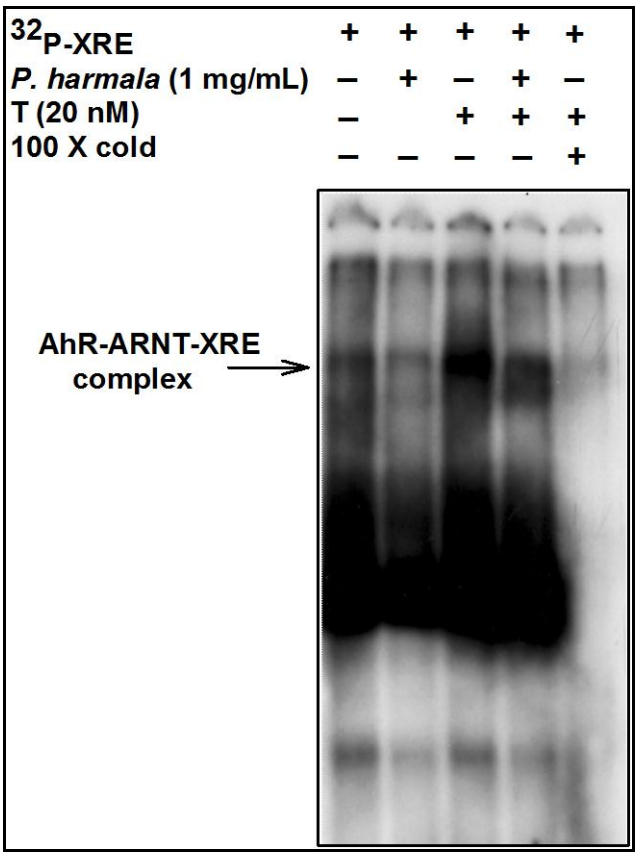


Fig. 3.5. Effect of *P. harmala* extract on AhR activation and transformation. *In vitro* AhR activity was measured by EMSA using guinea pig hepatic cytosolic extracts. Cytosolic extracts were incubated for 30 min with *P. harmala* extract (1 mg/mL) before TCDD (T, 20 nM) addition. The mixtures were further incubated for 2 h, and were tested for binding activity to a [γ-^{32}P]-labelled XRE consensus oligonucleotide for additional 15 min. The products of this binding were separated on a 4% polyacrylamide gel. The specificity of binding was confirmed by incubating the cytosolic extracts treated with TCDD with 100-fold molar excess of cold XRE. AhR-ARNT-XRE complex formed on the gel was visualized by autoradiography. One representative of three experiments is shown.

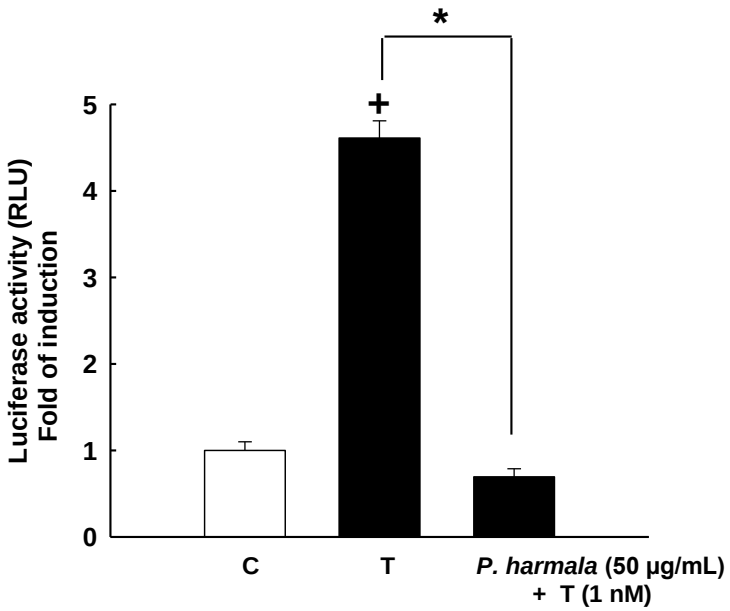


Fig. 3.6. Effect of *P. harmala* extract on XRE-dependent luciferase activity. Hepa 1c1c7 cells were transiently transfected with XRE-luciferase transporter plasmid pGudLuc1.1. Thereafter, cells were treated with *P. harmala* extract (50 µg/mL) for 30 min before the addition of TCDD (T, 1 nM) for 24 h. Cells were lysed and luciferase activity was measured as relative light unit (RLU) and reported as fold induction relative to control (mean ± S.E.M., n = 4). (+) $P<0.05$ compared with control (C), (*) $P<0.05$ compared with T.

3.1.6. Effect of *P. harmala* Extract on Human Hepatoma HepG2 Cell Viability

In order to test whether the effect of the plant extract is specific to murine hepatoma cells, we examined its effect in human hepatoma HepG2 cells. Therefore, we tested the effect of the plant extract on HepG2 cell viability using the MTT assay. Our results demonstrated that the plant extract did not affect HepG2 cell viability up to 50 μg/mL in presence or absence of TCDD (1 nM) (Fig. 3.1B). However, the highest concentration tested, 100 μg/mL, significantly decreased the cell viability by 23%, and 27% in absence and presence of TCDD, respectively (Fig. 3.1B). Therefore, similar to Hepa 1c1c7 cells, we have chosen 1, 10 and 50 μg/mL as safe concentrations for the following experiments.

3.1.7. Effect of *P. harmala* Extract on TCDD-Mediated Induction of CYP1A1 in HepG2 Cells

To test the effect of *P. harmala* extract on CYP1A1 mRNA, HepG2 cells were incubated with increasing concentrations of the plant extract for 30 min before addition of TCDD (1 nM) for 6 h and CYP1A1 mRNA was quantified using real-time PCR. Our results showed that TCDD significantly induced CYP1A1 mRNA by 33-fold (Fig. 3.7A) whereas pre-treatment with *P. harmala* extract significantly reduced TCDD-mediated induction of CYP1A1 mRNA by 44%, and 53% with 10 and 50 μg/mL, respectively (Fig. 3.7A). Res (25 μM) was used as positive control and it decreased the TCDD-mediated induction of CYP1A1 mRNA by 96% (Fig. 3.7A).

To examine whether the effect of the plant extract was translated to a relevant effect at protein and catalytic activity levels, cells were incubated with increasing concentrations of the plant extract for 30 min before addition of TCDD (1 nM) for 24 h and the CYP1A1 protein and catalytic activity were determined using Western blot and 7ER as substrate, respectively. Our data showed that *P. harmala* extract significantly decreased TCDD-mediated induction of CYP1A1 protein by 81% and 94% with 10 and 50 μg/mL, respectively (Fig. 3.7B). Moreover, *P. harmala* extract significantly decreased TCDD-mediated induction of CYP1A1 catalytic activity by 42%, 92%, and 98% with 1, 10, and 50 μg/mL, respectively (Fig. 3.7C). Additionally, Res significantly decreased TCDD-mediated induction of CYP1A1 catalytic activity by 95% (Fig. 3.7C). Taken together, these data demonstrate that *P. harmala* has a potent effect on TCDD-mediated induction of CYP1A1 in human hepatoma HepG2 cells. The effect on CYP1A1 in human hepatoma HepG2 cells was more pronounced than that observed in murine hepatoma Hepa 1c1c7 cells.

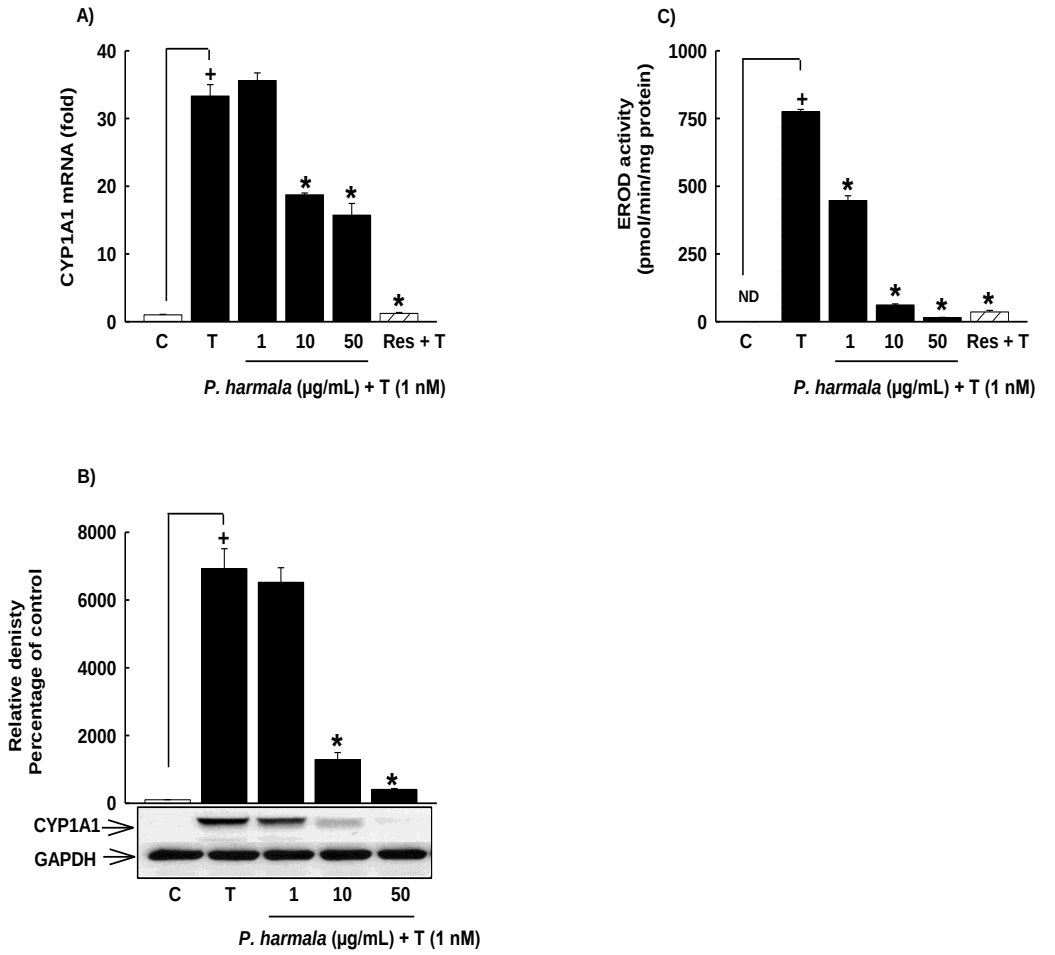


Fig. 3.7. Effect of *P. harmala* extract on CYP1A1 mRNA, protein, and catalytic activity in HepG2 cells. Cells were incubated with increasing concentrations of *P. harmala* extract (1, 10, and 50 µg/mL) or Res (25 µM) 30 min before the addition of TCDD (T, 1nM) for an additional 6 h for mRNA or 24 h for protein and catalytic activity. (A) The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Protein was separated on 10% SDS-PAGE and CYP1A1 protein was determined using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. (C) CYP1A1 catalytic activity was determined using CYP1A1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (ND): not detected.

3.1.8. Characterization and Quantification of the Extract Active Components

Characterization and quantification of the extract active components were carried out using liquid chromatography (LC)-electron spray ionization (ESI)-mass spectrometry (MS) (LC-ESI-MS) technique. Harmine (retention time = 3.4 min) and harmaline (retention time = 6.8 min) were found to be the most abundant ions in the plant extract, corresponding to m/z = 212.9 and 214.9 ion, respectively. Harmine possesses one double bond more than harmaline which allowed their separation through the use of the reversed phase C18 HPLC column and linear gradient mobile phase. Standard curves of authentic standards of harmine and harmaline were established and their amounts in the extract were calculated using harman (m/z = 182.9, retention time = 3.2 min) as an internal standard. It was found that *P. harmala* fruiting tops extract contains 7% and 4.85% (w/w) of harmine and harmaline, respectively.

3.2. Effect of Harman on the Carcinogen-Activating Enzyme CYP1A1 and the AhR Signaling Pathway

*The results of this section have been published in "Toxicol Appl Pharmacol. 2010; **249**:55-64.".*

3.2.1. Effect of Harman on HepG2 Cell Viability

To determine the optimal concentrations to use in our studies, harman was tested for potential cytotoxicity in HepG2 cells using the MTT assay. Our results showed that incubation of HepG2 cells with increasing concentrations of harman (0-100 μM) for 24 h did not significantly affect the cell viability up to 50 μM (Fig. 3.8). However, the highest concentration of harman (100 μM) significantly decreased HepG2 cell viability to 80% (Fig. 3.8.). Therefore, we have chosen concentrations between 1-50 μM as safe concentrations for the following experiments.

3.2.2. Time- and Concentration-Dependent Effect of Harman on CYP1A1 mRNA in HepG2 Cells

To investigate whether harman can affect the CYP1A1 mRNA, cells were incubated with either harman (25 μM) for different time points (Fig. 3.9A) or with increasing concentrations of harman (1-50 μM) for 6 h (Fig. 3.9B). The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Our data showed that harman significantly increased the gene expression of *CYP1A1* in a time-dependent manner. The onset of induction was as

early as 1 h after the addition of harman and remained elevated for at least 6 h after harman treatment (Fig 3. 9A). In addition, harman significantly increased *CYP1A1* gene expression in a concentration-dependent manner by 13-, 72-, 130- and 225-fold with harman concentrations of 1, 10, 25 and 50 μM, respectively (Fig. 3. 9B). The positive control, TCDD (1nM), significantly increased CYP1A1 mRNA by 495-fold (Fig. 3. 9B).

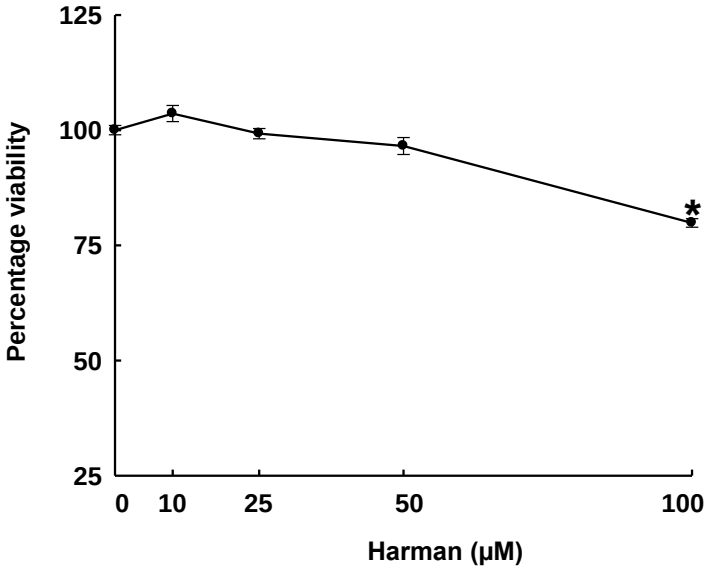


Fig. 3.8. Effect of harman on HepG2 cell viability. Cell viability was tested 24 h after treatment with increasing concentrations of harman (1-100 μM) using the MTT assay. Data were expressed as percentage of control, which was set at 100%, ± S.E.M. (n = 5). (*) $P < 0.05$ compared with control.

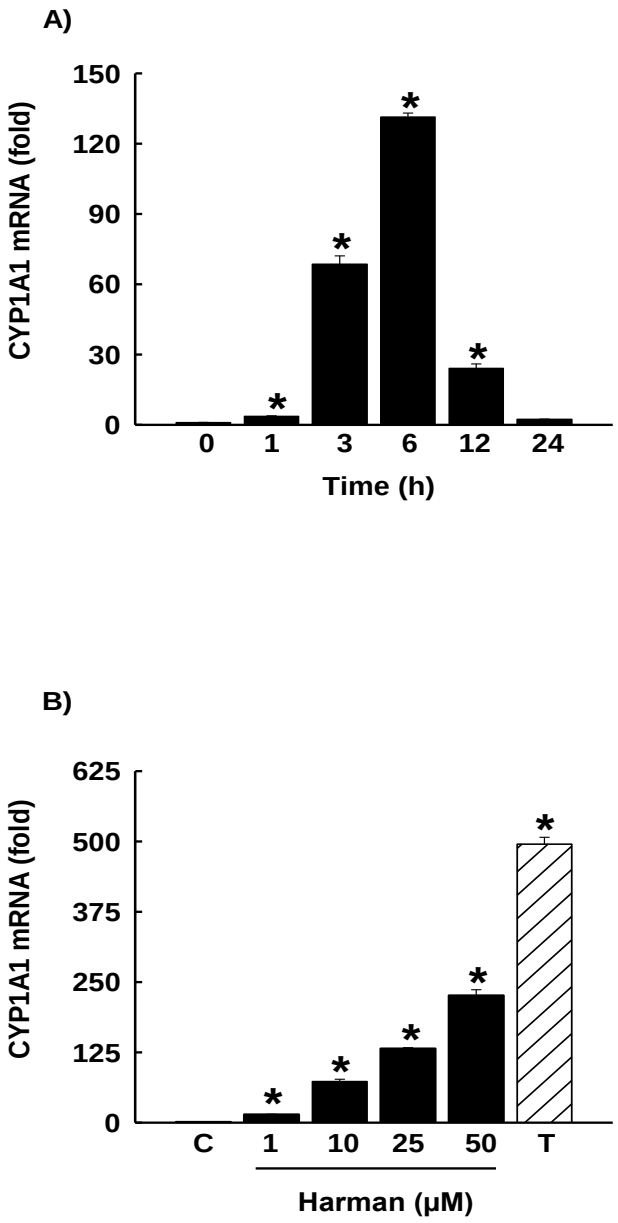


Fig. 3.9. Effect of harman on CYP1A1 mRNA in HepG2 cells. Cells were incubated with either harman (25 μM) for different time points (A) or increasing concentrations of harman (1-50 μM) or TCDD (T, 1 nM) as a positive control for 6 h (B). The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (*) $P < 0.05$ compared with control (C).

3.2.3. Effect of Harman on CYP1A1 Protein and Catalytic Activity Levels in HepG2 Cells

Western blot analysis was carried out to examine whether the obtained induction on CYP1A1 mRNA level was translated to a relevant effect at the protein level. Figure 3.10A shows that harman caused a significant concentration-dependent induction of the CYP1A1 protein by 9.7-, 18- and 48-fold when incubated for 24 h with harman concentrations of 10, 25 and 50 μM, respectively (Fig. 3.10A). Moreover, the positive control, TCDD (1nM), significantly increased CYP1A1 protein by 160-fold (Fig. 3.10A).

To determine whether the effect of harman on CYP1A1 mRNA and protein is translated to CYP1A1 catalytic activity, the cells were incubated with increasing concentrations of harman (1-50 μM) for 24 h and CYP1A1 catalytic activity was determined using the EROD assay. Our results showed that harman induced CYP1A1 catalytic activity in a concentration-dependent manner by 4.4-, 20.3- and 33.8-fold with harman concentrations of 10, 25 and 50 μM, respectively (Fig. 3.10B). Moreover, the positive control, TCDD, significantly induced CYP1A1 catalytic activity by 770-fold (Fig. 3.10B).

To examine the role of AhR in the induction of CYP1A1 activity by harman, HepG2 cells were pre-incubated with the AhR antagonist Res (20 μM) for 2 h before the treatment with harman or TCDD for an additional 24 h. Our results showed that Res significantly reduced the induction of CYP1A1 catalytic activity mediated either by harman or the positive control TCDD (Fig. 3.10B).

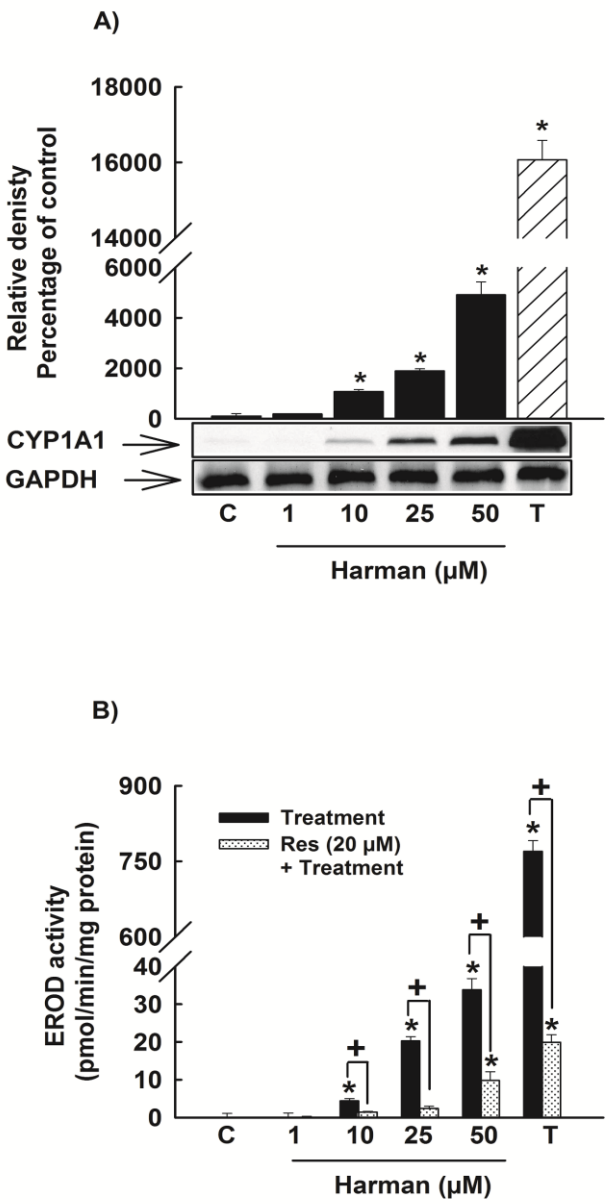


Fig. 3.10. Effect of harman on CYP1A1 protein (A) and catalytic activity (B) in HepG2 cells. Cells were treated with increasing concentrations of harman (1-50 μM) or TCDD (T, 1nM) as a positive control for 24 h. (A) Cells were then harvested and CYP1A1 protein was determined using Western blot analysis. The graph represents the average optical density (± S.E.M.) of bands from three different experiments as a percentage of control. (B) CYP1A1 activity was determined using CYP1A1-dependent EROD assay. To investigate the role of AhR in the induction of CYP1A1 by harman, the cells were incubated with Res (20 μM) for 2 h prior to the treatment with harman for an additional 24 h. Values represent mean activity ± S.E.M. (n = 8). (*) $P < 0.05$ compared with control (C), (+) $P < 0.05$ compared with the treatment.

3.2.4. Effect of AhR siRNA on Harman-Mediated Induction of CYP1A1 Protein and Catalytic Activity Levels

We took a genetic approach to confirm the role of AhR in the harman-mediated induction of CYP1A1. For this purpose, HepG2 cells were transfected with human AhR siRNA or silencer negative control siRNA for 48 h, and then the medium was replaced with fresh medium containing harman (25 μM) for an additional 24 h. Our results showed that AhR siRNA significantly decreased AhR protein by 78% compared to control (Fig. 3.11A). On the other hand, harman (25 μM) was able to increase CYP1A1 protein and activity levels by 16- and 30-fold, respectively (Fig. 3.11B & 3.11C). When the cells were transfected with AhR siRNA and then treated with harman (25 μM) there was a statistically significant decrease in CYP1A1 protein and activity levels by 75% and 80%, respectively (Fig. 3.11B & 3.11C). Moreover, the specificity of transfection was confirmed by the use of silencer negative control that did not affect the induction level caused by harman either in protein or catalytic activity levels (Fig. 3.11B & 3.11C).

3.2.5. Effect of Harman on CYP1A1 mRNA and Catalytic Activity Levels in Rat H4IIE Cells

To test whether the induction of CYP1A1 by harman is species specific, we tested the effect of harman on CYP1A1 at mRNA and activity levels in rat hepatoma H4IIE cells. Cells were incubated with harman (25 μM) for 6 h and the amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to β-actin reference gene. Our data showed that harman (25 μM) and TCDD

(1nM) significantly induced CYP1A1 mRNA by 79- and 424.6-fold, respectively (Fig. 3.12A). A similar effect was obtained with CYP1A1 activity when the cells were incubated with harman for 24 h. Harman (25 μM) and TCDD (1 nM) significantly induced CYP1A1 activity by 9- and 162-fold, respectively (Fig. 3.12B).

