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XENOBIOTICS-INDUCED PHOSPHORYLATIONS OF MDM2

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ABSTRACT

The aim of the present study was to characterize the Mdm2 and p53 responses induced by DNA damaging xenobiotics and how these responses can be modified by non-genotoxic xenobiotics. During control conditions cellular levels of p53 are low. The level is regulated by Mdm2 and Mdm2 and p53 forms an autoregulatory loop. When DNA is damaged, the p53-Mdm2 feedback loop is perturbed, and the cellular levels of p53 are increased so that p53 can regulate the transcriptional activity of proteins mediating responses such as apoptosis or cell cycle arrest. DNA damage may induce posttranslational modifications on p53 and Mdm2, such as phosphorylation or dephosphorylation induced by different kinases. These posttranslational modifications may modify the functions of Mdm2 and p53. This study focuses on Mdm2 phosphorylation within the epitopes of antibody 2A10, and phosphorylation of Mdm2 on Ser166.

Both TCDD and the cholesterol lowering drugs statins were shown to attenuate the p53 stabilization in response to DNA damaging agents in HepG2 cells and in rodents. TCDD is a persistent, bioaccumulating pollutant. It is a carcinogen without being genotoxic. The detailed mechanisms for cancer induction are unknown, however. Statins are drugs used to treat hypercholesterolemia, and have also anticarcinogenic properties. The attenuation of p53 DNA damage response was associated with