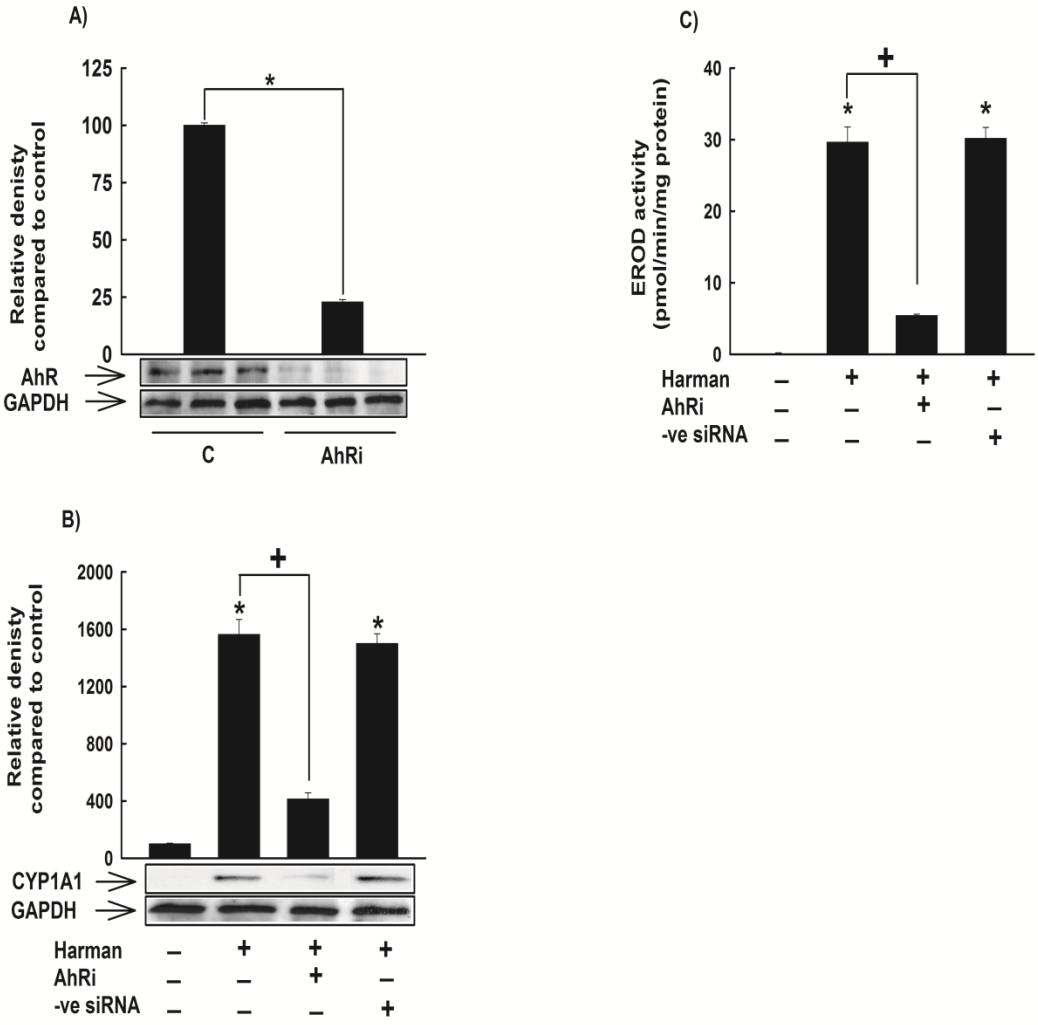


Fig. 3.11. Effect of AhR siRNA on harman-mediated induction of CYP1A1 protein and catalytic activity. HepG2 cells were transiently transfected with AhR siRNA (AhRi, 140 nM) or silencer select negative control siRNA (- ve siRNA, 140 nM) for 48 h. (A) Protein (50 µg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary AhR antibody for 24 h at 4°C, followed by 3 h incubation with secondary antibody at room temperature. AhR protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to GAPDH signal, which was used as loading control (n=3). (B and C) Forty-eight h post-transfection, medium was replaced with fresh medium containing harman (25 µM) for an additional 24 h to determine CYP1A1 protein (B) and catalytic activity levels (C) as described under Materials and Methods section (n=3). (*) $P < 0.05$ compared with control (C); (+) $P < 0.05$ compared with respective harman treatment.

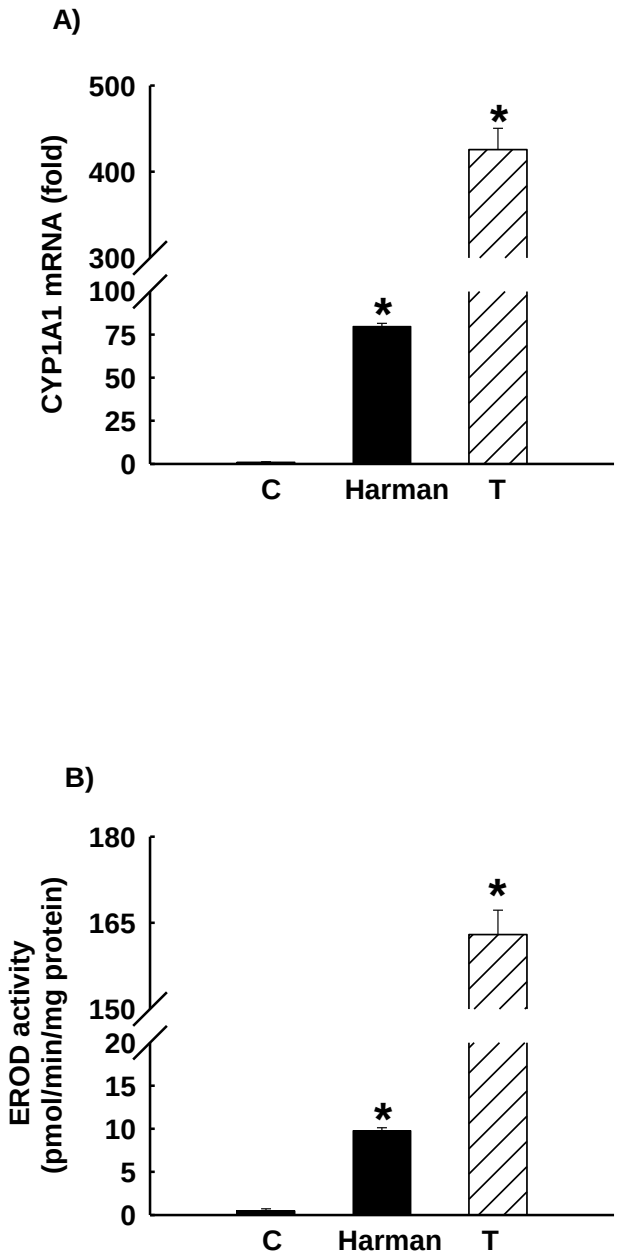


Fig. 3.12. Effect of harman on CYP1A1 mRNA (A) and catalytic activity (B) in rat hepatoma H4IIE cells. (A) Cells were incubated with harman (25 μM) or TCDD (T, 1 nM) for 6 h, and the amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Cells were incubated with harman (25 μM) or TCDD (T, 1 nM) for 24 h and CYP1A1 activity was determined using CYP1A1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (*) $P < 0.05$ compared with control (C).

3.2.6. Transcriptional Induction of CYP1A1 Gene by Harman

To investigate the mechanism involved in the induction of CYP1A1 mRNA by harman, HepG2 cells were treated with the transcription inhibitor, Act-D, prior to the treatment with harman. If the obtained effect of harman was through stabilization of CYP1A1 mRNA, there will be no effect of Act-D on the induced level of CYP1A1 mRNA caused by harman. Figure 3.13A shows that treatment of the cells with Act-D completely abolished the obtained induction of CYP1A1 mRNA level in response to harman. These results indicate the requirement of *de novo* RNA synthesis for harman-mediated induction of CYP1A1 and suggest the involvement of a transcriptional mechanism.

In an attempt to explore the effect of harman on the AhR-dependent transcriptional activation, HepG2 cells were transiently transfected with the XRE-driven luciferase reporter gene. Our results showed that harman significantly induced the luciferase activity in a concentration-dependent manner by 9.4-, 18.1- and 33.6-fold, compared to the control, with harman concentrations of 10, 25 and 50 μM, respectively (Fig. 3.13B). Moreover, TCDD (1 nM) caused a significant induction of luciferase activity by 310-fold compared to the control (Fig. 3.13B).

In order to test the ability of harman to directly activate the cytosolic AhR and subsequent DNA binding, EMSA was performed on untreated guinea pig hepatic cytosol incubated either with vehicle, harman or the positive control, TCDD, for 2 h. Figure 3.13C shows that both harman (1 mM) and the positive control, TCDD (20 nM), induced the activation of AhR and the formation of AhR-ARNT-XRE complex. The specificity of the binding was confirmed by

competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabelled XRE (Fig. 3.13C).

3.2.7. Post-Transcriptional Modification of CYP1A1 mRNA by Harman

The level of mRNA expression is a function of both the transcription rate and the elimination rate through processing or degradation. Therefore, we tested the effect of harman on the stability of CYP1A1 mRNA transcripts, using the Act-D-chase experiment. If harman stabilizes CYP1A1 mRNA, an increase in the $t_{1/2}$ would be expected. Our results showed that neither harman (25 μM) nor TCDD (1 nM) significantly affected the stability of CYP1A1 mRNA at the constitutive level, where the vehicle-treated cells showed a CYP1A1 mRNA with a $t_{1/2}$ of 4.47 ± 0.4 h (Fig. 3.14A). On the other hand, the $t_{1/2}$ of CYP1A1 mRNA for harman- and TCDD-treated cells was 4.83 ± 0.1 h and 5.22 ± 0.2 h, respectively (Fig. 3.14A). At the inducible level, treatment of the cells with TCDD alone for 6 h resulted in a CYP1A1 mRNA with a $t_{1/2}$ of 4.45 ± 0.45 h (Fig. 3.14B). Furthermore, harman did not alter the $t_{1/2}$ of the TCDD-induced CYP1A1 mRNA, which was 4.99 ± 0.35 h (Fig. 3.14B).

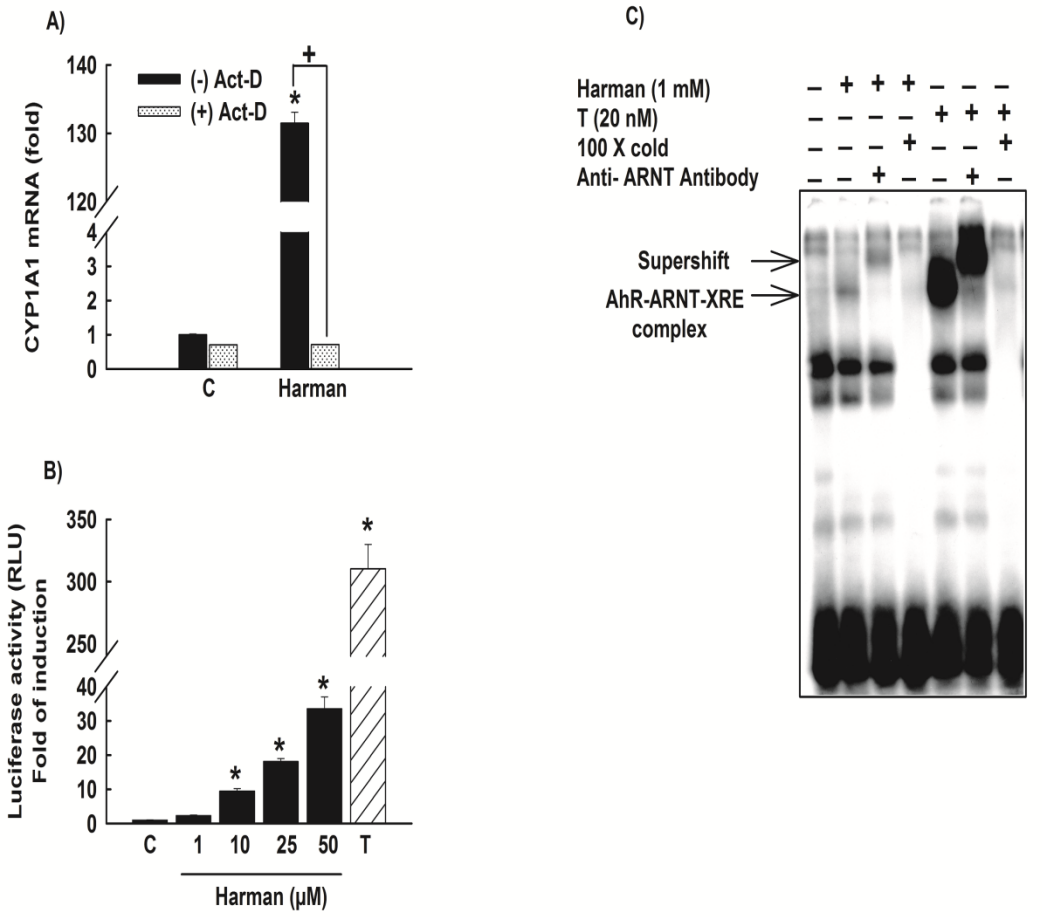


Fig. 3.13. Transcriptional regulation of CYP1A1 by harman. (A) HepG2 cells were incubated with the transcription inhibitor Act-D (5 μg/mL) for 30 min before exposure to harman (25 μM). The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) HepG2 cells were transiently transfected with XRE-luciferase reporter plasmid pGudLuc 6.1. Thereafter, the cells were treated with increasing concentrations of harman (1-50 μM) or TCDD (T, 1nM) for 24 h. Cells were lysed and luciferase activity was reported as fold of relative light unit (RLU) (mean ± S.E.M., n = 4). (C) *In vitro* AhR activity was measured by EMSA using guinea pig hepatic cytosolic extracts. Cytosolic extracts were incubated for 2 h either with DMSO, harman (1 mM), or T (20 nM). The mixtures were tested for binding activity to a [γ-^{32}P]-labelled XRE consensus oligonucleotide for additional 15 min. The products of this binding were separated on a 4% polyacrylamide gel. The specificity of the binding was confirmed by competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabelled XRE. AhR-ARNT-XRE complex formed on the gel was visualized by autoradiography. One representative of three experiments is shown. (*) $P < 0.05$ compared with control (C), (+) $P < 0.05$ compared with harman (25 μM).

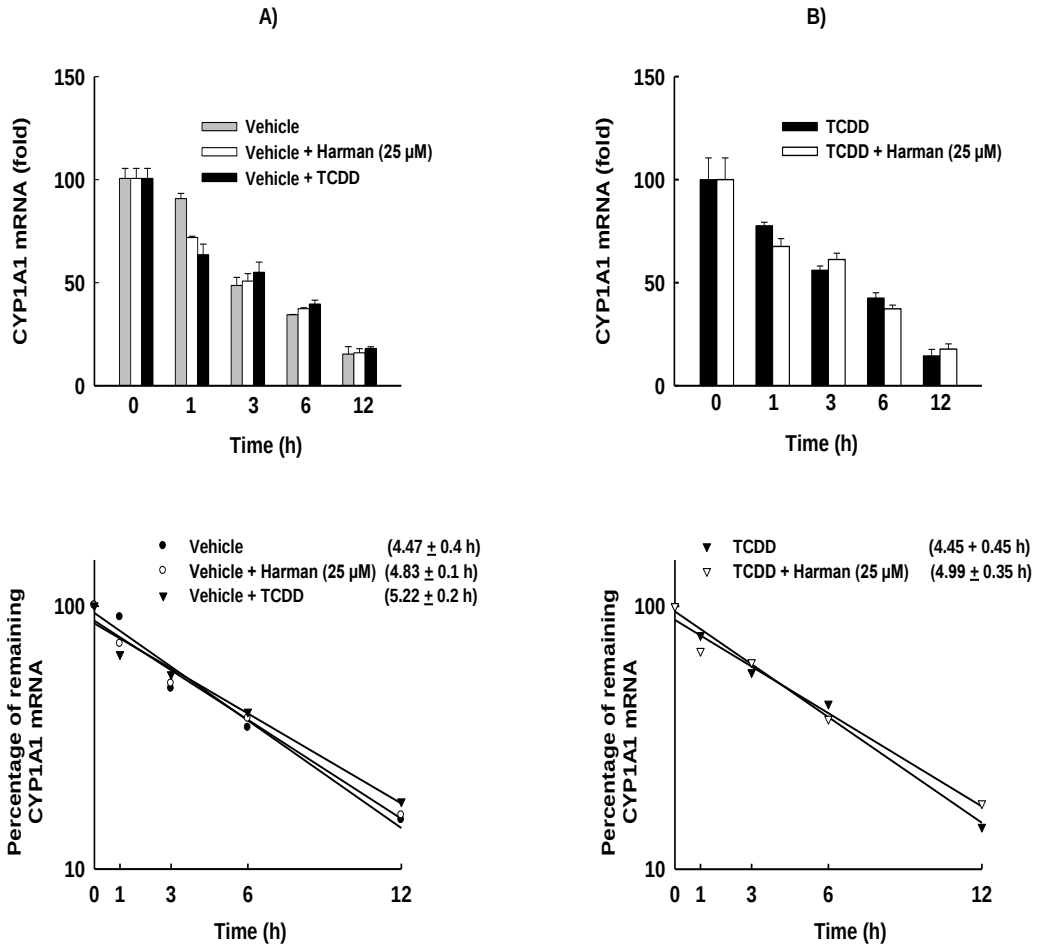


Fig. 3.14. Effect of harman on CYP1A1 mRNA stability using real-time PCR. (A) At the constitutive level, HepG2 cells were treated with either harman (25 μM), or TCDD (T, 1 nM) just after treatment with Act-D (5 μg/mL) to inhibit further RNA synthesis. (B) At the inducible level, HepG2 cells were induced with TCDD (1 nM) for 6 h; thereafter, Act-D (5 μg/mL) was added immediately before treatment with harman (25 μM). Total RNA was extracted at 0, 1, 3, 6 and 12 h after incubation with treatments and subjected to real-time PCR. mRNA decay curves were analysed individually, and the $t_{1/2}$ was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of mRNA amount, expressed as a percentage of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean $t_{1/2}$ (mean ± S.E.M., n = 3). Each of the slopes was significant at $P < 0.05$.

3.2.8. Post-Translational Modification of CYP1A1 Protein by Harman

Harman induced CYP1A1 at the protein level, which raises the question whether this effect might be due to the increase of its translation or the stabilization of CYP1A1 protein. Therefore, we tested the effect of harman on CYP1A1 protein stability using CHX-chase experiments. Figure 3.15. shows that CYP1A1 protein induced by TCDD degraded with a $t_{1/2}$ of 6.78 ± 0.41 h. Harman did not alter the stability of TCDD-induced CYP1A1 protein which degraded with a $t_{1/2}$ of 7.14 ± 0.11 h (Fig. 3.15.). Furthermore, treatment of cells with harman for 24 h caused an induction of CYP1A1 protein that was degraded with a $t_{1/2}$ of 7.22 ± 0.29 h, which is not statistically different from the $t_{1/2}$ of the TCDD-induced CYP1A1 protein (Fig. 3.15.).

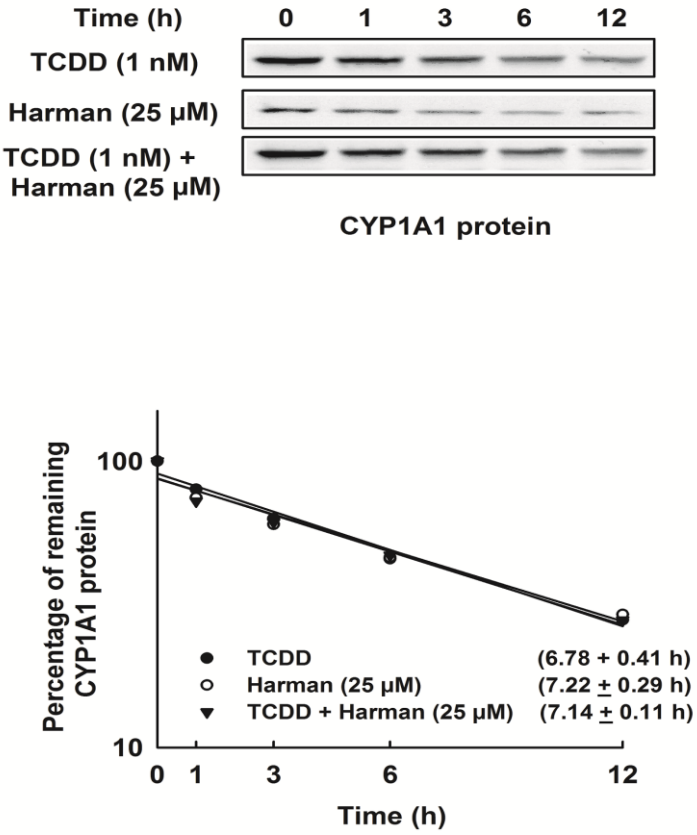


Fig. 3.15. Effect of harman on the CYP1A1 protein stability using Western blot analysis. HepG2 cells were treated with TCDD (T, 1 nM) for 24 h. Thereafter, the cells were washed and incubated in fresh media containing harman (25 μM) plus the protein translation inhibitor, CHX (10 μg/mL). Moreover, to determine the $t_{1/2}$ of CYP1A1 protein induced by harman, cells were treated with harman (25 μM) for 24 h immediately prior to treatment with CHX (10 μg/mL). CYP1A1 protein was determined by Western blot analysis as described in the Materials and Methods section. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls (data not shown). All protein decay curves were analysed individually. The $t_{1/2}$ was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot of protein amount, expressed as a percentage of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean $t_{1/2}$ (mean ± S.E.M., n = 3). Each of the slopes was significant at $P < 0.05$.

3.3. Effect of Harmine and Harmol on Dioxin-Mediated Induction of CYP1A1 and AhR Signaling Pathway

*Most of the results of this section have been published in "Toxicol Lett. 2012; **208**:51-61.".*

3.3.1. Effect of Harmine and Harmol on Cell Viability

To determine the nontoxic concentrations, harmine and harmol were tested for their potential cytotoxicity in HepG2 and Hepa 1c1c7 cells. Our results showed that harmine and harmol did not affect HepG2 cell viability up to 12.5 µM (Fig. 3.16A & 3.16B). However, the highest concentration of harmine (25 µM) significantly decreased HepG2 cell viability to 60% in presence or absence of TCDD (Fig. 3.16A). Moreover, harmol (25 µM) significantly decreased HepG2 cell viability in presence of TCDD to 85% (Fig. 3.16B). Similarly, incubation of Hepa 1c1c7 cells with increasing concentrations of harmine or harmol for 24 h did not significantly affect the cell viability up to 12.5 µM (Fig. 3.16C & 3.16D). However, harmine (25 µM) significantly decreased Hepa 1c1c7 cell viability in presence of TCDD to 83% (Fig. 3.16C). Therefore, we have chosen the concentrations between 0.5-12.5 µM for both harmine and harmol as safe concentrations for the following experiments.

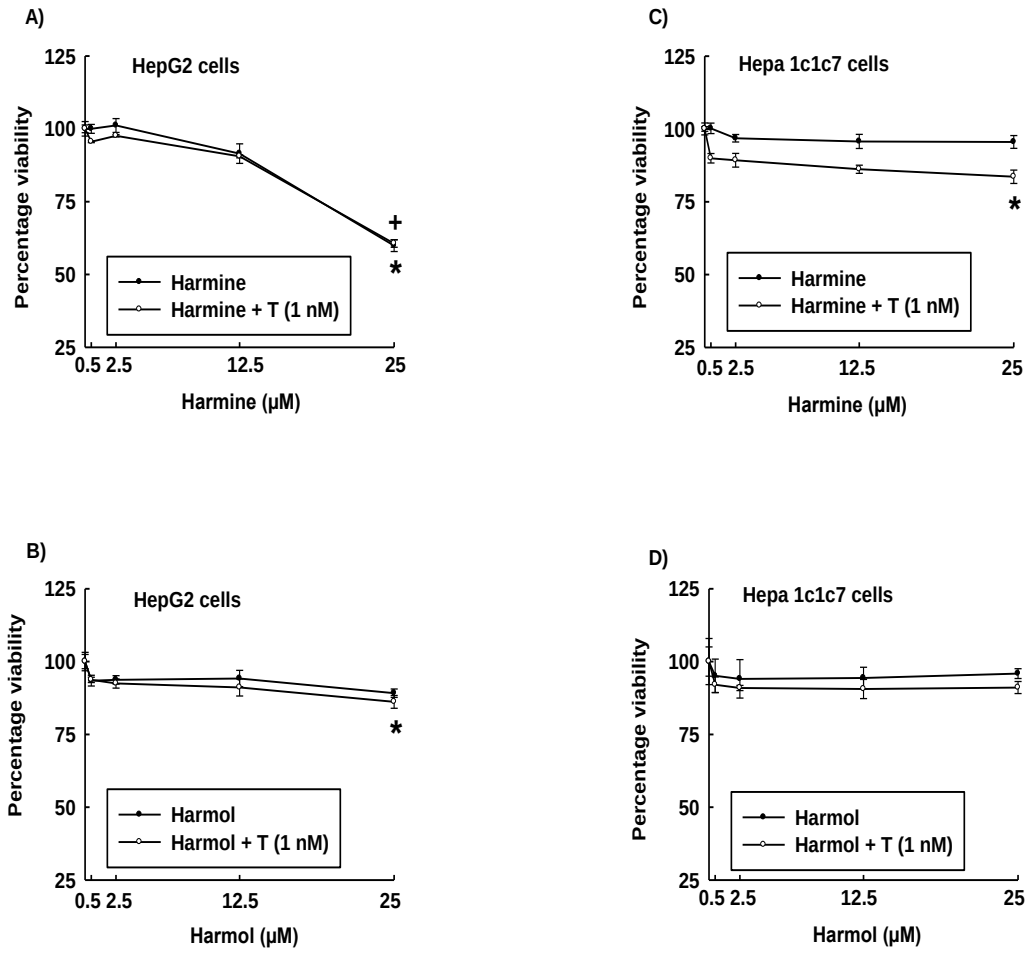


Fig. 3.16. Effect of harmine and harmol on HepG2 and Hepa 1c1c7 cell viability. The effect of increasing concentrations of harmine and harmol (0-25 μM) on HepG2 cell viability (A & B) and Hepa 1c1c7 cell viability (C & D) was tested using the MTT assay. Data were expressed as percentage of control, which was set at 100%, ± S.E.M. (n = 5). (+) $P < 0.05$ compared with control, (*) $P < 0.05$ compared with TCDD (T).

3.3.2. Effect of Harmine and Harmol on Dioxin-Mediated Induction of CYP1A1 mRNA, Protein, and Catalytic Activity Levels in HepG2 Cells

To investigate whether harmine alters the CYP1A1 mRNA level, HepG2 cells were pre-incubated with increasing concentrations of harmine (0.5-12.5 μM) for 30 min before the addition of TCDD (1 nM) for 6 h. Thereafter, CYP1A1 mRNA was quantified using real-time PCR. Our data showed that harmine significantly decreased the TCDD-mediated induction of CYP1A1 mRNA in a concentration-dependent manner by 27%, 64%, and 88% with harmine concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.17A).

Western blot analysis was employed to determine the effect of harmine on the expression of CYP1A1 at the protein level. Consistent with the mRNA results, harmine showed a significant concentration-dependent decrease in TCDD-mediated induction of CYP1A1 protein by 32%, 68%, and 90% with harmine concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.17B). To determine whether harmine has a similar effect on the CYP1A1 catalytic activity, HepG2 cells were incubated with increasing concentrations of harmine (0.5-12.5 μM) 30 min before the addition of TCDD (1 nM) for 24 h. Thereafter, CYP1A1 catalytic activity was determined using the EROD assay. Our results showed that harmine significantly decreased the TCDD-mediated induction of the CYP1A1 catalytic activity by 64%, 88%, and 95% with harmine concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.17C). To determine whether the effect of harmine is AhR ligand specific, we tested the effect of harmine on two other AhR ligands, namely, 3MC (0.25 μM) and *β*NF (10 μM). Our results showed that harmine significantly

decreased the induction of CYP1A1 by 41% and 66% in the presence of *β*NF and 25% and 41% in the presence of 3MC, with harmine concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.17C). The order of inhibition of harmine against different AhR ligands was TCDD > *β*NF > 3MC (Fig. 3.17C).

To investigate whether the effect of harmine is not due to its active metabolite, we examined the effect of its metabolite, harmol, on the CYP1A1 mRNA level in human HepG2 cells. Our results showed that harmol significantly decreased the TCDD-mediated induction of CYP1A1 mRNA in HepG2 cells in a concentration-dependent manner by 23% and 56% with harmol concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.18A).

Similarly, harmol significantly decreased the TCDD-mediated induction of CYP1A1 at the protein level by 25% and 45% with harmol concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.18B). At the CYP1A1 catalytic activity level, harmol decreased all AhR ligand-mediated induction of CYP1A1 catalytic activity in HepG2 cells. Harmol showed a higher activity against TCDD, where it significantly decreased the induction level of CYP1A1 catalytic activity by 75%, 89%, and 97% with harmol concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.18C). Moreover, harmol significantly decreased the induction of CYP1A1 catalytic activity by 41% and 91% in the presence of *β*NF and by 12% and 66% in the presence of 3MC, with harmol concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.18C). The order of inhibition of harmol against different AhR ligands was similar to harmine: TCDD > *β*NF > 3MC (Fig. 3.18C). Furthermore,

neither harmine nor harmol alone significantly affected CYP1A1 at the catalytic activity level in HepG2 cells (data not shown).

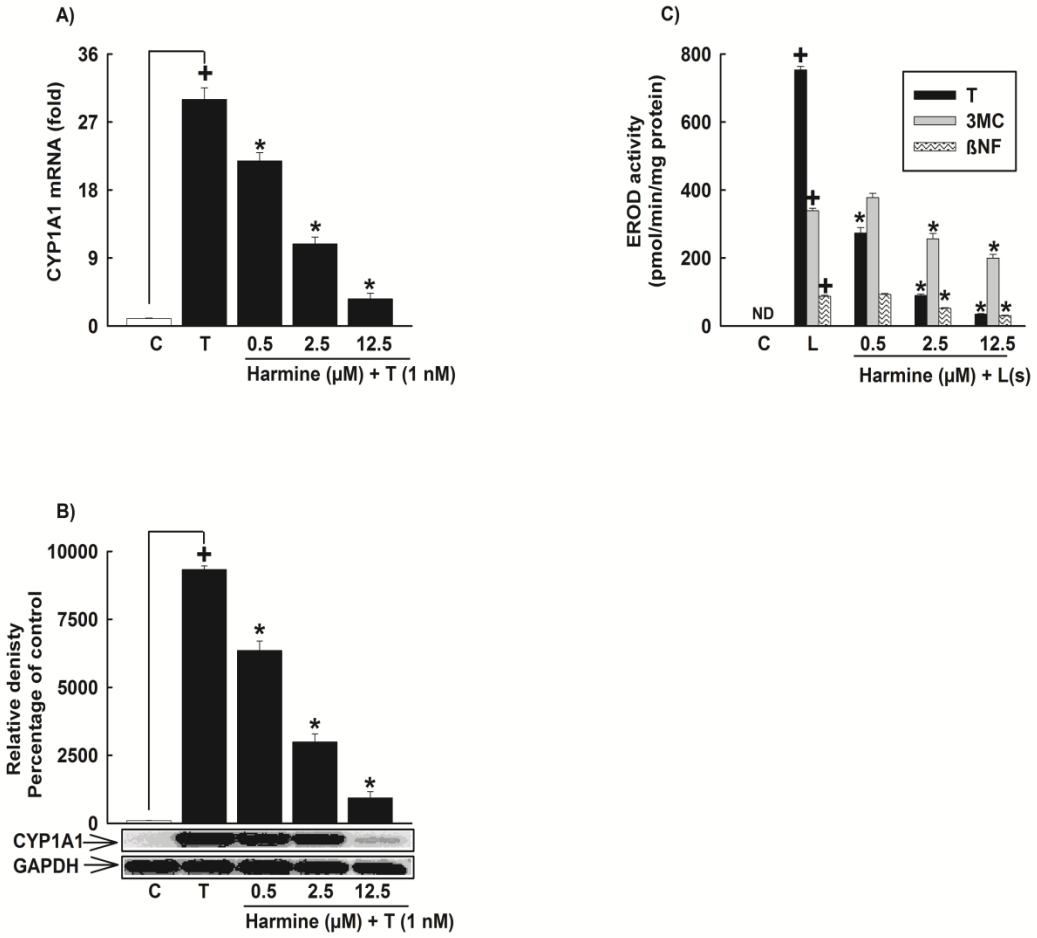


Fig. 3.17. Effect of harmine on CYP1A1 mRNA, protein, and catalytic activity in HepG2 cells. Cells were incubated with increasing concentrations of harmine (0.5-12.5 μM) 30 min before the addition of TCDD (T, 1nM) and for an additional 6 h for mRNA or 24 h for protein and catalytic activity. (A) The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to β-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Protein was separated on 10% SDS-PAGE and CYP1A1 protein was determined using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. (C) CYP1A1 activity was determined using CYP1A1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (L); ligand. (ND): not detected.

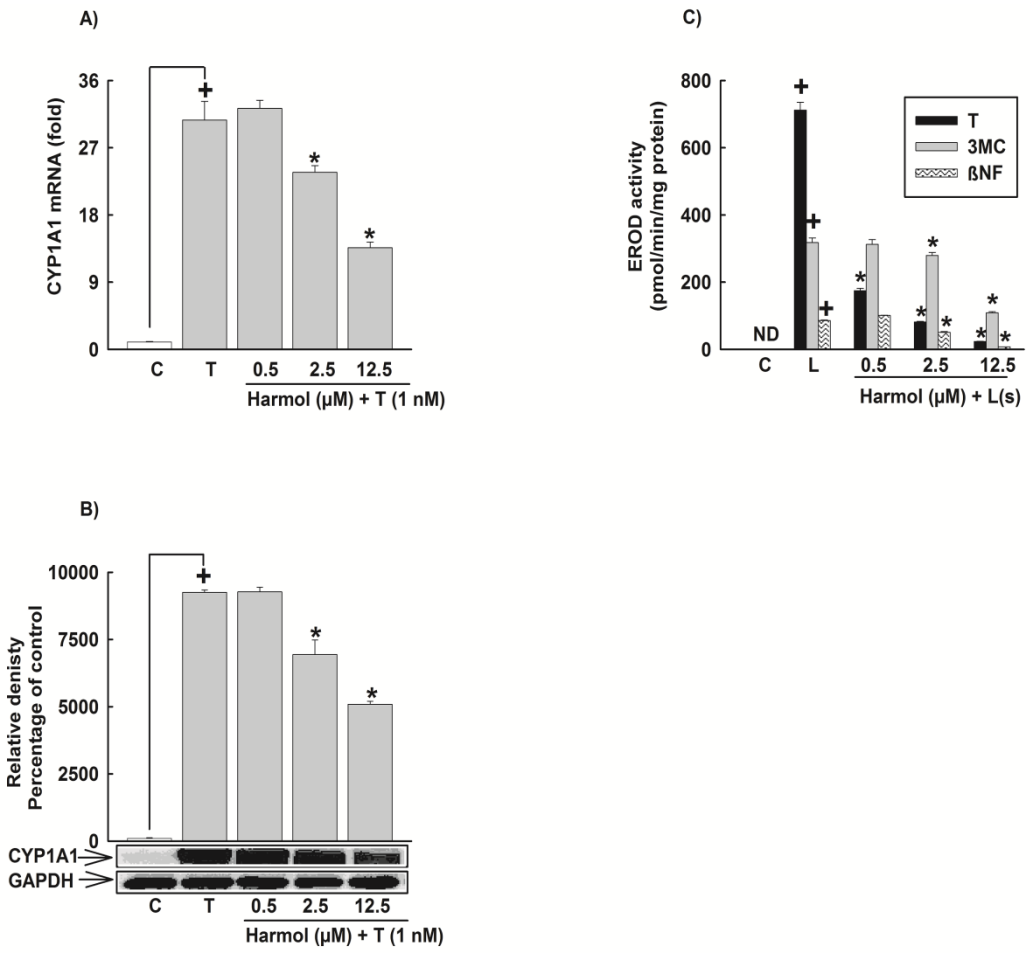


Fig. 3.18. Effect of harmol on CYP1A1 mRNA, protein, and catalytic activity in HepG2 cells. Cells were incubated with increasing concentrations of harmol (0.5-12.5 μM) 30 min before the addition of TCDD (T, 1nM) and for an additional 6 h for mRNA or 24 h for protein and catalytic activity. (A) The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Protein was separated on 10% SDS-PAGE and CYP1A1 protein was determined using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. (C) CYP1A1 activity was determined using CYP1A1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (L): ligand. (ND): not detected.

3.3.3. Effect of Harmine and Harmol on Dioxin-Mediated Induction of Cyp1a1 mRNA, Protein, and Catalytic Activity Levels in Hepa 1c1c7 Cells

In an attempt to explore whether the effect of harmine and harmol is species specific, we examined their effect on TCDD-mediated induction of Cyp1a1 using murine hepatoma cells, Hepa 1c1c7. Our results showed that harmine decreased the TCDD-mediated induction of Cyp1a1 mRNA by 25% and 29% with concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.19A). Furthermore, harmol showed a more pronounced effect than harmine in Hepa 1c1c7 where it decreased the TCDD-mediated induction of Cyp1a1 mRNA by 53%, 63%, and 71% with harmol concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.20A).

At the protein level, both compounds significantly inhibited the TCDD-mediated induction of Cyp1a1 in a concentration-dependent manner. Harmine significantly decreased the TCDD-mediated induction of Cyp1a1 protein by 18%, 33%, and 35% with harmine concentrations of 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.19B). Moreover, harmol showed a more pronounced effect than harmine, where it decreased the TCDD-mediated induction of Cyp1a1 protein by 44%, 62%, and 86% with 0.5, 2.5, and 12.5 μM, respectively (Fig. 3.20B).

At the catalytic activity level, both harmine and harmol significantly decreased the Cyp1a1 catalytic activity induced by all tested AhR ligands in a concentration-dependent manner. According to the percentage inhibition of the induced Cyp1a1 catalytic activity level, the order of inhibition of harmine against different AhR ligands was *β*NF > TCDD > 3MC (Fig. 3.19C). In contrast to

harmine, the order of inhibition of harmol against different AhR ligands was TCDD > 3MC > βNF (Fig. 3.20C). Similar to HepG2 cells, neither harmine nor harmol alone significantly affected Cyp1a1 at the catalytic activity level in Hepa 1c1c7 cells (data not shown).

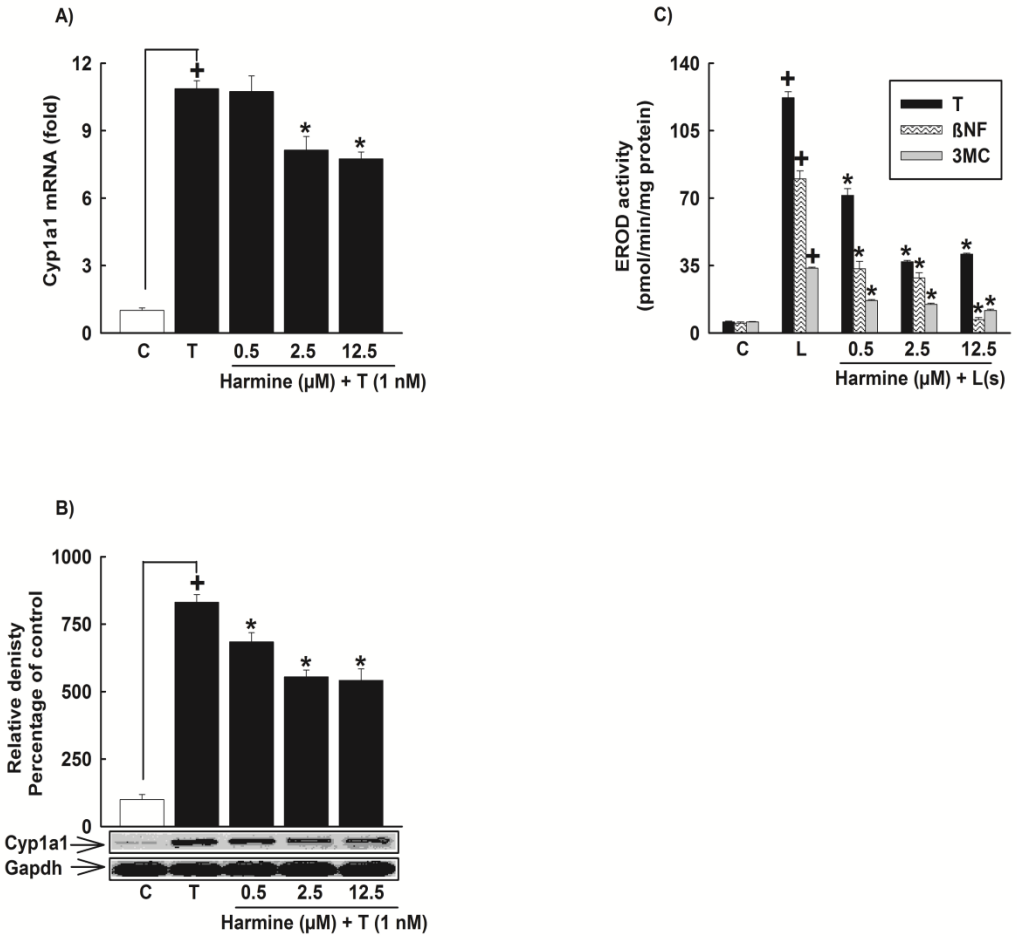


Fig. 3.19. Effect of harmine on Cyp1a1 mRNA, protein, and catalytic activity in Hepa 1c1c7 cells. Cells were incubated with increasing concentrations of harmine (0.5-12.5 μM) 30 min before the addition of TCDD (T, 1nM) and for an additional 6 h for mRNA or 24 h for protein and catalytic activity. (A) The amount of Cyp1a1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Protein was separated on 10% SDS-PAGE and Cyp1a1 protein was determined using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. (C) Cyp1a1 activity was determined using Cyp1a1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (L): ligand.

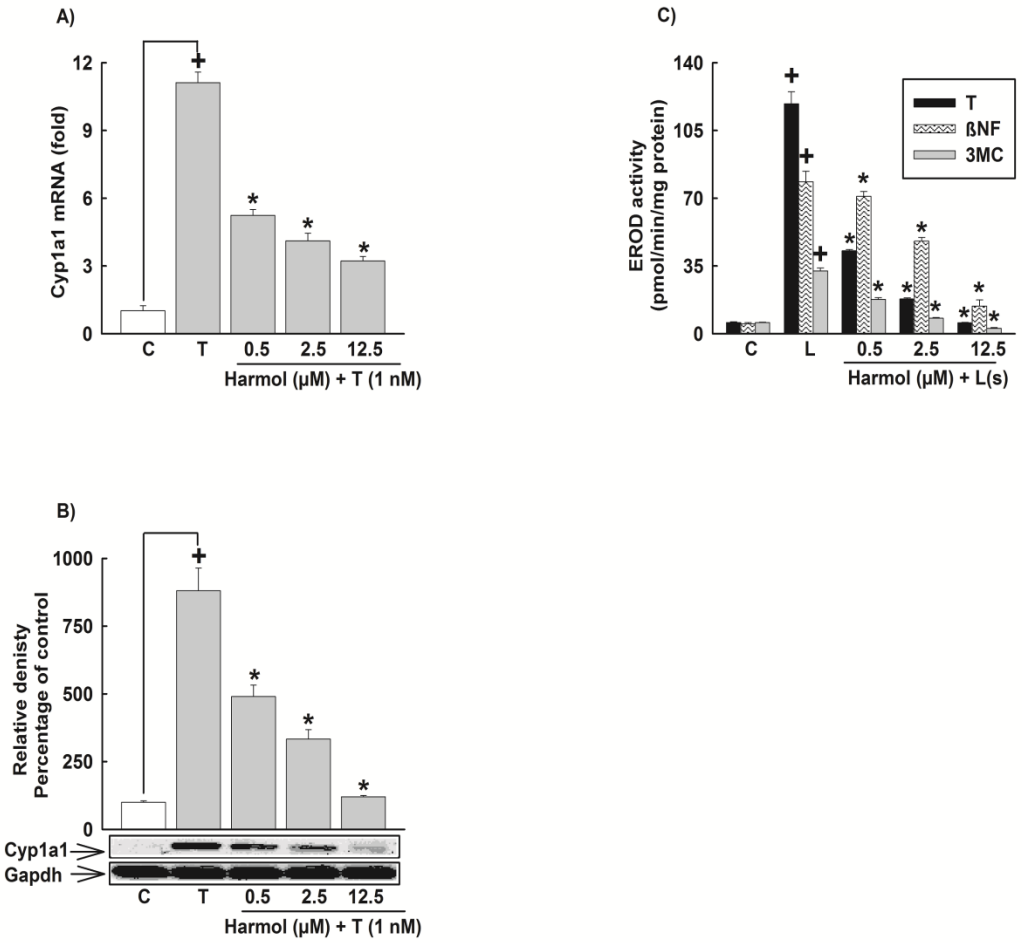


Fig. 3.20. Effect of harmol on Cyp1a1 mRNA, protein, and catalytic activity in Hepa 1c1c7 cells. Cells were incubated with increasing concentrations of harmol (0.5-12.5 μM) 30 min before the addition of TCDD (T, 1nM) and for an additional 6 h for mRNA or 24 h for protein and catalytic activity. (A) The amount of Cyp1a1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Values represent the mean of fold change ± S.E.M. (n=4). (B) Protein was separated on 10% SDS-PAGE and Cyp1a1 protein was determined using the enhanced chemiluminescence method. The intensity of bands was normalized to Gapdh signals, which was used as loading control. One of three representative experiments is shown. (C) Cyp1a1 activity was determined using Cyp1a1-dependent EROD assay. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (L): ligand.

3.3.4. Transcriptional Effect of Harmine and Harmol on *CYP1A1* Gene

In an attempt to explore the effect of harmine and harmol on the AhR-dependent transcriptional activation, HepG2 cells were transiently co-transfected with the XRE-driven luciferase reporter gene and renilla luciferase vector, which was used for normalization of transfection efficiency. Our results showed that TCDD alone significantly induced the luciferase activity by 1300% as compared with the control (Fig. 3.21A) whereas neither harmine nor harmol alone significantly affected the luciferase activity relative to control (Fig. 3.21A). On the other hand, harmine and harmol significantly decreased the TCDD-induced luciferase activity by 42% and 22%, respectively (Fig. 3.21A).

In order to test the ability of harmine and harmol to directly interfere with AhR activation and subsequent DNA binding to XRE, EMSA was performed using untreated guinea pig hepatic cytosol incubated either with vehicle (DMSO), harmine (250 μM) or harmol (250 μM) in the absence and presence of TCDD (20 nM) for 2 h. Figure 3.21B shows that both harmine and harmol (250 μM) alone did not alter the AhR activity, while TCDD (20 nM) alone induced the AhR activity and the formation of AhR-ARNT-XRE complex. On the other hand, pre-incubation of guinea pig cytosolic extracts with harmine or harmol significantly inhibited the TCDD-mediated activation of AhR and the formation of AhR-ARNT-XRE complex (Fig. 3.21B). The specificity of the binding was confirmed by the competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabeled XRE (Fig. 3.21B).

To determine whether harmine or harmol are direct ligands for the AhR, a ligand competition binding assay using HAP was performed (Fig. 3.22.). In this assay, we used untreated guinea pig and mouse hepatic cytosols to study the binding ability of harmine and harmol to AhR from two different species. Moreover, the total binding is the overall binding of [^{3}H]TCDD to cytosolic AhR protein. However, to account for the non-specific binding that happens not through the AhR or not through the ligand-binding center of the AhR, reactions were conducted in the presence of 100-fold excess of the competitor. We have chosen TCDF rather than TCDD because of its higher solubility as TCDD would not be soluble at 200 nM. Thus, the specific binding of [^{3}H]TCDD to the AhR was calculated by subtracting the non-specific binding from the total binding. Our results demonstrated that harmine at concentrations of 1 μM and 25 μM was able to significantly displace [^{3}H]TCDD (2 nM) by 19% and 74%, and by 20% and 63% using guinea pig and mouse cytosols, respectively (Fig. 3.22A & 3.22B). To a lower degree, harmol displaced [^{3}H]TCDD (2 nM) by 6% and 19%, and by 9% and 25% using guinea pig and mouse cytosols, respectively (Fig. 3.22A & 3.22B). The effect was significant for all tested concentrations of harmol except for the 1 μM concentration in the guinea pig cytosol treatment (Fig. 3.22A).

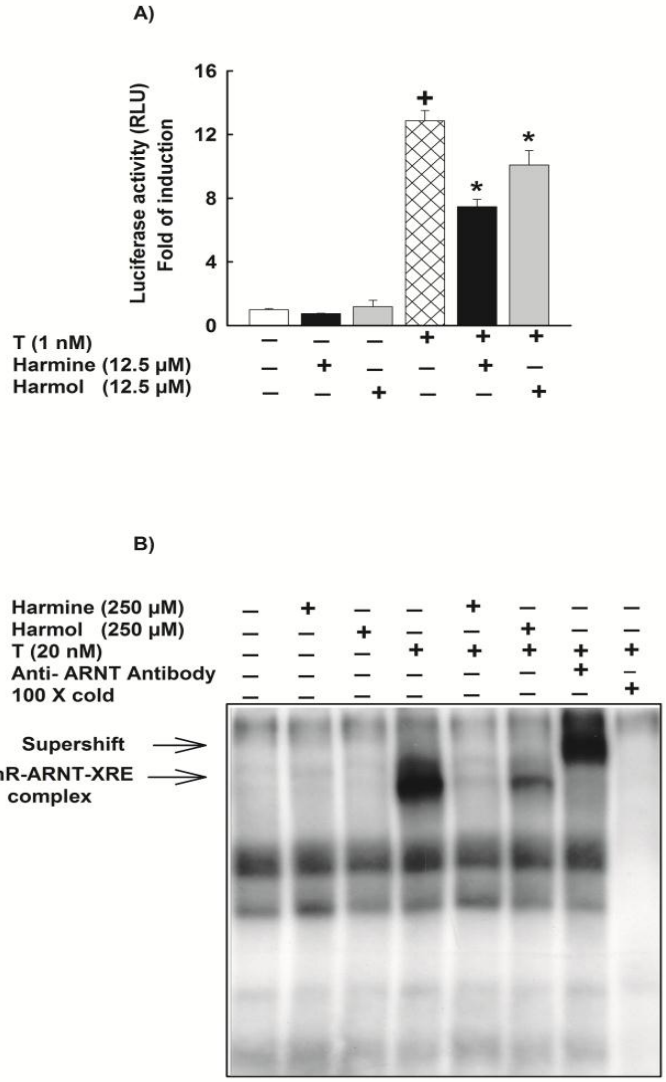


Fig. 3.21. Effect of harmine and harmol on XRE-dependent luciferase activity and AhR activation using EMSA. (A) HepG2 cells were transiently co-transfected with XRE-luciferase reporter plasmid pGudLuc 6.1. and renilla luciferase control plasmid pRL-CMV. Cells were treated with DMSO, harmine (12.5 µM) or harmol (12.5 µM) 30 min before the addition of TCDD (T, 1nM) for an additional 24 h. Cells were lysed and luciferase activity was reported as relative light unit (RLU) fold of firefly luciferase to renilla luciferase (Fluc/Rluc) (mean ± S.E.M., n = 4). (+) $P < 0.05$ compared with Control (C), (*) $P < 0.05$ compared with T. (B) *In vitro* AhR activity was measured by EMSA using guinea pig hepatic cytosolic extracts. Cytosolic extracts were incubated with DMSO, harmine (250 µM) or harmol (250 µM) for 30 min before the addition of TCDD (20 nM) for 2 h. The mixtures were tested for binding activity to a [γ-^{32}P]-labeled XRE consensus oligonucleotide for an additional 15 min. The products of this binding were separated on a 4% polyacrylamide gel. The specificity of the binding was confirmed by competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabeled XRE. AhR-ARNT-XRE complex formed on the gel was visualized by autoradiography. One representative of three experiments is shown.

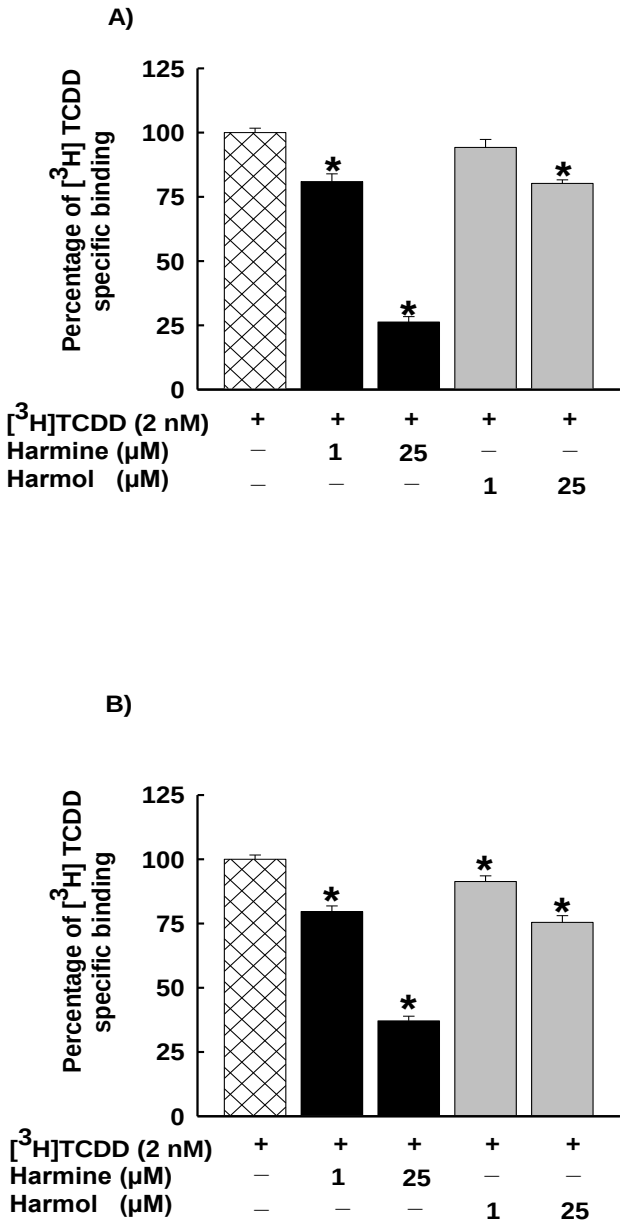


Fig. 3.22. AhR ligand binding ability of harmine and harmol. (A) Guinea pig hepatic cytosol (2 mg/mL) or (B) C57BL/6 mouse hepatic cytosols (2 mg/mL) were incubated with [^{3}H]TCDD (2 nM) alone (total binding), [^{3}H]TCDD (2 nM) and TCDF (200 nM, 100-fold excess of competitor, nonspecific binding), or [^{3}H]TCDD (2 nM) in the presence of increasing concentrations of harmine or harmol (1 and 25 μM) and the samples were analyzed by the HAP assay as described under Materials and Methods section. Values were adjusted for nonspecific binding and expressed as percentage specific binding relative to the absence of a competitor ligand. Values were presented as the mean ± S.E.M. (n = 9). (*) $P < 0.05$ compared with [^{3}H]TCDD.

3.3.5. Post-Transcriptional Effect of Harmine and Harmol

The level of gene expression is regulated by the transcription rate and the elimination rate through processing or degradation. Therefore, we tested the effect of harmine and harmol on the stability of CYP1A1 mRNA transcripts in HepG2 cells, using the Act-D-chase experiment. If the effect of harmine or harmol on CYP1A1 involves post-transcriptional effects through destabilization of CYP1A1 mRNA, a decrease in CYP1A1 mRNA $t_{1/2}$ would be expected. Our results showed that TCDD-induced CYP1A1 mRNA degraded with a $t_{1/2}$ of 4.9 ± 0.4 h (Fig. 3.23A). Moreover, treatment with harmine or harmol did not significantly alter CYP1A1 mRNA $t_{1/2}$ which was 5.4 ± 0.26 h and 4.9 ± 0.04 h, respectively (Fig. 3.23A).

3.3.6. Post-Translational Effect of Harmine and Harmol

The effect of harmine and harmol on the activity level was much higher than that obtained with the protein level, which raises the possibility of post-translational modifications. Therefore, we tested the effect of harmine and harmol on CYP1A1 protein stability in HepG2 cells using a CHX-chase experiment. Figure 3.23B shows that CYP1A1 protein induced by TCDD degraded with a $t_{1/2}$ of 6.4 ± 0.13 h. Furthermore, harmine and harmol significantly reduced the stability of CYP1A1 protein which degraded with half-lives of 2.5 ± 0.14 h and 2.2 ± 0.23 h, respectively (Fig. 3.23B).

In order to elucidate the underlying mechanisms of the post-translational modifications of harmine and harmol, we tested the role of ubiquitin-proteasomal

pathway. HepG2 cells were treated with TCDD (1 nM) for 24 h, and thereafter cells were washed three times with PBS and incubated with fresh media containing CHX (10 μg/mL) alone, CHX (10 μg/mL) and harmine (2.5 μM) or CHX (10 μg/mL) and harmol (2.5 μM) in the absence and presence of the proteasomal inhibitor, MG-132 (0.5 μM). Total protein was extracted after 6 h and CYP1A1 protein was determined using Western blot analysis. Our data showed that MG-132 alone did not significantly affect the level of CYP1A1 protein (Fig. 3.24A). On the other hand, MG-132 significantly prevented the degradation of CYP1A1 protein for CHX and harmine- or CHX and harmol-treated cells (Fig. 3.24A).

In addition, we tested the direct inhibitory effect of harmine and harmol on CYP1A1 enzyme. HepG2 cells were treated for 24 h with TCDD (1 nM), thereafter, the cells were washed twice with PBS and harmine or harmol (2.5 μM) in assay buffer were further incubated for 15 min before the addition of the substrate (7ER, 2 μM final concentration). The remaining CYP1A1 activity was measured using the EROD assay. Figure 3.24B shows that harmine and harmol possess direct inhibitory effects on CYP1A1 enzyme, where harmine (2.5 μM) and harmol (2.5 μM) significantly inhibited the CYP1A1 activity by 50%, and 75%, respectively (Fig. 3.24B).

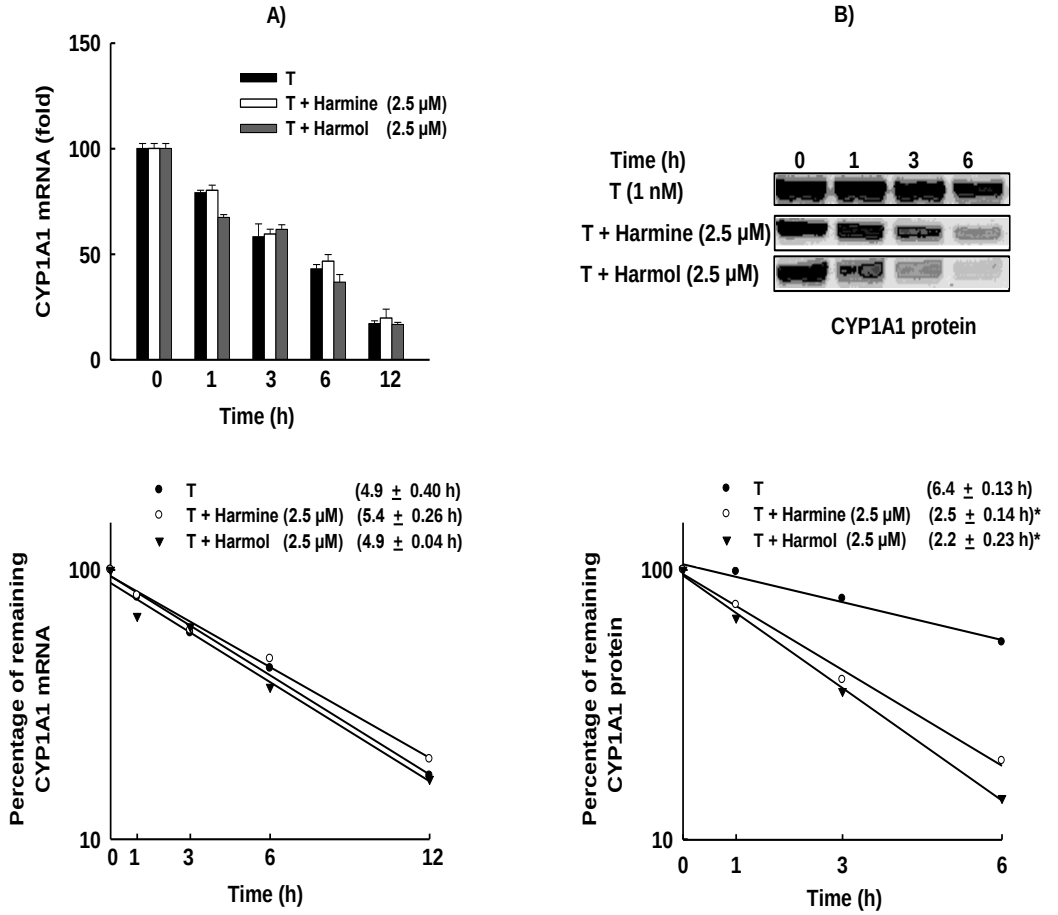


Fig. 3.23. Effect of harmine and harmol on CYP1A1 mRNA and protein stability. HepG2 cells were treated with TCDD (T, 1 nM) for 6 h for mRNA stability and 24 h for protein stability assays. Thereafter, the cells were washed and incubated with fresh media containing harmine (2.5 μM) or harmol (2.5 μM) plus Act-D (5 μg/mL, the mRNA synthesis inhibitor) or CHX (10 μg/mL, the protein translation inhibitor). (A) Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with harmine or harmol and subjected to real-time PCR. (B) Protein was separated on 10% SDS-PAGE and CYP1A1 protein was determined using the enhanced chemiluminescence method. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls (data not shown). mRNA and protein decay curves were analyzed individually, and the $t_{1/2}$ was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot, expressed as a percentage of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean $t_{1/2}$ (mean ± S.E.M., n = 3). (*) $P < 0.05$ compared with T. Each of the slopes was significant at $P < 0.05$.

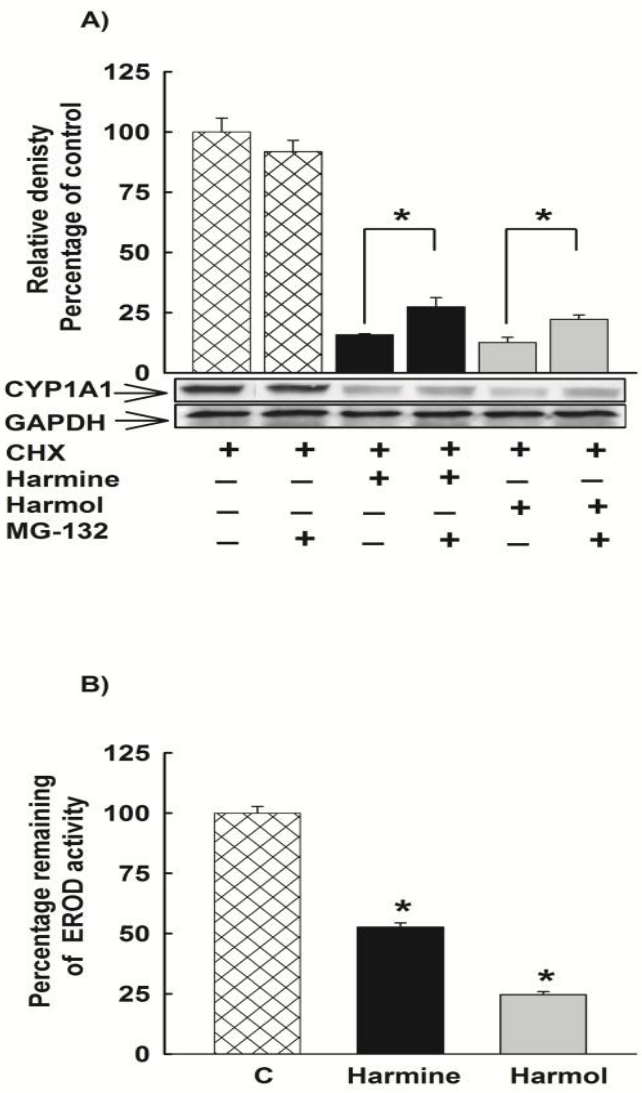


Fig. 3.24. Post-translational modifications of CYP1A1 by harmine and harmol. (A) Effect of the proteasomal inhibitor MG-132 on the reduced CYP1A1 protein stability by harmine and harmol. HepG2 cells were treated with TCDD (1 nM) for 24 h. Thereafter, cells were washed three times with PBS and incubated with fresh media containing CHX alone (10 μg/mL, the protein translation inhibitor), CHX (10 μg/mL) and harmine (2.5 μM) or CHX (10 μg/mL) and harmol (2.5 μM) in the absence and presence of the proteasomal inhibitor MG-132 (0.5 μM). After 6 h incubation, total protein was extracted and CYP1A1 protein was determined using Western blot analysis. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls. Values represent mean of relative densities and expressed as percentage of control (vehicle-treated cells) ± S.E.M. One of three representative experiments is shown. (*) $P < 0.05$ compared with the relevant treatment. (B) The direct inhibitory effects of harmine and harmol on CYP1A1 enzyme. HepG2 cells were pre-treated with TCDD (1nM) for 24 h, and thereafter media were removed, and the cells were washed three times with PBS before harmine (2.5 μM) and harmol (2.5 μM) in assay buffer were added at 15 min prior to the addition of 7ER (2 μM final concentration) for the EROD measurement. Results are expressed as percentage of remaining EROD activity (mean ± S.E.M, n = 8). (*) $P < 0.05$ compared with control (C).

3.4. Effect of Harmaline and Harmalol on Dioxin-Mediated Induction of CYP1A1 and AhR Signaling Pathway

*The results of this section have been published in "Food Chem Toxicol. 2012; **50**:353-362.".*

3.4.1. Effect of Harmaline and Harmalol on Cell Viability

To determine the nontoxic concentrations that will be used in our study, increasing concentrations of harmaline and harmalol were incubated with HepG2 cells in presence and absence of TCDD (1 nM) for 24 h. Our results demonstrated that neither harmaline nor harmalol (0-25 μM) significantly affected cell viability when incubated with human hepatoma cells for 24 h either in presence or absence of TCDD (Fig. 3.25.).

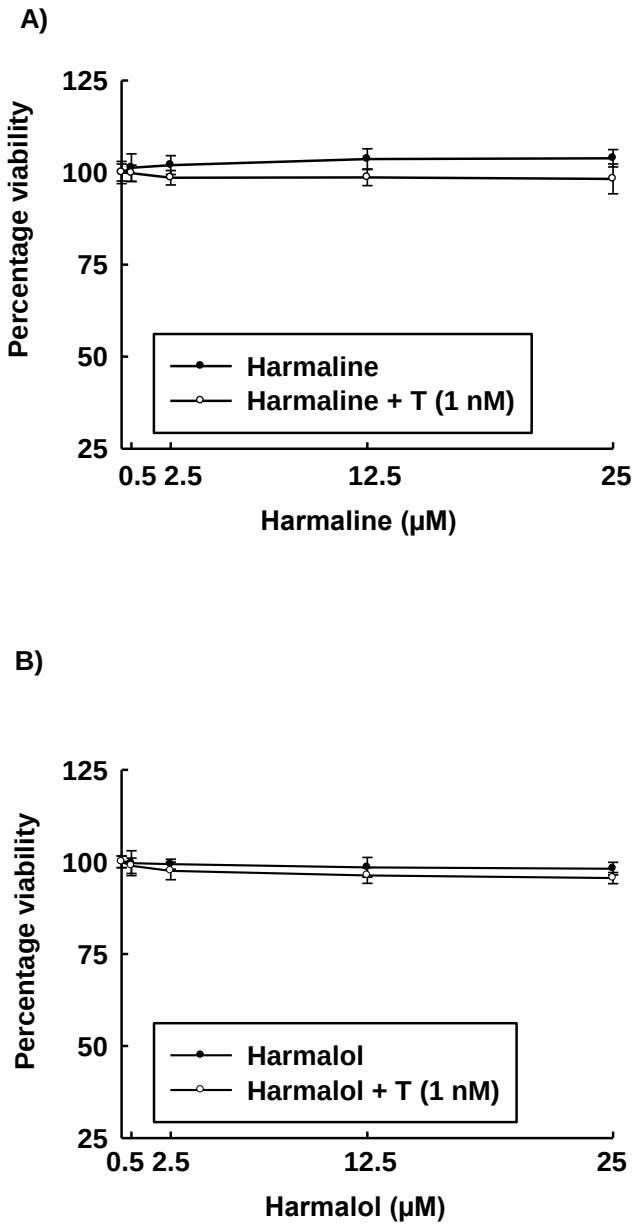


Fig. 3.25. Effect of harmaline and harmalol on HepG2 cell viability. The effect of different concentrations of harmaline (A) and harmalol (B) on HepG2 cell viability was tested using the MTT assay. Data were expressed as percentage of control, which was set at 100%, ± S.E.M. (n = 5). (+) $P < 0.05$ compared with control, (*) $P < 0.05$ compared with TCDD (T).

3.4.2. Effect of Harmaline on Dioxin-Mediated induction of CYP1A1 mRNA, Protein, and Catalytic Activity Levels in HepG2 Cells

To investigate the ability of harmaline to alter the CYP1A1 mRNA level, HepG2 cells were pre-incubated with increasing concentrations of harmaline (0.5-12.5 μM) for 30 min before the addition of TCDD (1 nM) and incubation for 6 h. Real-time PCR was employed to quantify CYP1A1 mRNA level. As shown in Fig 3.26A, harmaline significantly decreased the TCDD-mediated induction of CYP1A1 mRNA in a concentration-dependent manner by 28% and 43% with harmaline concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.26A). The positive control, Res (12.5 μM) significantly decreased the TCDD-mediated induction of CYP1A1 mRNA by 97% (Fig. 3.26A).

In order to assess the effect of harmaline on CYP1A1 protein level, HepG2 cells were pre-incubated with increasing concentrations of harmaline (0.5-12.5 μM) for 30 min before the incubation with TCDD (1 nM) for 24 h. Thereafter, the level of CYP1A1 protein was measured using Western blot analysis. Our results showed that harmaline significantly decreased the TCDD-mediated induction of CYP1A1 protein in a concentration-dependent manner, where it showed 66% and 76% decrease in CYP1A1 protein with harmaline concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.27A). To test whether the effect of harmaline is translated to the CYP1A1 catalytic activity, HepG2 cells were pre-incubated with increasing concentrations of harmaline (0.5-12.5 μM) for 30 min before the incubation with TCDD (1 nM) for 24 h. Thereafter, CYP1A1 catalytic activity was determined using the EROD assay. Our results showed that

harmaline significantly decreased the TCDD-mediated induction of the CYP1A1 catalytic activity by 67%, 80% and 90% with harmaline concentrations of 0.5, 2.5 and 12.5 μM, respectively (Fig. 3.28A). Res (12.5 μM) significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity by 96% (Fig. 3.28A).

To test whether the inhibitory effects of harmaline are not AhR ligand specific, HepG2 cells were pre-incubated with increasing concentrations of harmaline before the addition of B[a]P (1 μM). Thereafter, CYP1A1 catalytic activity was determined using 7ER as a substrate. Figure 3.29A shows that harmaline decreased the B[a]P-mediated induction of the CYP1A1 catalytic activity by 5%, 7%, and 20% with harmaline concentrations of 0.5, 2.5 and 12.5 μM, respectively (Fig. 3.29A). However, the effect was only significant at the highest concentration tested, 12.5 μM (Fig. 3.29A). Furthermore, Res (12.5 μM) significantly decreased the B[a]P-mediated induction of CYP1A1 catalytic activity by 29% (Fig. 3.29A). In addition, harmaline alone did not affect the constitutive CYP1A1 at the catalytic activity level in HepG2 cells (Fig. 3.29A).

3.4.3. Effect of Harmalol on Dioxin-Mediated induction CYP1A1 mRNA, Protein, and Catalytic Activity Levels in HepG2 Cells

In an attempt to explore whether the effect of harmaline is due to its active metabolite, we examined the effect of harmalol on the CYP1A1 mRNA level in human HepG2 cells. Our results showed that harmalol significantly decreased the TCDD-mediated induction of CYP1A1 mRNA in HepG2 cells in a concentration-dependent manner by 43% and 50% with harmalol concentrations of 2.5 and 12.5

μM, respectively (Fig. 3.26B). Res (12.5 μM) was used as a positive control, where it showed a significant decrease of TCDD-mediated induction of CYP1A1 mRNA by 97% (Fig. 3.26B).

Consistent with the CYP1A1 mRNA results, harmalol significantly decreased the TCDD-mediated induction of CYP1A1 at the protein level by 64% and 80% with harmalol concentrations of 2.5 and 12.5 μM, respectively (Fig. 3.27B). At the CYP1A1 catalytic activity level, harmalol showed a higher effect, where it significantly decreased the induction level of CYP1A1 catalytic activity by 62%, 82% and 91% with harmalol concentrations of 0.5, 2.5 and 12.5 μM, respectively (Fig. 3.28B). Res (12.5 μM) significantly decreased the TCDD-mediated induction of CYP1A1 catalytic activity by 96% (Fig. 3.28B). Furthermore, harmalol significantly decreased the B[a]P-mediated induction of CYP1A1 catalytic activity in HepG2 cells in a concentration-dependent manner by 31%, 36% and 48% with harmalol concentrations of 0.5, 2.5 and 12.5 μM, respectively (Fig. 3.29B). Furthermore, Res (12.5 μM) decreased the B[a]P-mediated induction of CYP1A1 by 29% (Fig. 3.29B). Additionally, neither harmalol (0.5-12.5 μM) nor Res (12.5 μM) alone significantly increased the constitutive CYP1A1 at the catalytic activity level in HepG2 cells (Fig. 3.29B).

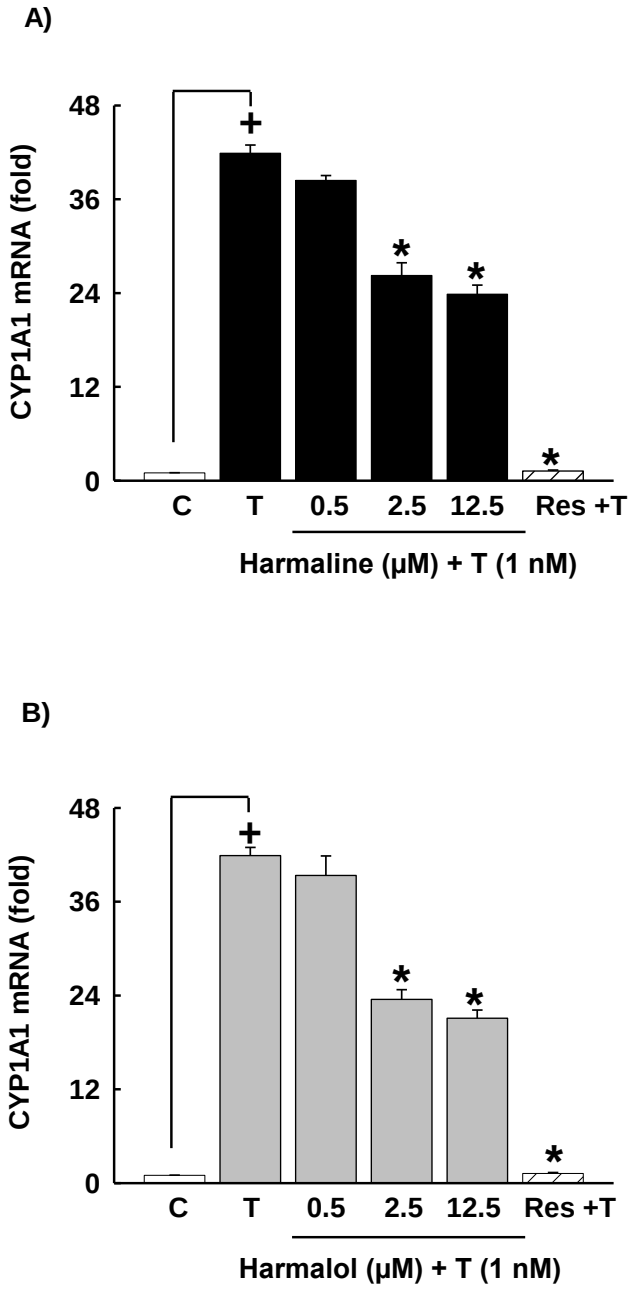


Fig. 3.26. Effect of harmaline and harmalol on CYP1A1 mRNA in HepG2 cells. Cells were pre-incubated with increasing concentrations of harmaline (A) or harmalol (B) for 30 min before the addition of TCDD (T, 1nM) and incubation for an additional 6 h. The amount of CYP1A1 mRNA was quantified using real-time PCR and normalized to *β*-actin reference gene. Res (12.5 μM) was used as a positive control. Values represent the mean of fold change ± S.E.M. (n=4). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

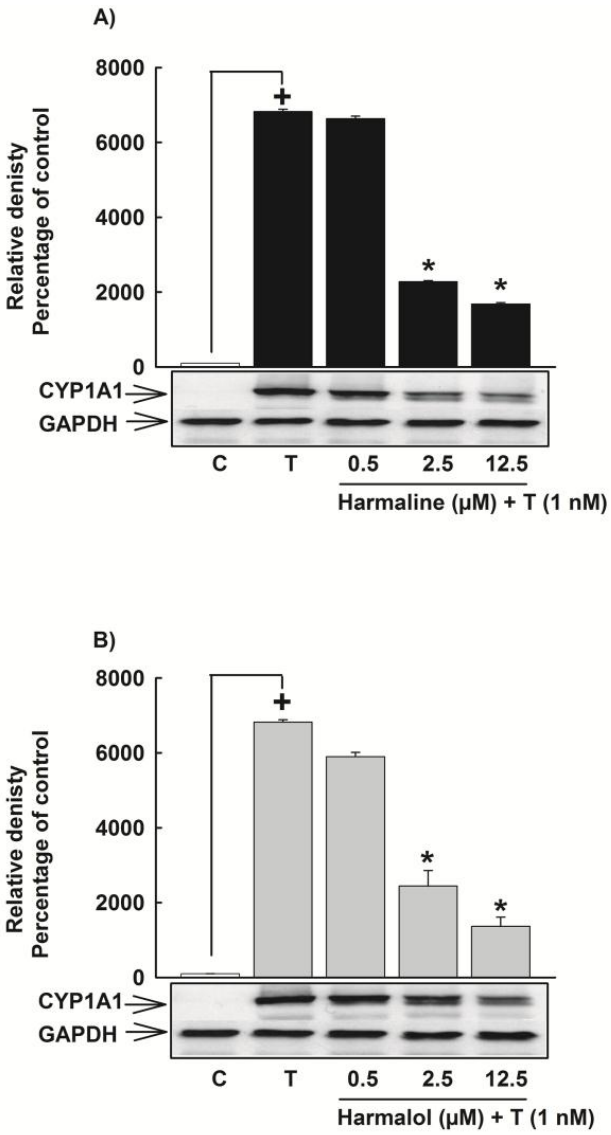


Fig. 3.27. Effect of harmaline and harmalol on CYP1A1 protein in HepG2 cells. Cells were pre-incubated with increasing concentrations of harmaline (A) or harmalol (B) for 30 min before the addition of TCDD (T, 1nM) and incubation for an additional 24 h. Protein was separated on 10% SDS-PAGE and CYP1A1 protein was detected using the enhanced chemiluminescence method. The intensity of bands was normalized to GAPDH signals, which was used as loading control. One of three representative experiments is shown. (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

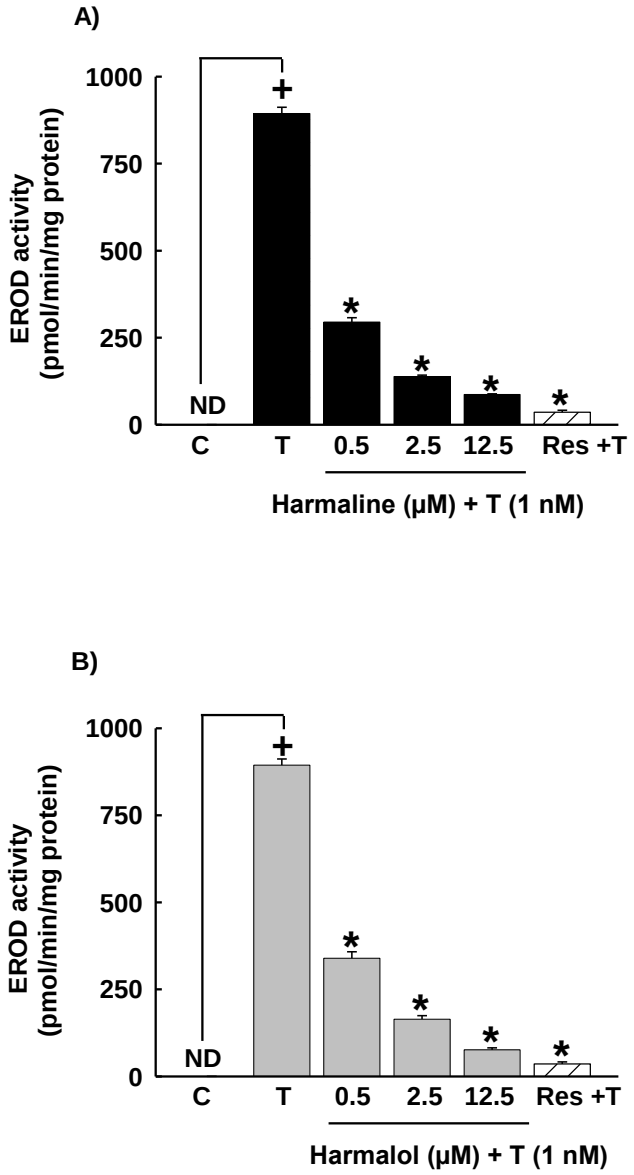


Fig. 3.28. Effect of harmaline and harmalol on TCDD-mediated induction of CYP1A1 catalytic activity in HepG2 cells. Cells were pre-incubated with increasing concentrations of harmaline (A) or harmalol (B) for 30 min before the addition of TCDD (T, 1nM) and incubation for an additional 24 h. The amount of CYP1A1 activity was determined using a CYP1A1-dependent EROD assay. Res (12.5 μM) was used as a positive control. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T, (ND): Not detected.

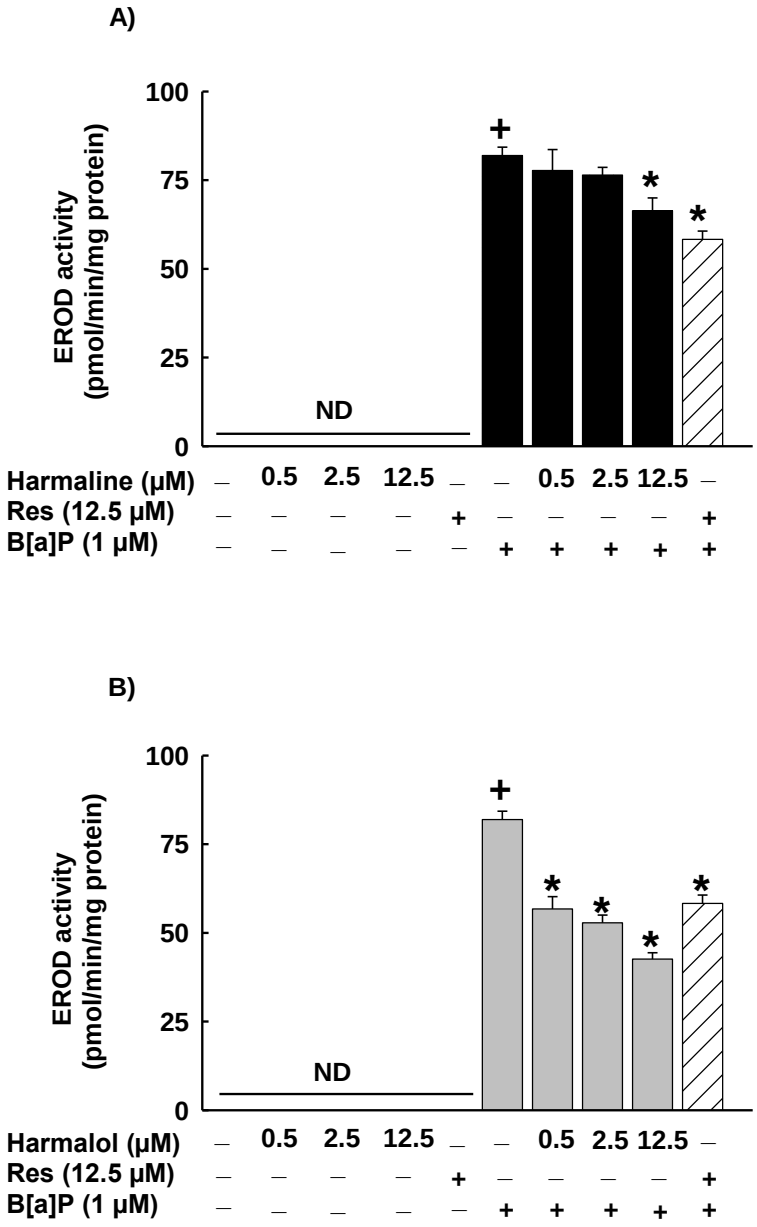


Fig. 3.29. Effect of harmaline and harmalol on B[a]P-mediated induction of CYP1A1 catalytic activity in HepG2 cells. Cells were incubated with increasing concentrations of harmaline (A) or harmalol (B) in the absence and presence of B[a]P (1 μM) for 24 h. The amount of CYP1A1 activity was determined using a CYP1A1-dependent EROD assay. Res (12.5 μM) was used as a positive control. Values represent mean activity ± S.E.M. (n = 8). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with B[a]P, (ND): Not detected.

3.4.4. Transcriptional Effects of Harmaline and Harmalol on *CYP1A1* gene

To investigate the underlying mechanisms for the effect of harmaline and harmalol on CYP1A1, we tested the role of transcriptional mechanisms. For this purpose, HepG2 cells were transiently co-transfected with the XRE-driven luciferase reporter gene and renilla luciferase vector, which was used for normalization of transfection efficiency. Our results demonstrated that neither harmaline nor harmalol alone significantly affected the level of luciferase activation relative to controls (Fig. 3.30A). On the other hand, TCDD alone significantly induced the luciferase activity by 1300% as compared with the control. Of interest, harmaline and harmalol significantly decreased the TCDD-induced luciferase activity by 30% and 27%, respectively (Fig. 3.30A).

In an attempt to elucidate the direct effect of harmaline and harmalol on AhR activation and the subsequent DNA binding to XRE, EMSA was performed on untreated guinea pig hepatic cytosol incubated either with vehicle, harmaline or harmalol in the absence and presence of TCDD (20 nM) for 2 h. Similar to luciferase results, both harmaline and harmalol (250 μM) alone did not alter the AhR activity (Fig. 3.30B). TCDD (20 nM) alone induced the AhR activity and the formation of AhR-ARNT-XRE complex. On the other hand, pre-incubation of cytosolic extracts with harmaline or harmalol significantly inhibited the TCDD-mediated activation of AhR and the formation of the AhR-ARNT-XRE complex (Fig. 3.30B). The specificity of the binding was confirmed by the competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabeled XRE (Fig. 3.30B).

To examine whether harmaline or harmalol are direct ligands for the AhR, a ligand competition binding assay was performed using untreated guinea pig hepatic cytosols (Fig. 3.31.). Our results showed that harmaline at concentrations of 25 μM and 50 μM was able to significantly displace [^{3}H]TCDD (2 nM) by 28% and 35%, respectively (Fig. 3.31.). On the other hand, harmalol did not show a significant displacement of [^{3}H]TCDD (Fig. 3.31.).

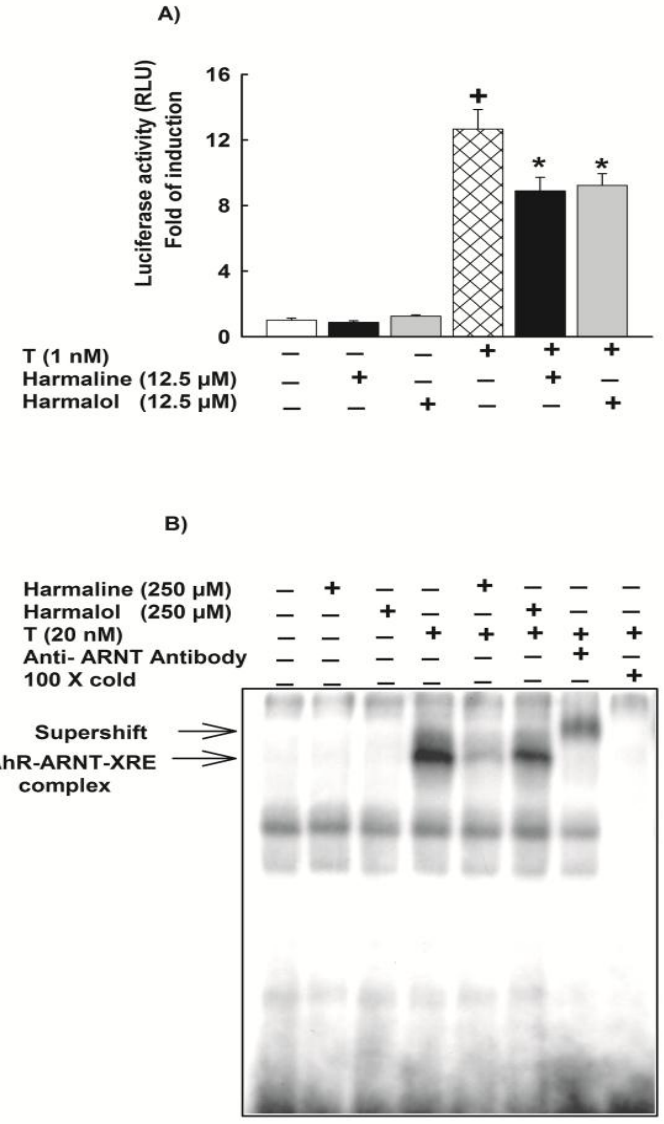


Fig. 3.30. Effect of harmaline and harmalol on XRE-dependent luciferase activity and AhR activation using EMSA. (A) HepG2 cells were transiently co-transfected with XRE-luciferase reporter plasmid pGudLuc 6.1. and renilla luciferase control plasmid pRL-CMV. Cells were treated with vehicle, harmaline (12.5 µM) or harmalol (12.5 µM) for 30 min before the addition of TCDD (T, 1nM) and incubation for an additional 24 h. Cells were lysed and luciferase activity was reported as relative light unit (RLU) fold of firefly luciferase to renilla luciferase (Fluc/Rluc) (mean ± S.E.M., n = 4). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T. (B) *In vitro* AhR activity was measured by EMSA using guinea pig hepatic cytosolic extracts. Cytosolic extracts were pre-incubated with vehicle, harmaline (250 µM) or harmalol (250 µM) for 30 min before the addition of TCDD (20 nM) and incubation for 2 h. The mixtures were tested for binding activity to a [γ-^{32}P]-labeled XRE consensus oligonucleotide for an additional 15 min. The products of this binding were separated on a 4% polyacrylamide gel. The specificity of the binding was confirmed by competition assays using anti-ARNT antibody or a 100-fold molar excess of unlabeled XRE. AhR-ARNT-XRE complex formed on the gel was visualized by autoradiography. One representative of three experiments is shown.

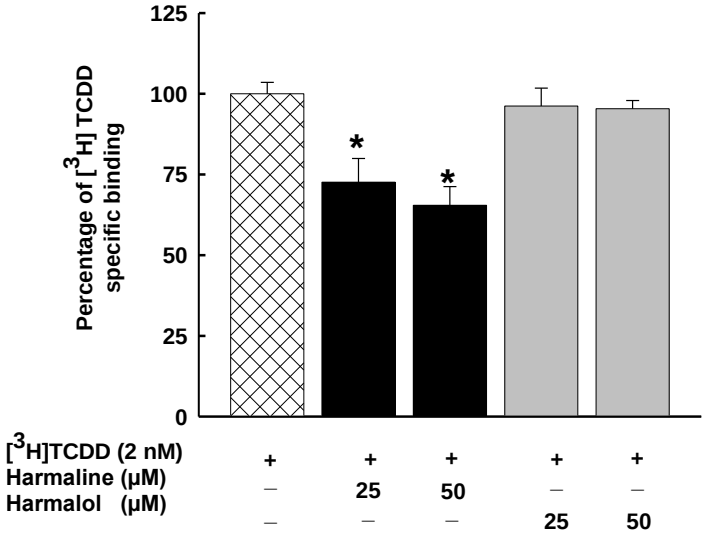


Fig. 3.31. AhR ligand binding ability of harmaline and harmalol. Untreated guinea pig hepatic cytosol (2 mg/mL) was incubated with [^{3}H]TCDD (2 nM) alone (total binding), [^{3}H]TCDD (2 nM) and TCDF (200 nM, 100-fold excess of competitor, nonspecific binding), or [^{3}H]TCDD (2 nM) in the presence of increasing concentrations of harmaline or harmalol (25, and 50 μM) and the samples were analyzed by the HAP assay as described under Materials and Methods section. Values were adjusted for nonspecific binding and expressed as percentage specific binding relative to the absence of a competitor ligand. Values were presented as the mean ± S.E.M. (n = 9). (*) $P < 0.05$ compared with [^{3}H]TCDD.

3.4.5. Post-Transcriptional Modification of CYP1A1 mRNA by Harmaline and Harmalol

To investigate the post-transcriptional modifications, we tested the effect of harmaline and harmalol on the stability of CYP1A1 mRNA transcripts in HepG2 cells, using the Act-D-chase experiment. If the effect of harmaline or harmalol involves destabilization of CYP1A1 mRNA, a decrease in the $t_{1/2}$ would be expected. Our results showed that TCDD-induced CYP1A1 mRNA degraded with a $t_{1/2}$ of 4.9 ± 0.4 h (Fig. 3.32A). Moreover, treatment with harmaline or harmalol did not significantly alter CYP1A1 mRNA $t_{1/2}$ which was 5.4 ± 0.2 h and 4.3 ± 0.1 h, respectively (Fig. 3.32A).

3.4.6. Post-Translational Modification of CYP1A1 Protein by Harmaline and Harmalol

The effect of harmaline and harmalol on the protein and the activity levels was much higher than that obtained with the mRNA level, which raises the possibility of post-translational modification. Therefore, we tested the effect of harmaline and harmalol on CYP1A1 protein stability in HepG2 cells using CHX-chase experiment. Our results showed that CYP1A1 protein induced by TCDD degraded with a $t_{1/2}$ of 6.4 ± 0.1 h (Fig. 3.32B). Furthermore, harmaline and harmalol significantly reduced the stability of CYP1A1 protein which degraded with half-lives of 2.1 ± 0.3 h and 2.5 ± 0.5 h, respectively (Fig. 3.32B).

To further investigate the underlying mechanisms of the post-translational modifications of harmaline and harmalol, we tested the role of the ubiquitin-

proteasomal pathway. Therefore, HepG2 cells were treated with TCDD (1 nM) for 24 h, and thereafter cells were washed three times with PBS and incubated with fresh media containing CHX (10 μg/mL) alone, CHX (10 μg/mL) and harmaline (2.5 μM) or CHX (10 μg/mL) and harmalol (2.5 μM) in the absence and presence of the proteasomal inhibitor, MG-132 (0.5 μM). After 6 h incubation, total protein was extracted and CYP1A1 protein was detected using Western blot analysis. Figure 3.33A shows that MG-132 alone did not affect the level of CYP1A1 protein. On the other hand, MG-132 significantly prevented the degradation of CYP1A1 protein for CHX and harmaline- or CHX and harmalol-treated cells (Fig. 3.33A).

Furthermore, the direct effect of harmaline or harmalol on CYP1A1 enzyme was determined. In this regard, HepG2 cells were treated for 24 h with TCDD (1 nM), and thereafter the cells were washed twice with PBS and harmaline (2.5 μM) or harmalol (2.5 μM) in assay buffer and were further incubated for 15 min before the addition of the substrate (7ER, 2 μM final concentration). The remaining CYP1A1 activity was detected using the EROD assay. Figure 3.33B shows that harmaline and harmalol possess direct inhibitory effects on CYP1A1 enzyme, where they significantly inhibited the CYP1A1 activity by 18% and 65%, respectively (Fig. 3.33B).

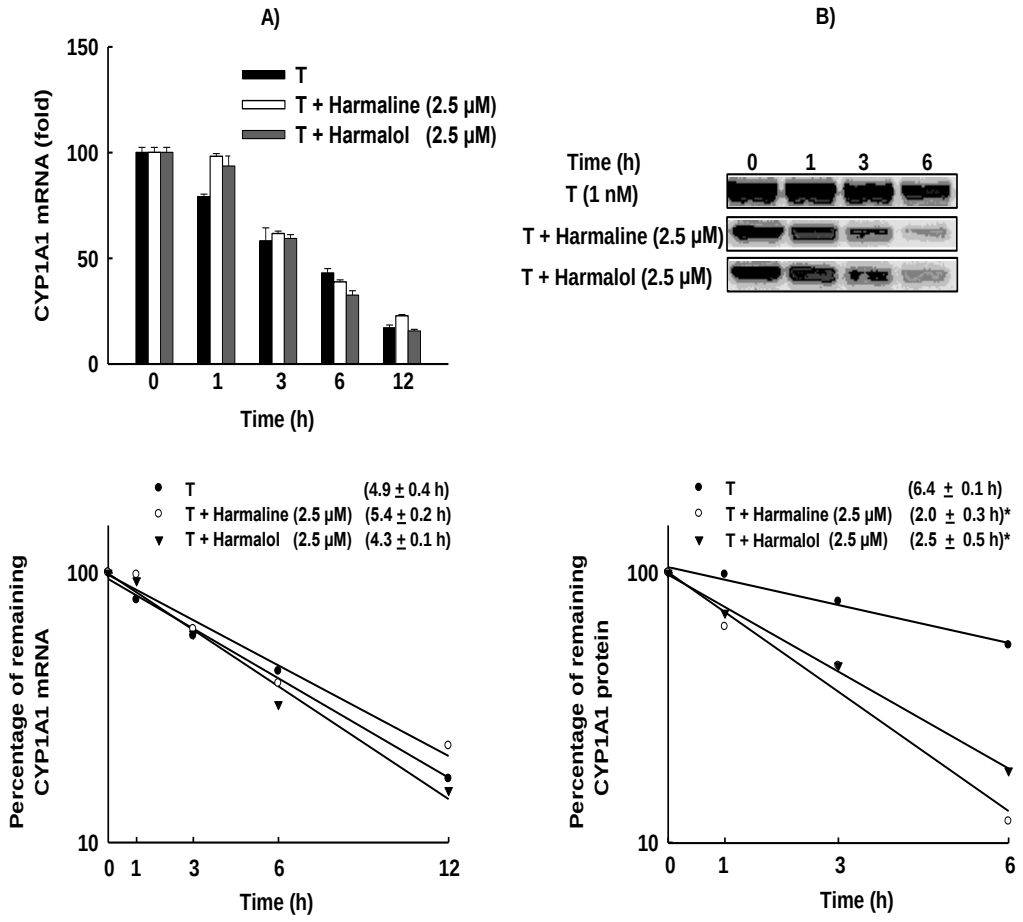


Fig. 3.32. Effect of harmaline and harmalol on CYP1A1 mRNA and protein stability. HepG2 cells were treated with TCDD (T, 1 nM) and incubated for 6 h for mRNA stability and 24 h for protein stability assays. Thereafter, the cells were washed and incubated with fresh media containing harmaline (2.5 μM) or harmalol (2.5 μM) plus Act-D (5 μg/mL, the mRNA synthesis inhibitor) or CHX (10 μg/mL, the protein translation inhibitor). (A) Total RNA was extracted at 0, 1, 3, 6, and 12 h after incubation with harmaline or harmalol and subjected to real-time PCR. (B) Protein was separated on 10% SDS-PAGE and CYP1A1 protein was determined using the enhanced chemiluminescence method. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls (data not shown). mRNA and protein decay curves were analyzed individually, and the $t_{1/2}$ was estimated from the slope of a straight line fitted by linear regression analysis ($r^2 \geq 0.9$) to a semilog plot, expressed as a percentage of treatment at time = 0 h (maximum, 100%) level, versus time. The half-lives obtained from three independent experiments were then used to calculate the mean $t_{1/2}$ (mean ± S.E.M., n = 3). (*) $P < 0.05$ compared with T. Each of the slopes was significant at $P < 0.05$.

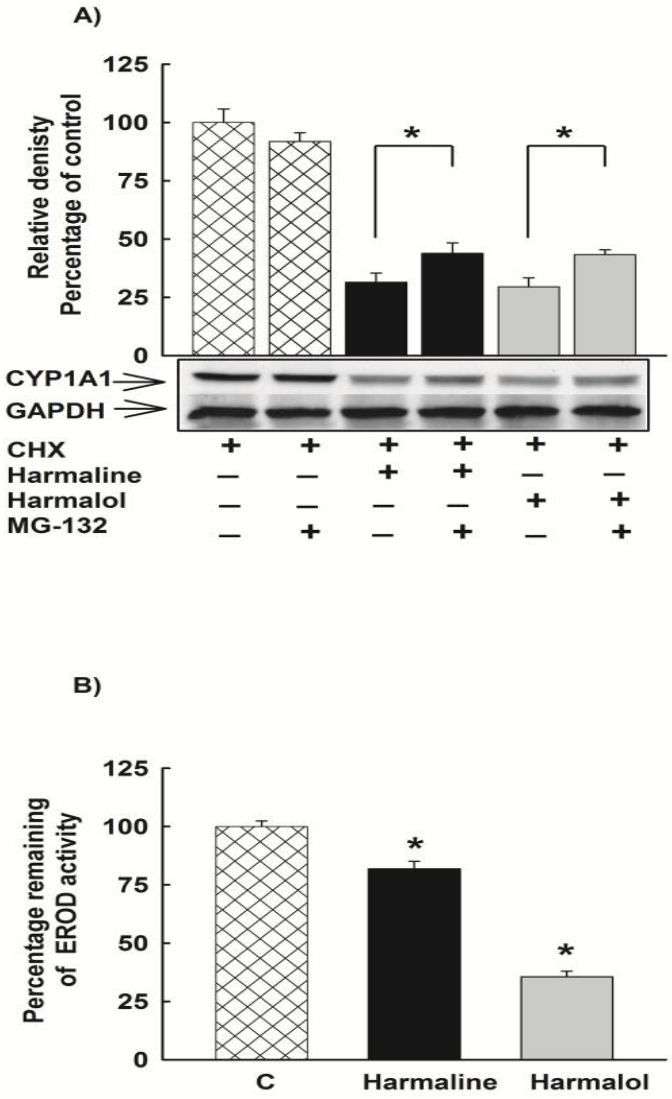


Fig. 3.33. Post-translational modifications of CYP1A1 by harmaline and harmalol. (A) Effect of the proteasomal inhibitor MG-132 on the reduced CYP1A1 protein stability by harmaline and harmalol. HepG2 cells were treated with TCDD (1 nM) for 24 h. Thereafter, cells were washed three times with PBS and incubated with fresh media containing CHX alone (10 μg/mL, the protein translation inhibitor), CHX (10 μg/mL) and harmaline (2.5 μM) or CHX (10 μg/mL) and harmalol (2.5 μM) in the absence and presence of the proteasomal inhibitor MG-132 (0.5 μM). After 6 h incubation, total protein was extracted and CYP1A1 protein was determined using Western blot analysis. The intensities of CYP1A1 protein bands were normalized to GAPDH signals, which were used as loading controls. Values represent mean of relative densities and expressed as percentage of control (vehicle-treated cells) ± S.E.M. One of three representative experiments is shown. (*) $P < 0.05$ compared with the relevant treatment. (B) The direct inhibitory effects of harmaline and harmalol on CYP1A1 enzyme. HepG2 cells were pre-treated with TCDD (1nM) for 24 h, and thereafter media were removed, and the cells were washed three times with PBS before harmaline (2.5 μM) and harmalol (2.5 μM) in assay buffer were added at 15 min prior to the addition of 7ER (2 μM final concentration) for the EROD measurement. Results are expressed as percentage of remaining EROD activity (mean ± S.E.M, n = 8). (*) $P < 0.05$ compared with control (C).

3.5. Effect of Harmine and Harmaline on Dioxin-Mediated Induction of Cyp1a1 in the Livers and Lungs of C57BL/6 Mice

The results of this section have been submitted for publication in "J Biomed Biotechnol 2012; under review".

3.5.1. Effect of Harmine and Harmaline on Cyp1a1 mRNA, Protein, and Enzymatic Activity Levels in C57BL/6 Mice Livers

Our results showed that treatment of mice with dioxin significantly increased the level of Cyp1a1 mRNA by approximately 200,000% compared to the control group. Moreover, treatment with harmine significantly decreased dioxin-mediated induction of Cyp1a1 mRNA by 15% (Fig. 3.34A).

To examine whether the effect of harmine on hepatic Cyp1a1 mRNA is translated to a relevant effect at protein and enzymatic activity levels, microsomal fractions were isolated from livers and the effect of harmine on Cyp1a protein and Cyp1a1 enzymatic activity was determined using Western blot analysis and 7ER as a substrate, respectively. Our results showed that dioxin induced Cyp1a protein by 250% relative to the control group. On the other hand, treatment of harmine significantly decreased dioxin-mediated induction of Cyp1a protein by 17% (Fig. 3.34B). Moreover, dioxin induced Cyp1a1 enzymatic activity by 2,000% relative to the control group, whereas harmine treatment significantly decreased dioxin-mediated Cyp1a1-dependent enzymatic activity by 60% (Fig. 3.34C).

On the other hand, our results showed that harmaline decreased the level of dioxin-mediated induction of Cyp1a1 mRNA by 9%; however, the effect was

not significant (Fig. 3.35A). Furthermore, harmaline significantly decreased Cyp1a protein by 20% and Cyp1a1 enzymatic activity by 32% using Western blotting and 7ER as a substrate, respectively (Fig. 3.35B & 3.35C). Collectively, both alkaloids decreased dioxin-mediated induction of Cyp1a1 in liver tissues; however, harmine showed a more pronounced effect.

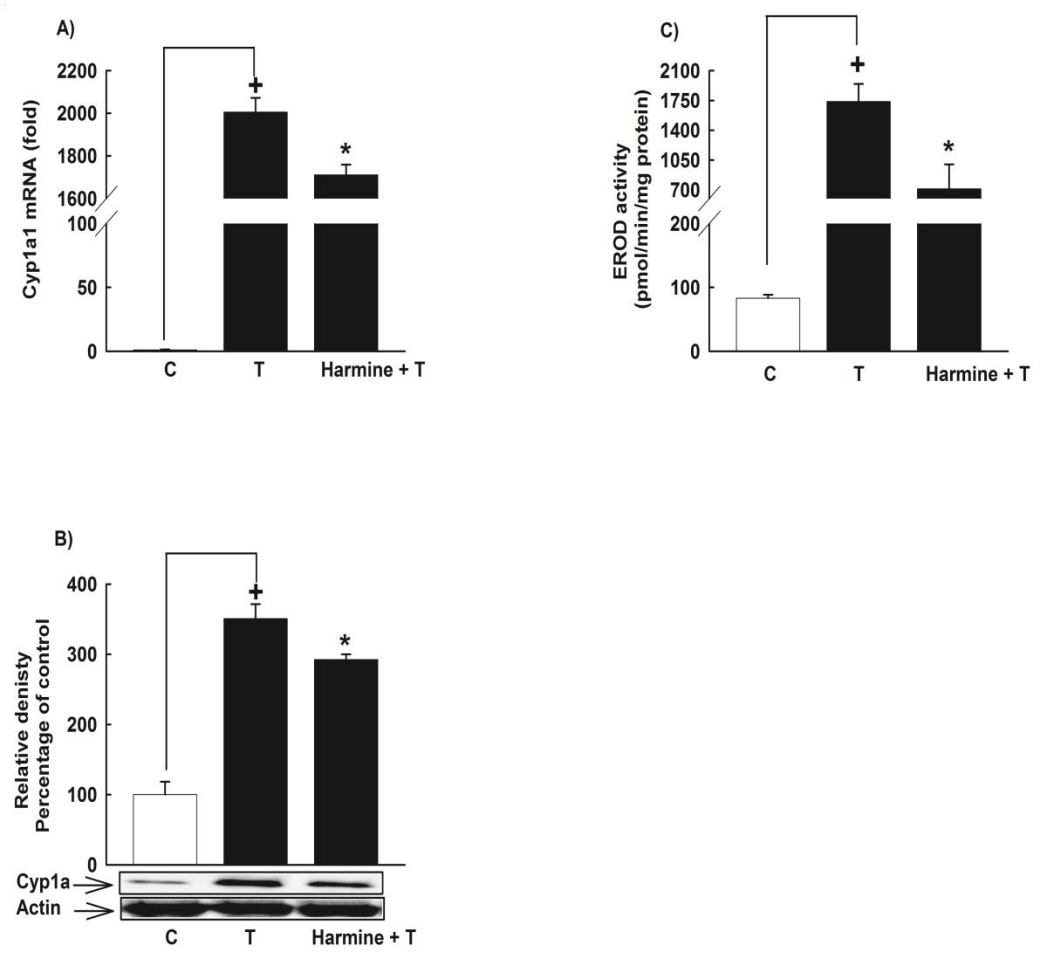


Fig. 3.34. Effect of harmine on dioxin-mediated induction of hepatic Cyp1a1 at mRNA (A), protein (B) and catalytic activity (C) in C57BL/6 mice. Mice were distributed into several groups, receiving the vehicle (weight-matched control), dioxin (T), or dioxin and harmine. After 14 h from dioxin treatment, mice were sacrificed and the livers were isolated. (A) Total RNA was isolated using TRIzol reagent and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR and normalized to *18S* reference gene. (B) Cyp1a protein was determined using Western blot analysis. Microsomal protein (2 µg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary anti-mouse Cyp1a antibody for 2 h at room temperature or primary rabbit anti-mouse actin for 24 h at 4°C. Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies namely, goat anti-mouse IgG for Cyp1a or goat anti-rabbit IgG for actin detection for another 1 h. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to actin signal, which was used as loading control. (C) Cyp1a1 catalytic activity was determined in microsomal fractions using 7ER as a substrate. Values represent the mean of fold change ± S.E.M. (n=6). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

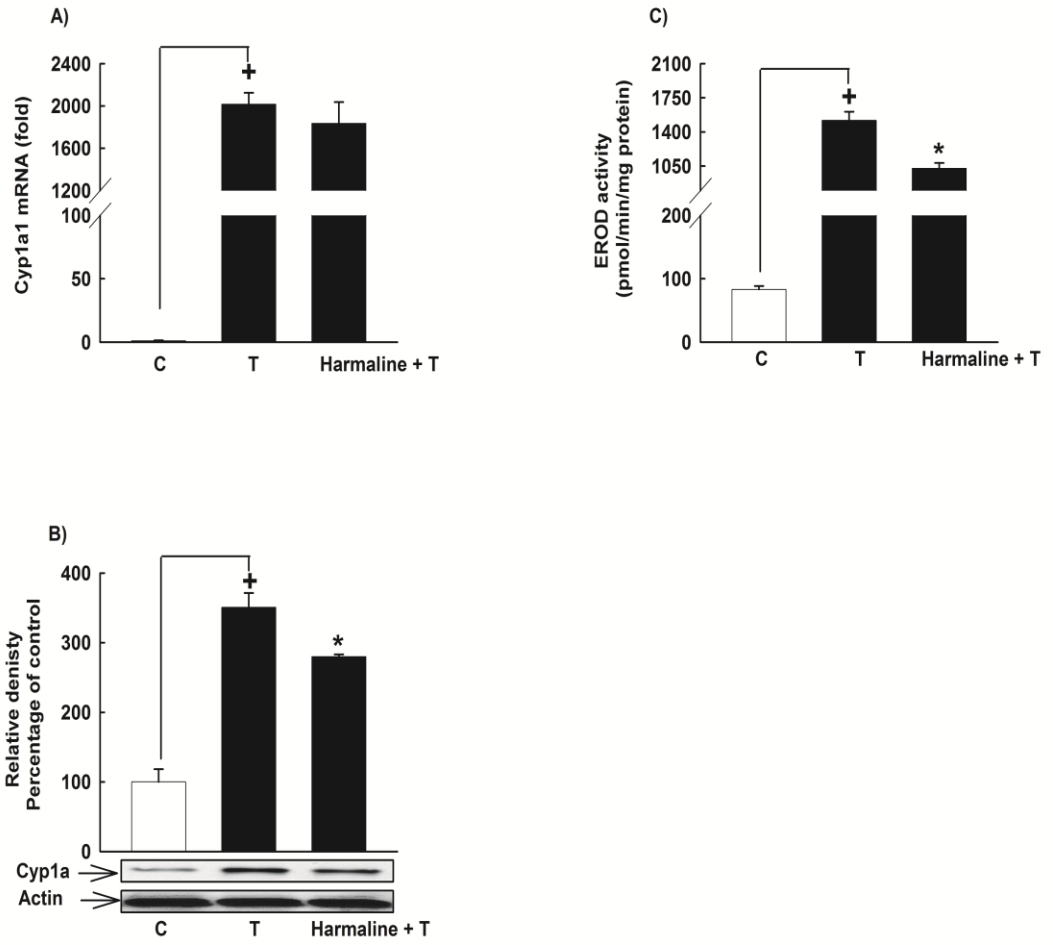


Fig. 3.35. Effect of harmaline on dioxin-mediated induction of hepatic Cyp1a1 at mRNA (A), protein (B) and catalytic activity (C) in C57BL/6 mice. Mice were distributed into several groups, receiving the vehicle (weight-matched control), dioxin (T), or dioxin and harmaline. After 14 h from dioxin treatment, mice were sacrificed and the livers were isolated. (A) Total RNA was isolated using TRIzol reagent and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR and normalized to *18S* reference gene. (B) Cyp1a protein was determined using Western blot analysis. Microsomal protein (2 µg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary anti-mouse Cyp1a antibody for 2 h at room temperature or primary rabbit anti-mouse actin for 24 h at 4°C. Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies, namely, goat anti-mouse IgG for Cyp1a or goat anti-rabbit IgG for actin detection for another 1 h. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to actin signal, which was used as loading control. (C) Cyp1a1 catalytic activity was determined in microsomal fractions using 7ER as a substrate. Values represent the mean of fold change ± S.E.M. (n=6). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

3.5.2. Effect of Harmine and Harmaline on Cyp1a1 mRNA, Protein, and Enzymatic Activity Levels in C57BL/6 Mice Lungs

In an effort to examine whether the effect of harmine and harmaline is not specific to liver tissues, lung tissues were isolated and the effect of both alkaloids on dioxin-mediated induction of Cyp1a1 was determined at mRNA, protein and enzymatic activity levels. Our results showed that dioxin significantly induced the lung Cyp1a1 mRNA by 43,000% compared to control group, whereas harmine significantly decreased dioxin-mediated induction of lung Cyp1a1 mRNA by 44% (Fig. 3.36A). Moreover, dioxin caused induction of lung Cyp1a protein and Cyp1a1 enzymatic activity by 440% and 792%, respectively (Fig. 3.36B & 3.36C). On the other hand, harmine significantly decreased dioxin-mediated induction of lung Cyp1a protein and enzymatic activity by 43% and 60%, respectively (Fig. 3.36B & 3.36C). Taken together, these data demonstrate that the effect of harmine on dioxin-mediated Cyp1a1 is similar in liver and lung tissues.

Similar to harmine, we tested the effect of harmaline on dioxin-mediated induction of lung Cyp1a1 at mRNA, protein and enzymatic activity levels. Our results demonstrated that harmaline decreased dioxin-mediated induction of lung Cyp1a1 mRNA by 34% (Fig. 3.37A), whereas, it significantly decreased dioxin-mediated Cyp1a protein and Cyp1a1 enzymatic activity by 44% and 40%, respectively (Fig. 3.37B & 3.37C). Taken together, these data demonstrate that the effect of harmaline on dioxin-mediated Cyp1a1 is almost the same in lung and liver tissues. Similar to liver tissue, harmine showed a more pronounced effect in

decreasing dioxin-mediated induction of lung Cyp1a1 than that observed with harmaline.

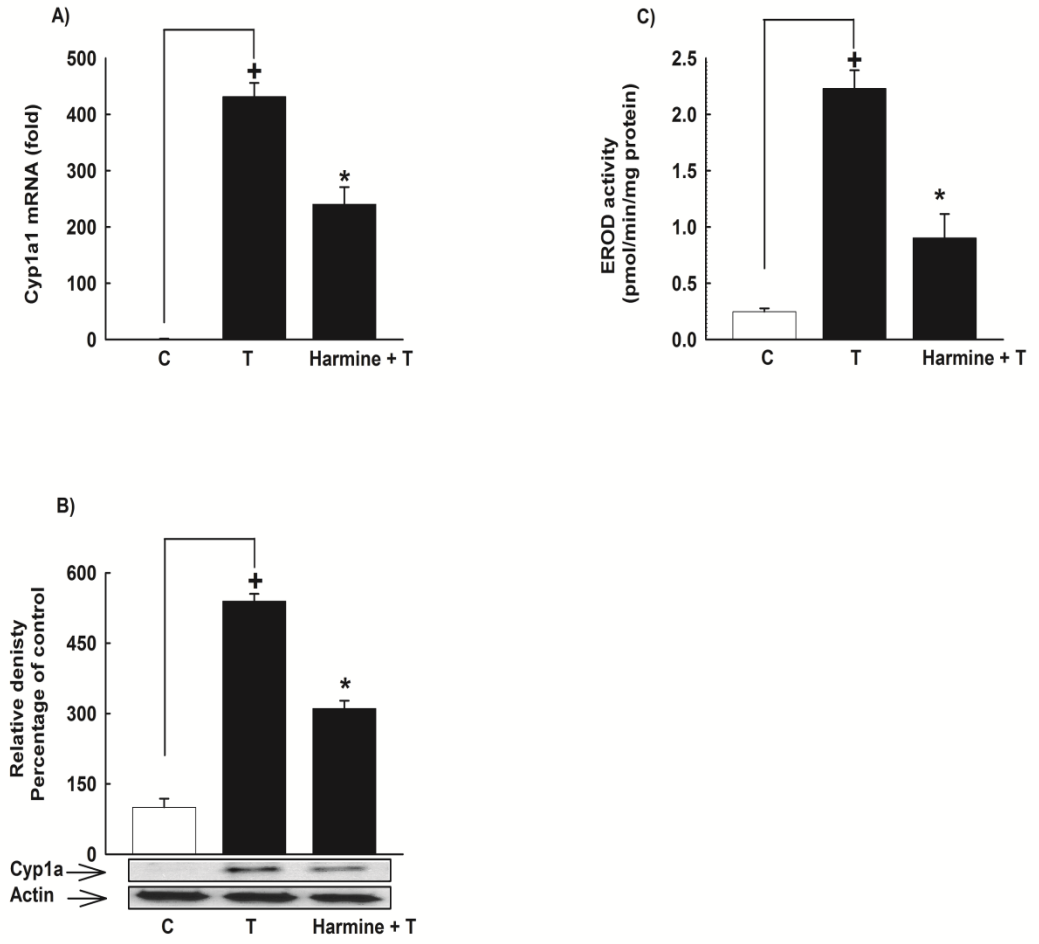


Fig. 3.36. Effect of harmine on dioxin-mediated induction of lung Cyp1a1 at mRNA (A), protein (B) and catalytic activity (C) in C57BL/6 mice. Mice were distributed into several groups, receiving the vehicle (weight-matched control), dioxin (T), or dioxin and harmine. After 14 h from dioxin treatment, mice were sacrificed and the lungs were isolated. (A) Total RNA was isolated using TRIzol reagent and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR and normalized to *18S* reference gene. (B) Cyp1a protein was determined using Western blot analysis. Microsomal protein (2 µg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary anti-mouse Cyp1a antibody for 2 h at room temperature or primary rabbit anti-mouse actin for 24 h at 4°C. Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies, namely, goat anti-mouse IgG for Cyp1a or goat anti-rabbit IgG for actin detection for another 1 h. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to actin signal, which was used as loading control. (C) Cyp1a1 catalytic activity was determined in microsomal fractions using 7ER as a substrate. Values represent the mean of fold change ± S.E.M. (n=6). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

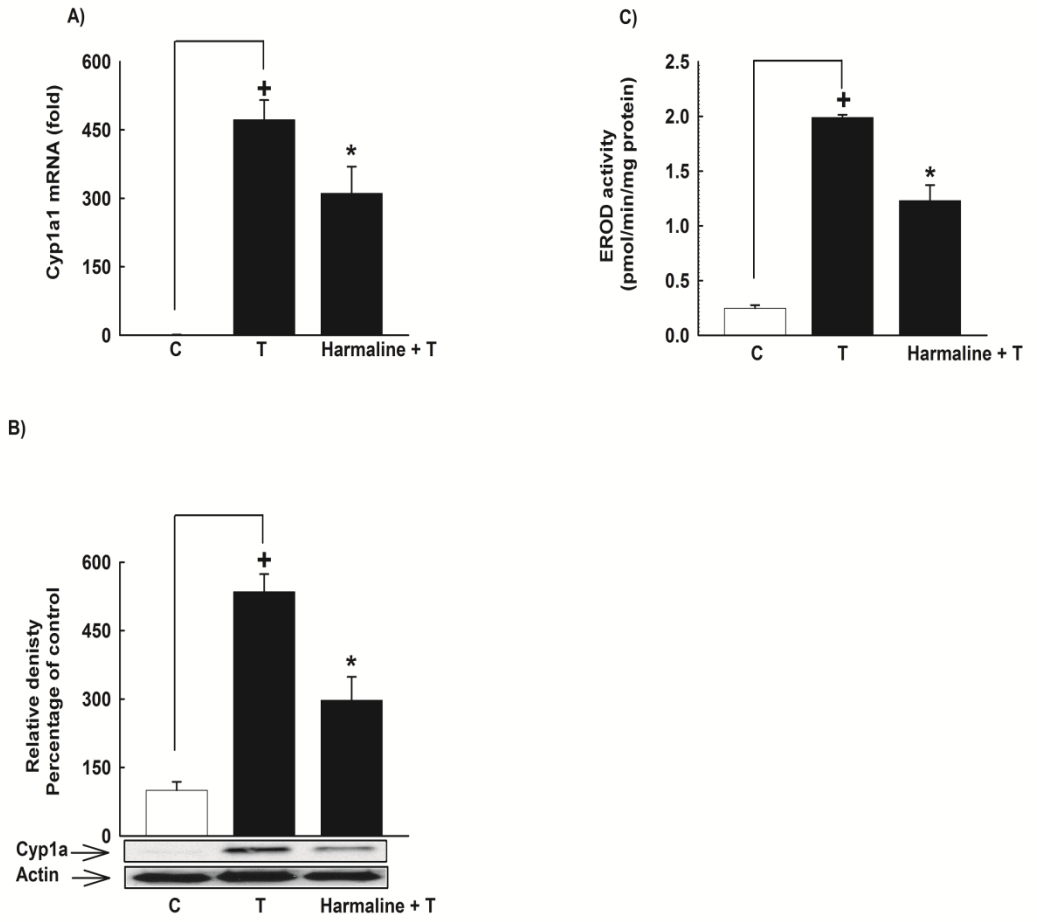


Fig. 3.37. Effect of harmaline on dioxin-mediated induction of lung Cyp1a1 at mRNA (A), protein (B) and catalytic activity (C) in C57BL/6 mice. Mice were distributed into several groups, receiving the vehicle (weight-matched control), dioxin (T), or dioxin and harmaline. After 14 h from dioxin treatment, mice were sacrificed and the lungs were isolated. (A) Total RNA was isolated using TRIzol reagent and microsomal fractions were isolated using ultracentrifugation. The level of Cyp1a1 mRNA was determined using real-time PCR and normalized to *18S* reference gene. (B) Cyp1a protein was determined using Western blot analysis. Microsomal protein (2 µg) was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Protein blots were blocked overnight at 4°C and then incubated with a primary anti-mouse Cyp1a antibody for 2 h at room temperature or primary rabbit anti-mouse actin for 24 h at 4°C. Finally, the membranes were incubated with peroxidase-conjugated secondary antibodies, namely, goat anti-mouse IgG for Cyp1a or goat anti-rabbit IgG for actin detection for another 1 h. Cyp1a protein was detected using the enhanced chemiluminescence method. The intensity of the bands was normalized to actin signal, which was used as loading control. (C) Cyp1a1 catalytic activity was determined in microsomal fractions using 7ER as a substrate. Values represent the mean of fold change ± S.E.M. (n=6). (+) $P < 0.05$ compared with control (C), (*) $P < 0.05$ compared with T.

CHAPTER 4 - DISCUSSION

4.1. *Peganum harmala* Extract Inhibits Dioxin-Mediated Induction of CYP1A1 through AhR-Dependent Mechanism

There are several herbal plants and plant constituents capable of decreasing the induced levels of CYP1A1 (Amakura et al 2002). In this context, it has been reported previously that traditional Chinese herbal plants such as *Oldenlandia diffusa* and *Scutellaria barbata* are used for treatment of liver, lung and rectal tumors. Their antitumor effects were strongly correlated with their capacity to inhibit *β*NF-induced CYP1A1 activity (Wong et al 1993). In addition, Res, a polyphenolic compound found in red wine, grapes and grape juice, was found to have chemopreventative properties by inhibiting human CYP1A1 (Chun et al 1999, Lee and Safe 2001).

Therefore, the aim of this study was to test the capacity of a well-known traditional herbal plant in the Middle East, Asia, and North Africa to interfere with TCDD-mediated AhR-regulated signal transduction. As a measure of its protective effects against TCDD-mediated toxicity and carcinogenicity, we studied the effects of *Peganum harmala* on TCDD-mediated induction of the *CYP1A1* gene expression and hypothesized that it would inhibit that induction.

From ancient times, *Peganum harmala* has been known for its valuable therapeutic effects. It has been used for treatment of different diseases including tumors in Moroccan and Iranian traditional remedies (Lamchouri et al 1999, Sobhani et al 2002). The antitumor properties of *Peganum harmala* extract and harmine (one of its active alkaloids) were confirmed previously, and several mechanisms such as inhibition of cycline dependent kinases and topoisomerases

have been proposed to explain these effects (Jimenez et al 2008, Li et al 2007, Sobhani et al 2002). However, the effect of *Peganum harmala* extract on TCDD-mediated AhR-regulated signal transduction as a measure of its possible chemopreventative and/or antitumor effects was not previously studied.

The active constituents of *Peganum harmala* seed extract are *β*-carboline alkaloids, namely, harmine, and harmaline. Two previous studies reported the amount of harmine and harmaline in *Peganum harmala* seeds using high performance thin layer chromatography. In the first study, the amount of harmine and harmaline was estimated to be 5.5% and 7.9% (w/w), respectively (Sobhani et al 2002). In the second study, harmine and harmaline concentrations were reported to be 0.44% and 0.097% (w/w), respectively (Pulpati et al 2008). Furthermore, a recent study using HPLC demonstrated that harmine and harmaline are the active ingredients of *Peganum harmala* seeds with relative abundances of 4.3% and 5.6% (w/w), respectively (Herraiz et al 2010). Our results for quantification of the active alkaloids in *Peganum harmala* extract differ from these trials. The amount obtained for harmine and harmaline was 7% and 4.85% (w/w), respectively. The discrepancy between our results and those reported earlier (Herraiz et al 2010, Pulpati et al 2008, Sobhani et al 2002), might be attributed to the different techniques used and/or season of harvesting. In the current study, we used a LC-ESI-MS technique, one of the most sensitive techniques, to quantify the active ingredients of *Peganum harmala* extract. On the other hand, harman was not detected in *Peganum harmala* extract. Therefore, it was used as an internal standard.

In the current study, we demonstrated that *Peganum harmala* extract is capable of reducing the TCDD-mediated induction of Cyp1a1 mRNA in a concentration-dependent manner in murine hepatoma Hepa 1c1c7 cells. The effect of *Peganum harmala* extract on TCDD-mediated induction of Cyp1a1 protein was similar to that obtained with mRNA results, suggesting a transcriptional mechanism. In addition, *Peganum harmala* extract reduced the TCDD-mediated induction of Cyp1a1 enzyme activity with all the tested AhR ligands in Hepa 1c1c7 cells. The transcriptional mechanism for Cyp1a1 regulation was supported by two experiments; first, *Peganum harmala* extract inhibited AhR activation and transformation using *in vitro* EMSA of guinea pig hepatic cytosol. Second, *Peganum harmala* extract significantly inhibited TCDD-mediated induction of AhR-dependent luciferase reporter gene assay. On the other hand, the inhibitory effect of *Peganum harmala* extract on Cyp1a1 catalytic activity level appeared to be larger than what would be expected from a transcriptional effect, suggesting that a post-transcriptional mechanism is involved.

Several studies demonstrated that the effects of AhR agonists and antagonists are species-specific (Zhang et al 2003, Zhou et al 2003). Therefore, HepG2 cells were utilized to examine whether the effect of *Peganum harmala* extract on Cyp1a1 in murine Hepa 1c1c7 cells can also be extended in human hepatoma cells. Interestingly, *Peganum harmala* extract significantly decreased the TCDD-mediated induction of CYP1A1 at mRNA, protein and catalytic activity levels. The effect was more pronounced than that observed in murine hepatoma Hepa 1c1c7 cells. Despite the fact that human AhR is known to have a

lower affinity than murine AhR for prototypical AhR ligands such as TCDD (Harper et al 1988, Ramadoss and Perdew 2004), several indole compounds, such as indirubin, showed higher binding and activation of human AhR relative to murine AhR (Flaveny et al 2009, Schroeder et al 2010). Moreover, indirubin showed a higher level of induction of CYP1A1 in human than in murine hepatocytes (Flaveny et al 2009). The presence of several indole alkaloids in *Peganum harmala* may explain its selective higher effect on human CYP1A1 than on its murine counterpart. Similar to Hepa 1c1c7 cells, the inhibitory effects of *Peganum harmala* extract on CYP1A1 catalytic activity is much higher than that observed on CYP1A1 mRNA l, suggesting the involvement of a post-transcriptional mechanism. In this context, it has been previously reported that harmine and harmaline can be metabolized by human CYP1A1 to demethylated metabolites, harmol and harmalol, respectively (Yu et al 2003).

In the current study, we suggested that the effect of *Peganum harmala* extract on the induced CYP1A1 level may be due to the presence of harmine and harmaline alkaloids that can interfere with the metabolic activation of different pro-carcinogens. The antitumor effect of *Peganum harmala* extract is well documented and the relative potencies for harmine and harmaline were reported previously for cytotoxicity and topoisomerase I inhibition activity. The order of their relative potencies is harmine > harmaline (al-Allaf et al 1999, Sobhani et al 2002). This effect is attributed to the planar structure of harmine which is absent in the harmaline structure (Sobhani et al 2002).

In conclusion, the present study demonstrates for the first time that *Peganum harmala L*. inhibits the dioxin-mediated induction of CYP1A1. Thus, these results suggest that *Peganum harmala* may protect against TCDD- and other AhR ligands-mediated effects including carcinogenicity.

4.2. Harman Induces CYP1A1 Enzyme Through an AhR-Dependent Mechanism

Recent studies have demonstrated the wide structural diversity of AhR ligands/agonists and the existence of AhR ligands in several foods and commercial consumer product extracts (Amakura et al 2003, Bohonowych et al 2008). To date, numerous chemicals have been identified as AhR ligands (Denison et al., 2002). Most of them, the "classical" ligands, including PAHs and HAHs, share the structural features of being planar, aromatic, and hydrophobic (Denison and Nagy, 2003). Furthermore, a relatively large number of AhR ligands whose structures and physiochemical characteristics differ from classical ligands have been identified (Denison and Nagy, 2003). Most of these "nonclassical" AhR ligands have a low affinity to the AhR and are relatively weak inducers of CYP1A1, compared with TCDD. A wide range of structural diversity in AhR ligands indicates that a greater spectrum of chemicals can interact with and activate this receptor than previously thought (Denison and Nagy, 2003). Here, our data provide strong evidence that harman, which has a β-carboline structure, is a weak ligand of AhR compared with TCDD, and can directly induce *CYP1A1* gene expression and enzymatic activity in an AhR-dependent manner. In

agreement with our results, it has been previously reported that harman is a weak inducer of AhR activity in MCF7 and H4IIE cells (Hummerich et al 2004, Levitt et al 1977).

AhR has been fully characterized regarding its role in mediating the biochemical activation and toxic effects of a variety of environmental contaminants such as dioxins and related dioxin-like compounds, mainly PAHs and HAHs (Bohonowych et al 2008). It has been previously reported that the genotoxicity associated with cigarette smoke condensate (a source of B[a]P and harman) in mice was abolished by the AhR antagonist MNF (Dertinger et al 2001). AhR ligands can induce carcinogenesis through two main mechanisms; the first group contains agents that contribute directly to carcinogenesis through their metabolism post-AhR binding and causing DNA adducts such as B[a]P, whereas the other mechanism involves agents, such as TCDD, which contribute indirectly to carcinogenesis by inducing persistent CYP1A1 activation that increases the likelihood of other pro-carcinogens being activated (Bradshaw et al 2002).

The harmful effects of most of the chemical carcinogens are attributed to metabolic activation by CYP enzymes to highly reactive metabolites. These reactive metabolites have been proven to cause cancers in several experimental animals as well as humans. CYP1A1 is one of the main CYP enzymes involved in metabolic activation of several environmental contaminants such as PAHs (Mikstacka et al 2008). CYP1A1 is mainly regulated by an AhR-dependent mechanism and its carcinogenic potential in bio-activation of PAHs has been well documented *in vitro* and *in vivo* (Androutsopoulos et al 2009). The biological

importance of CYP1A1 not only involves the metabolic activation of several pro-carcinogens, but also includes the metabolic clearance and elimination of several contaminants. Numerous PAHs are substrates for CYP1A1 and are rapidly metabolized by elevated CYP1A1 levels (Androutsopoulos et al 2009). In this context, harman was found to be a substrate of CYP1A1/1A2 and is metabolized mainly to two major metabolites when incubated with human liver microsomes, namely 3- and 6-hydroxyharman (Herraiz et al 2008). This is in agreement with the common features of AhR ligands which are known to induce CYP1A1 that participates in their metabolism as a method of their detoxification and clearance (Ma and Lu 2007).

Harman is a known mutagenic, co-mutagenic and carcinogenic compound, and high levels of harman are associated with colon cancer (Louis et al 2008, Umezawa et al 1978, Yamashita et al 1988). Therefore, the aim of this study was to investigate the effect of harman on the AhR signaling pathway as a possible mechanism by which harman induces its toxic effects.

Harman is an alkaloid formed inside the body and found in higher levels in cigarette smoke and in several foods and drinks such as certain traditional plants, coffee, soy sauce and over-cooked foods. The daily exposure to harman was calculated previously and was found to range from tens to hundreds of micrograms and can be increased several fold depending on smoking, eating and drinking habits (Herraiz 2004, Pfau and Skog 2004). Several studies determined the human plasma level of harman and it was found to be from 0.004 to 0.3 µg/L (Kuhn et al 1996, Louis et al 2008). The estimated plasma level is much lower

than the daily amount consumed by humans. This lower plasma level may be due to the short $t_{1/2}$ of harman and the possibility of accumulation in body fat; the estimated plasma levels of harman have generally disregarded the dietary sources (Pfau and Skog 2004). However, patients with colon cancers were found to have higher plasma levels of harman than normal patients (Louis et al 2008).

In the current study, we tested the effect of harman on the AhR signaling pathway in HepG2 cells. We found that harman significantly induced CYP1A1 at mRNA in a concentration- and time-dependent manner. Moreover, harman induced CYP1A1 mRNA at early time point, 1 h, suggesting the involvement of a transcriptional mechanism. Pre-treatment of HepG2 cells with the RNA polymerase inhibitor Act-D completely blocked the harman-mediated increase of CYP1A1 mRNA, indicating that harman induced *de novo* mRNA synthesis and suggesting that transcriptional activation of CYP1A1 is required. Similar to mRNA, harman induced CYP1A1 at protein and catalytic activity levels in a concentration-dependent manner. In agreement with our results, it was previously reported that harman significantly induced CYP1A1 mRNA in human breast cancer MCF7 cells (Hummerich et al 2004).

To examine whether the harman-mediated induction of CYP1A1 is species specific, we tested the effect of harman on other cells from a different species: H4IIE rat hepatoma cell line. As with HepG2 cells, harman significantly induced CYP1A1 at mRNA and catalytic activity levels in rat H4IIE cells. In agreement with our results, harman was found previously to induce CYP1A1 mRNA and CYP1A1 catalytic activity in H4IIE cells (Hummerich et al 2004).

Because CYP1A1 transcription is regulated by the AhR, we investigated whether harman is an AhR ligand. First, we examined the induction of CYP1A1 catalytic activity mediated by harman in the presence of a known AhR antagonist; Res. Our results showed that Res significantly inhibited the harman-mediated induction of CYP1A1 catalytic activity, suggesting the involvement of an AhR-dependent mechanism. Second, we used siRNA specific for human AhR to determine its role in the induction mediated by harman. We found that silencing the AhR significantly reduced the induction caused by harman at the protein and activity levels, implying a direct role for the AhR in harman-mediated induction of CYP1A1.

To confirm the transcriptional effects of harman, we tested the effect of harman on XRE-driven luciferase reporter gene that regulates the transcription of CYP1A1. Our results demonstrated that harman significantly increased the XRE-driven luciferase activity in a concentration-dependent manner and further confirmed the transcriptional regulation of CYP1A1 by harman. In addition, we examined the ability of harman to transform and activate the cytosolic AhR to its DNA binding form using guinea pig hepatic cytosol. As shown in the EMSA, harman increased the AhR binding to XRE and further confirmed that harman is a ligand for the AhR.

To examine whether harman induces CYP1A1 solely through a transcriptional mechanism, we tested the effect of harman on the post-transcriptional and post-translational levels in HepG2 cells using Act-D- and CHX-chase experiments, respectively. Our results demonstrated that harman did

not significantly alter the stability of CYP1A1 mRNA and protein levels, indicating that post-transcriptional and post-translational mechanisms are not involved in harman-mediated effect.

Several mechanisms were postulated to explain the harmful effects of harman, including mutagenicity, co-mutagenicity and carcinogenicity. It was reported previously that harman causes DNA injury through intercalation with DNA (Balon et al 1999). Furthermore, it was reported that harman can cause mutations through inhibition of DNA relaxation by affecting topoisomerases I and II (Funayama et al 1996). Moreover, it is well known that AhR ligands cause the induction of CYP1A1, leading to a higher level of oxidative stress. In this context, it has been previously reported that oxidative stress might participate in the mutagenic effect of several AhR ligands such as TCDD (Matsumura 2003, Yoshida and Ogawa 2000). This may explain the mechanism by which harman induces mutagenicity. Moreover, being an AhR ligand, harman can stimulate the metabolic activation of numerous heterocyclic aromatic compounds, leading to formation of several DNA adducts. In agreement with this hypothesis, it was previously reported that aniline and toluidine (present in cigarette smoke and certain vegetables) can bind to harman inside the body and form mutagenic compounds capable of producing DNA adducts (Totsuka et al 2004).

Taken all together, the results provided here present the first evidence that harman directly modulates the expression of CYP1A1 through an AhR-dependent pathway by acting as an AhR ligand. In addition, induction of CYP1A1 by harman may be directly or indirectly involved in harman-induced carcinogenesis.

4.3. Transcriptional and Post-Translational Inhibition of Dioxin-Mediated Induction of CYP1A1 by Harmine and Harmol

The current study provides the first mechanistic evidence that harmine and its metabolite, harmol, significantly inhibit the induction of CYP1A1 by dioxin at the transcriptional and post-translational levels.

AhR activation can result in several biological and toxic effects that depend on the type of AhR ligand (Bradshaw and Bell 2009). The effect of AhR ligands on cell cycle, inflammation, and cancer cell proliferation raised the therapeutic potential for activators/inhibitors of the AhR signaling pathway (Bradshaw and Bell 2009, Zhao et al 2010). Dioxins are metabolically stable AhR ligands that produce a spectrum of TCDD-like AhR-dependent toxicity and carcinogenicity (Mandal 2005). Accordingly, AhR has been used as a target for screening of new chemopreventative agents (Puppala et al 2008). Numerous AhR antagonists have shown promising results against several carcinogen-activating agents. It has been previously reported that the genotoxicity associated with B[a]P in mice was inhibited by AhR antagonists such as MNF and Res (Dertinger et al 2001, Revel et al 2003). However, several AhR antagonists lack specificity and can act as partial agonists; therefore, the search for new AhR antagonists is still in progress (Puppala et al 2008, Signorelli and Ghidoni 2005, Zhou and Gasiewicz 2003).

Harmine is metabolized in the liver and extra-hepatic tissues to its metabolite, harmol, by the cytochrome P450s, mainly CYP2D6 and CYP1A2 (Fig.1.5.) (Yu et al 2003). CYP2D6 is known to have a great polymorphic

variability between individuals that might affect the pharmacological effects and the toxicity of harmine (Callaway 2005). Harmol was found to be conjugated and excreted in human urine mainly as harmol glucuronide (Slotkin et al 1970). Furthermore, it has been demonstrated, using human liver microsomes, that harmine and harmol competitively inhibit CYP2D6 enzyme; however, the median inhibitory concentration for both of them (39 μM for harmine, and 45 μM for harmol) is higher than the concentrations used in the current study (Zhao et al 2011).

It was previously reported that harmine and harmol possess antimutagenic and antigenotoxic effects in yeast and mammalian cells, respectively (Moura et al 2007). These effects have been correlated with the hydroxyl radical-scavenging and the antioxidant properties of harmine and harmol (Moura et al 2007). In our attempt to search for new chemopreventative agents from natural sources, we have shown that the extract of *Peganum harmala* fruiting tops decreased the TCDD-mediated induction of Cyp1a1 at mRNA, protein, and enzyme activity levels in the murine hepatoma Hepa 1c1c7 cell line (El Gendy et al 2010). Furthermore, we confirmed the effect using human HepG2 cells and we identified the AhR signaling pathway as the underlying mechanism of *Peganum harmala* extract. Additionally, harmine was identified as one of the main active ingredients of the plant extract (El Gendy et al 2010). However, the effect of harmine and harmol on dioxin-mediated induction of CYP1A1 has not been studied. Therefore, we determined the effect of harmine and its metabolite, harmol, in human

hepatoma HepG2 cells and compared their effect to that obtained in murine hepatoma Hepa 1c1c7 cells.

In the current study, we tested whether harmine and its metabolite, harmol, alter CYP1A1 in HepG2 cells. Our results showed that harmine significantly reduced the dioxin-mediated induction of CYP1A1 in a concentration-dependent manner at mRNA, protein, and activity levels. In order to test whether the effect of harmine is due to the parent compound or due to its active metabolite, harmol, we examined both compounds in HepG2 cells. Our results demonstrated that similar to harmine, harmol significantly reduced the dioxin-mediated induction of CYP1A1 in a concentration-dependent manner at mRNA, protein, and catalytic activity levels in HepG2 cells. However, the effect of harmine was more pronounced.

The fact that the response of AhR agonists/antagonists can vary between different species (Zhang et al 2003, Zhou et al 2003) prompted us to test the effect of harmine and harmol on murine hepatoma Hepa 1c1c7 cells. Our results showed that both harmine and harmol significantly reduced the dioxin-mediated induction of Cyp1a1 in a concentration-dependent manner at mRNA, protein, and catalytic activity levels in Hepa 1c1c7 cells. In contrast to HepG2 cells, the effect of harmol is greater than the effect of harmine in Hepa 1c1c7 cells. Several postulations have been proposed to explain the species-dependence of some of the AhR antagonists across AhR regulated genes. Of these, intrinsic variation in binding affinity for AhR and the differences in the rate of uptake and metabolism of AhR antagonists are different between different species (Murray et al 2010). In

this context, the differences between the effect of harmine and harmol among different species can be attributed to the structural differences between harmine and its metabolite, harmol. Harmine contains a methoxy group that is altered by metabolism to a hydroxy group in its metabolite, harmol. The differences in structure of harmine and harmol might affect the uptake and the binding of dioxin to AhR and the subsequent recruitment of different co-regulatory proteins among different species (Suzuki and Nohara 2007).

Furthermore, to examine whether the effect of harmine and harmol is AhR ligand specific, we examined the effect of harmine and harmol on ligand-mediated induction of CYP1A1 activity by two different AhR ligands, namely 3MC and βNF. Our results demonstrated that both harmine and harmol significantly inhibited induction of CYP1A1 activity by both AhR ligands in both human and murine hepatoma cell lines, suggesting that the effect of harmine and harmol is not AhR ligand dependent. It has been previously reported that AhR antagonists show variable inhibitory effects against different AhR ligands/agonists. In this context, it was previously reported that TMF antagonizes the effects of TCDD and its related HAH, as well as non-HAH effects (Murray et al 2010). On the other hand, CH223191 was found to preferentially inhibit the effects of HAH, but not other non-HAH compounds such as βNF on the AhR signaling pathway (Zhao et al 2010). Most importantly, both harmine and harmol inhibited the CYP1A1 activity induced by the tested AhR ligands in a similar order in human hepatoma HepG2 cells. However, harmine and harmol showed a different order of inhibition against the tested AhR ligands in murine hepatoma

Hepa 1c1c7 cells. The variation in potency of harmine and harmol against different AhR ligands could be attributed to the differences in structure between harmine and its metabolite, harmol. Harmine and harmol could act as selective AhR modulators and alter the AhR protein or the ligand binding domain on the AhR in such a way that they interact differently with the tested AhR ligands (Zhao et al 2010).

To investigate the underlying mechanisms of harmine and harmol against dioxin-mediated induction of CYP1A1, we tested whether harmine and harmol inhibit CYP1A1 at the transcriptional level. For this purpose we examined the effect of harmine and harmol on the AhR-dependent luciferase reporter assay and AhR activation and transformation using EMSA. As expected, harmine and harmol significantly inhibited the TCDD-induced AhR-dependent luciferase activity. Moreover, both harmine and harmol inhibited the TCDD-mediated activation and binding of AhR to the XRE using EMSA, confirming the involvement of a transcriptional mechanism. Most importantly, the result of the EMSA and luciferase assays demonstrate that neither harmine nor harmol alone significantly induced AhR activation when incubated with guinea pig hepatic cytosols or the AhR-dependent luciferase reporter assay. In the same context, both harmine and harmol alone did not affect CYP1A1 at the catalytic activity level in human and murine hepatoma cells (data not shown). This result suggests that harmine and harmol are not partial agonists for AhR at the concentrations used. To further study whether harmine and harmol are AhR ligands, a ligand competition binding assay using HAP was performed. The assay was carried out

using untreated guinea pig and mouse hepatic cytosols. Our data demonstrated that both harmine and harmol possess an AhR ligand binding affinity both in guinea pig and mouse cytosols. These data are in agreement with the results of EMSA and suggest that both harmine and harmol are AhR antagonists at the tested concentrations.

In addition, we investigated the role of post-transcriptional and post-translational mechanisms using Act-D- and CHX-chase experiments, respectively. Our results demonstrated that harmine and harmol did not alter the stability of CYP1A1 mRNA in HepG2 cells. However, both compounds significantly decreased the CYP1A1 protein stability in HepG2 cells as indicated by the lower $t_{1/2}$ of CYP1A1 protein after incubation either with harmine or harmol. We postulate that the presence of the methoxy or hydroxy group in harmine and harmol aromatic structures, respectively, plays a role in the stability of CYP1A1 protein by both compounds. This conclusion is substantiated by the fact that harman, an aromatic β-carboline which lacks those functioning groups, did not alter the stability of CYP1A1 protein in HepG2 cells (El Gendy and El-Kadi 2010).

Several mechanisms have been proposed to explain the protein degradation, including but not limited to ubiquitin-proteasomal, autophagy-lysosome and calpain pathways (Taguchi et al 2011). However, the ubiquitin-proteasomal pathway possesses an important role in CYP1A regulation (Pollenz 2007, Wiseman and Vijayan 2007). Therefore, we tested the effect of the ubiquitin-proteasomal pathway in the post-translational modifications of harmine

and harmol by using the proteasomal inhibitor MG-132. Our results showed that inhibition of the ubiquitin-proteasomal pathway significantly induced the CYP1A1 protein level of harmine- and harmol-treated cells, implying the involvement of the ubiquitin-proteasomal pathway in the post-translational modifications produced by harmine and harmol. Moreover, the direct inhibitory effect of harmine and harmol on CYP1A1 enzyme was tested. In this assay we incubated HepG2 cells with TCDD for 24 h to induce the level of CYP1A1 enzyme. Thereafter, harmine and harmol (2.5 μM) were added for 15 min before the addition of the substrate (7ER). Our data showed that harmine and harmol significantly reduced the level of CYP1A1 enzyme activity as measured by the EROD assay, suggesting that both compounds possess a direct inhibitory effect on CYP1A1 enzyme that participates in their post-translational modifications. In this context, it has been previously reported, using human liver microsomes, that CYP1A1 may participate in the metabolism of harmine to its metabolite harmol (Yu et al 2003).

In conclusion we demonstrated that harmine and harmol inhibit the dioxin-mediated induction of CYP1A1 at transcriptional and post-translational levels. Furthermore, these data may represent novel mechanisms by which harmine and its metabolite, harmol, inhibit the dioxin-mediated effects.

4.4. Harmaline and Harmalol Inhibit the Carcinogen-Activating Enzyme CYP1A1 via Transcriptional and Post-Translational Mechanisms

To our knowledge, the present study demonstrates for the first time that harmaline and its metabolite, harmalol, significantly inhibit the dioxin-mediated induction of CYP1A1 at the transcriptional and post-translational levels.

Harmaline and harmalol are common dihydro-*β*-carbolines that are widely distributed in the environment including alcoholic beverages and several plants such as *Peganum harmala* (Zygophyllaceae) (Herraiz et al 2010, Park et al 2010). Harmaline and harmalol possess antioxidant and hydroxyl radical-scavenging properties and they are more active than their aromatic *β*-carboline analogues harmine and harmol (Moura et al 2007, Tse et al 1991). Moreover, they inhibit lipid peroxidation in microsomal hepatic preparations (Tse et al 1991). The antioxidant activity of harmaline and harmalol depends mainly on the stabilization of the formed radical by several resonance structures (Moura et al 2007, Tse et al 1991). The antioxidant properties of harmaline and harmalol were postulated to be the main cause of their antimutagenic and antigenotoxic effects in yeast and mammalian cells, respectively (Moura et al 2007). Of particular note, several AhR ligands cause the induction of CYP1A1, leading to higher level of oxidative stress. In this context, it has been previously reported that oxidative stress might participate in the mutagenic effect of several AhR ligands such as TCDD (Matsumura 2003, Yoshida and Ogawa 2000). Therefore, because of their antioxidant properties, harmaline and harmalol can participate indirectly in the protective effects against dioxin-induced toxicity including carcinogenicity.

However, the direct effect of harmaline and harmalol on dioxin-induced CYP1A1 has not been studied before.

In the present study, we tested the effect of harmaline and its metabolite, harmalol, on the dioxin-mediated induction of CYP1A1 enzyme in human hepatoma HepG2 cells. The use of harmaline and its main metabolite was carried out for several reasons. First, to test whether or not the effect of harmaline on dioxin-mediated-induction of CYP1A1 is due to metabolic activation. Our results showed that both the drug and its metabolite possess similar inhibitory effects on CYP1A1 in a concentration-dependent manner at mRNA, protein and catalytic activity levels. The second reason for testing harmalol is the neuropharmacological side effects of harmaline (Abe and Yamada 2009). Finally, harmaline is metabolized in the liver and extra-hepatic tissues to its metabolite, harmalol, by CYP enzymes, mainly CYP2D6 and CYP1A2 (Fig. 1.5) (Wu et al 2009, Yu et al 2003). CYP2D6 is considered one of the most polymorphic phase I drug-metabolizing enzymes and is involved in the biotransformation of 20–30% of marketed drugs and some endogenous substrates (Wu et al 2009). Therefore, the pharmacological effects, clearance and toxic effects of harmaline can be affected by CYP2D6 status (Ho et al 1971, Yu et al 2003). In contrast, the metabolic pathway for harmalol does not involve CYP2D6, but it involves conjugation mainly with glucuronic acid and the resultant conjugate is excreted in urine and bile (Mulder and Hagedoorn 1974). Furthermore, it was reported that harmaline exhibited competitive inhibition against CYP2D6 in human liver microsomes, whereas harmalol did not show similar effects (Zhao et al 2011).

Harmaline concentrations used in this study (0.5-12.5 µM) are much lower than harmaline median inhibitory concentration for CYP2D6 enzyme (26 µM) (Zhao et al 2011).

In the current study, we have chosen human hepatoma HepG2 cells because it is considered as a useful model for investigating the regulation of CYP1A1 expression (Beedanagari et al 2009, Ciolino et al 1998a). In addition, we have chosen harmaline and harmalol (0.5, 2.5 and 12.5 µM) as safe, nontoxic concentrations to human HepG2 cells according to the cell viability test. In the current study, we demonstrated that harmaline and harmalol inhibit TCDD-mediated induction of CYP1A1 at mRNA, protein and catalytic activity levels in human HepG2 cells. Moreover, harmaline and harmalol significantly decreased the B[a]P-mediated induction of CYP1A1 at the catalytic activity level, suggesting that the inhibitory effect of harmaline and harmalol is not AhR ligand specific. In agreement with our results, we previously shown that the extract of *Peganum harmala* fruiting tops that possesses harmaline as one of its main active ingredients decreased the TCDD-mediated induction of Cyp1a1 in the murine hepatoma Hepa 1c1c7 cell line (El Gendy et al 2010). In contrast to our results, other *β*-carbolines such as rutaecarpine (found in *Rutaceae* and szechuan pepper), annomontine (found in *Annonaceae* and marine sponges of the order *Petrosiidae*) and xestomanzamine A (contained in *Petrosiidae* sponges), have been found to induce CYP1 enzyme activity (Haarmann-Stemmann et al 2010).

Interestingly, the effects of harmaline and harmalol on the CYP1A1 protein and the activity levels are much higher than the effects obtained at the

mRNA level, suggesting several mechanisms are involved in the obtained results. Therefore, to investigate the underlying mechanisms involved in the effects of harmaline and harmalol on CYP1A1, we tested whether harmaline and harmalol inhibit CYP1A1 at the transcriptional level. For this purpose we examined the effect of harmaline and harmalol on the AhR-dependent luciferase reporter assay and EMSA. Our results showed that harmaline and harmalol significantly inhibited the TCDD-induced AhR-dependent luciferase activity. Furthermore, both harmaline and harmalol inhibited the TCDD-mediated activation of AhR binding to the XRE using EMSA, confirming the involvement of a transcriptional mechanism. In agreement with the luciferase activity, neither harmaline nor harmalol alone significantly induced AhR activation or transformation when incubated with guinea pig hepatic cytosols using EMSA. Moreover, harmaline and harmalol alone did not affect CYP1A1 at the catalytic activity level in human HepG2 cells. These results suggest that both harmaline and harmalol are not partial agonists for AhR at the used concentrations. Furthermore, these results are in contrast to the aromatic β-carboline compound harman that showed an induction of CYP1A1 and luciferase activity in human HepG2 cells and an induction of the AhR activation and transformation in untreated guinea pig hepatic cytosols (El Gendy and El-Kadi 2010). These observations suggest that both harmaline and harmalol are AhR antagonists. To confirm that harmaline and harmalol are AhR antagonists, we performed a ligand competition binding assay. Our data showed that harmaline, but not harmalol, possesses the ability to displace [^{3}H]TCDD from the binding site on AhR. These data suggest that the

underlying mechanism of harmalol's inhibitory effect is due to other cascades in the process of AhR activation rather than a competing for the ligand-binding center of the AhR. We postulate two mechanisms responsible for the inhibitory effect of harmalol on AhR. First, harmalol may interact with a second binding site on the AhR rather than the TCDD-binding site (Ciolino et al 1998a). This AhR binding site has been proposed for AhR-dependent induction of CYP1A1 by carotenoids and primaquine. This was substantiated by the fact that these compounds did not show a significant competition with the [^{3}H]TCDD binding site on the AhR (Delescluse et al 2000, Fontaine et al 1999, Gradelet et al 1997). Second, harmalol may indirectly affect AhR (Ciolino et al 1998a). There are several proteins participating in the activation process of AhR, such as HSP90 (Palermo et al 2005, Pratt 1997), c-SRC (Enan and Matsumura 1996), the AhR-interacting protein (XAP2) (Ma and Whitlock 1997), PKC (Long et al 1998), and the proteins responsible for the recruitment of AhR or ARNT to the XRE of the corresponding genes (Beedanagari et al 2009). Harmalol may inhibit TCDD-mediated activation of AhR by affecting one or more of these target proteins without affecting the ligand binding site of the AhR.

Also, we investigated the role of post-transcriptional and post-translational mechanisms using Act-D- and CHX-chase experiments, respectively. Our results demonstrated that harmaline and harmalol did not alter the stability of CYP1A1 mRNA in HepG2 cells. However, both compounds significantly decreased the CYP1A1 protein stability in HepG2 cells. We postulate that the presence of methoxy or hydroxy groups in the uncompleted aromatic structures of harmaline

and harmalol, respectively, plays a role in the stability of CYP1A1 protein produced by both compounds. This conclusion is substantiated by the fact that harman, an aromatic β-carboline, which lacks those functioning groups, did not alter the stability of CYP1A1 protein in HepG2 cells (El Gendy and El-Kadi 2010).

In order to investigate the underlying mechanism behind the post-translational modifications of harmaline and harmalol, we tested the role of the ubiquitin-proteasomal pathway, which possesses a key role in CYP1A regulation (Pollenz 2007, Wiseman and Vijayan 2007). Therefore, we tested the effect of MG-132, a proteasomal inhibitor, on the reduced CYP1A1 protein stability caused by harmaline and harmalol. Our results demonstrated that inhibition of ubiquitin-proteasomal pathway significantly induced the CYP1A1 protein level of harmaline- and harmalol-treated cells, implying the involvement of the ubiquitin-proteasomal pathway in the post-translational modifications of harmaline and harmalol. In addition, we tested the direct inhibitory effect of harmaline and harmalol on CYP1A1 enzyme. Incubation of harmaline and harmalol for 15 min with HepG2 cells pre-treated with TCDD for 24 h significantly reduced the level of CYP1A1 enzyme activity as measured by EROD assay, suggesting that both compounds possess a direct inhibitory effect on CYP1A1 enzyme that participates in their post-translational modifications. In this context, it has been previously reported, using human liver microsomes, that CYP1A1 may participate in the metabolism of harmaline to its metabolite harmalol (Yu et al 2003).

In conclusion, harmaline and harmalol inhibit the dioxin-mediated induction of CYP1A1 at transcriptional and post-translational levels. Furthermore, these mechanisms may participate, at least in part, in the protective effects of both harmaline and its metabolite, harmalol, against dioxin-mediated toxicity.

4.5. Harmine and Harmaline Downregulate Dioxin-Induced Cyp1a1 in the Livers and Lungs of C57BL/6 Mice

The present study demonstrates for the first time that harmine and harmaline significantly decreased the dioxin-mediated induction of the carcinogen-activating enzyme Cyp1a1 livers and lungs of mice.

Cancer development is a multi-stage process that involves several factors. Inherited genetic factors can explain the incidence of 5-15% of most cancers, but environment and lifestyle are the major factors contributing to cancer development (Safe et al 2008). Dioxin is a widely distributed environmental pollutant that is usually released in the environment from several sources such as waste incinerators, ferrous and non-ferrous metal production, herbicide manufacturing and power generation (Marinkovic et al 2010). Several accidents and occupational exposures to dioxin demonstrated the role of dioxin in the increased risk of cancer incidence and mortality (Warner et al 2011). Dioxin is a metabolically stable AhR ligand, and several adverse effects of dioxin exposure are related to the persistent activation of the AhR signaling pathway. In agreement with this hypothesis are the results of experiments using transgenic mice in which AhR function has been compromised. It has been demonstrated that the dioxin-

mediated adverse effects are attenuated in mice possessing disrupted AhR function (Denison et al 2011, Gonzalez and Fernandez-Salguero 1998).

Harmine is an aromatic *β*-carboline compound that is structurally similar to its dihydro-*β*-carboline analogue, harmaline (Fig. 1.5). We have demonstrated that both harmine and harmaline are capable of inhibiting dioxin-mediated induction of Cyp1a1 in murine hepatoma Hepa 1c1c7 cells (El Gendy et al 2010). Moreover, we demonstrated that both compounds can as AhR antagonists. Finally, we confirmed that harmine and harmaline possess post-translational modification by which they reduce the CYP1A1 protein stability in human hepatoma HepG2 cells (El Gendy et al 2012a, El Gendy et al 2012b). Therefore, we hypothesized that the effect of harmine and harmaline can be translated *in vivo* using a responsive mice strain, C57BL/6 mice. Moreover, we tested whether or not the effect of harmine and harmaline can be demonstrated in other extra-hepatic tissues, using lungs as a representative tissue.

In the current study, we have chosen the C57BL/6 mouse strain as it contains a responsive AhR allele (AhR^b) (Denison et al 2011). Regarding the selection of dioxin dose, it is known that dioxin is a metabolically stable compound and its $t_{1/2}$ has been previously determined in mice to be around 20 days (Koshakji et al 1984). Several concentrations of dioxin has been examined previously for Cyp1a1 induction and AhR activation in the same mouse strain and it was demonstrated that 15 µg/kg (i.p.) provides a submaximal saturation/activation of the AhR (Nazarenko et al 2001). Additionally, harmine and harmaline doses have been selected according to their half-lives. It has been

demonstrated previously that harmine possesses a short $t_{1/2}$ in rodents estimated to be around 20 min, whereas harmaline possesses a relatively longer $t_{1/2}$, around 60 min (Zetler et al 1974). Therefore, we thought that multiple doses of both alkaloids would be advantageous. Most importantly, 10 mg/kg body weight for three doses has been selected based on preliminary experiments, in which we could not detect any effect with lower doses. On the other hand, it is well established that the major drawbacks of using these β-carboline alkaloids are their tremorgenic side effects (Cao et al 2007). In our study, slight to moderate tremors have been detected for harmine and harmaline in the first dose with higher effect with harmaline. However, these tremors decreased dramatically in the subsequent doses.

There are several reasons behind the choice of liver tissue in our study. First, we have previously studied the effect of harmine and harmaline using different human and murine hepatoma cells. Second, it is well established that dioxin is concentrated in the body mainly in adipose tissue and liver (Hu and Bunce 1999). Dioxin is sequestered in the liver by liver-specific microsomal binding proteins (Diliberto et al 1997). Third, active AhR is an important factor for developing dioxin-mediated hepatocellular toxicity (Nukaya et al 2010). Finally, the liver is the place of maximum metabolism and highest amounts of CYP enzymes, with a maximum level of CYP1A1 induction. On the other hand, lung has been selected in our study because it is one of the highly exposed organs to environmental pollutants through smoking and air pollutants. Furthermore,

several studies have correlated the induction of CYP1A1 enzymatic activity with the development of lung cancer (Oyama et al 2007).

In the current study, harmine and harmaline significantly decreased dioxin-mediated Cyp1a1 induction in mouse liver and lung. Harmine showed a greater effect than harmaline in both liver and lung. The differences between the effect of harmine and harmaline can be attributed to two main reasons. First, there is a structural difference between both alkaloids. Harmine has an aromatic planar structure that can enhance its binding ability to AhR, whereas harmaline possesses a co-planar structure. In this context, we have previously demonstrated that harmine efficiently displaced radiolabeled-TCDD in a ligand competition binding assay, whereas harmaline showed only a modest effect (El Gendy et al 2012a, El Gendy et al 2012b). Second, harmine and harmaline possess different pharmacokinetic parameters. It was estimated that the ability of harmine to concentrate in lung is more than that observed for harmaline in rodents (Zetler et al 1974).

In conclusion, harmine and harmaline decrease the dioxin-mediated induction of the carcinogen-activating enzyme Cyp1a1 in C57BL/6 mice liver and lung. These data provide the first evidence that harmine and harmaline can prevent the adverse effect of dioxins and other AhR ligands *in vivo*.

4.6. General Conclusion

Cancer is a major health problem worldwide and it comes as a second cause of death after heart diseases in North America (American Cancer Society

and Canadian Cancer Society). Several factors have been proposed to explain the process of carcinogenesis including lifestyle and exposure to environmental pollutants. It is important to note that most of environmental carcinogens are inert and they require metabolic activation to exert their toxic and carcinogenic effects. In the same context, AhR, a ubiquitous transcriptional factor, plays a key role in the process of chemical carcinogenesis. It regulates several enzymes, such as CYP1A1, that are capable of activating pro-carcinogens to their ultimate carcinogenic forms. Several AhR ligands, such as dioxins and B[a]P, have been demonstrated to cause numerous side effects including cancer. Most importantly, several AhR antagonists prevent the genotoxicity produced by AhR ligands in mice. Therefore, the AhR has been recognized as a target for new chemopreventative/antitumor agents.

In the present work, we have demonstrated that *Peganum harmala*, a common traditional plant in Middle East, Asia, and North Africa, significantly inhibited the dioxin-mediated induction of the carcinogen-activating enzyme CYP1A1 both in murine and human hepatoma cells. Moreover, we identified that the effect of *Peganum harmala* involves the modulation of the AhR signaling pathway. Additionally, we identified the *β*-carboline alkaloids harmine and harmaline as the active constituents of the plant extract. We concluded that *β*-carboline compounds can modulate the AhR signaling pathway.

The identified active alkaloids, harmine and harmaline, and their metabolites, harmol and harmalol, as well as harman, a widely distributed parent nucleus structure of harmine and harmol, were examined on the carcinogen-

activating enzyme CYP1A1 at the catalytic activity level. Out of the five compounds, only harman significantly induced CYP1A1 catalytic activity. Harman has mutagenic and co-mutagenic effects and higher plasma levels have been correlated to increased incidence of colon cancer. Therefore, we tested the effect of harman on the AhR signaling pathway. Our data demonstrated that harman significantly induced CYP1A1 at mRNA, protein and catalytic activity levels in HepG2 cells. In addition, harman significantly induced CYP1A1 at mRNA and catalytic activity levels in rat H4IIE cells, suggesting that the effect of harman is not species specific. Moreover, we confirmed that the induction of CYP1A1 by harman is AhR-dependent using AhR-dependent luciferase assay, *in vitro* EMSA, and a genetic approach using siRNA against AhR. We demonstrated that the effect of harman was mainly through a transcriptional mechanism as harman did not modulate CYP1A1 at post-transcriptional and post-translational levels. Moreover, the AhR antagonist, Res, inhibited the increase in CYP1A1 activity by harman and the RNA polymerase inhibitor, Act-D, completely abolished the CYP1A1 mRNA induction by harman, indicating a transcriptional activation.

On the other hand, the effect of harmine and harmaline and their metabolites harmol and harmalol was investigated on dioxin-mediated AhR-regulated signal transduction. The four alkaloids showed a significant decrease of dioxin-mediated induction of CYP1A1 at mRNA, protein and catalytic activity levels. The effect of alkaloids on CYP1A1 catalytic activity was much higher than that observed at mRNA levels, suggesting that several mechanisms are involved

in the obtained results. Moreover, the four compounds showed a similar decrease of CYP1A1 catalytic activity using different AhR ligands, suggesting that the effect is AhR-ligand independent. Additionally, we confirmed the role of AhR in the obtained effects using AhR-dependent luciferase assay and *in vitro* EMSA. Furthermore, harmine, harmol and harmaline significantly displaced radiolabeled-TCDD from the AhR binding site, suggesting that these compounds are AhR antagonists at the concentrations used. However, harmalol has been suggested to affect AhR signaling pathway indirectly (i.e. affecting other proteins participating in the activation of AhR or to interact with a second binding site on the AhR rather than the TCDD-binding site). Although harmine, harmol, harmaline and harmalol did not affect CYP1A1 post-transcriptionally, all compounds decreased the stability of CYP1A1 protein, suggesting a role of post-translational modifications. The post-translational modifications by the four compounds were confirmed by identifying a role of ubiquitin-proteasomal pathway as well as the direct inhibitory effect of the alkaloids on CYP1A1 enzyme. We proposed that the presence of a substitution at C7 of *β*-carboline structure might participate in the post-translational modification of harmine, harmol, harmaline and harmalol. This hypothesis is substantiated by the fact that harman, which lacks a substitution at C7 position, did not show any effect on CYP1A1 protein stability. Moreover, the complete aromatic structure is not essential in the obtained post-translational effect as both *β*-carbolines (harmine and harmol) and dihydro-*β*-carboline (harmaline and harmalol) possess a similar activity.

In order to test whether the *in vitro* effects of harmine and harmaline will be translated to relevant effects *in vivo*, harmine and harmaline were administered with dioxin to the AhR-responsive mouse strain, C57BL/6. Thereafter, the effect of harmine and harmaline on dioxin-mediated induction of Cyp1a1 mRNA, protein and activity was determined in mouse livers and lungs. Our results showed that harmine and harmaline significantly decreased the dioxin-mediated induction of Cyp1a1 in both the liver and lung tissues, with more pronounced effect of harmine in both tissues. The differences between harmine and harmaline may be attributed to the planar structure of harmine that affects its binding ability to AhR as well as the differences in the pharmacokinetic parameters between the two compounds.

In conclusion, our work demonstrates that *Peganum harmala* extract inhibited the dioxin-mediated induction of the carcinogen-activating enzyme CYP1A1 through an AhR-dependent mechanism. The active ingredients of the plant extract, harmine and harmaline and their metabolites, harmol and harmalol, are promising candidates for cancer chemoprevention and/or treatments through interfering with AhR activation as well as inhibiting CYP1A1 post-translationally. The effect of harmine and harmaline was confirmed *in vivo* using a responsive mouse strain. On the other hand, harman, the parent nucleus of harmine and harmol, was found to induce CYP1A1 enzyme through an AhR-dependent mechanism.

4.7. Future Directions

The results of the present work have highlighted the effect of *Peganum harmala* and its active alkaloids on dioxin-mediated effects including carcinogenicity. However, additional experiments need to be conducted in order to bring the obtained results into clinical practice.

(1) To investigate the effect of the active alkaloids on the formation of DNA adducts using several carcinogenic agents such as B[a]P.

(2) To investigate the chemopreventative and/or the antitumor effect of these active compounds on tumor initiation and progression *in vivo*.

(3) To synthesize different derivatives from the active alkaloid(s) and test their potential as chemopreventative and/or antitumor agents.

(4) To determine the pharmacokinetic parameters as well as the plasma concentration levels for the active alkaloids in mice.

CHAPTER 5 - REFERENCES

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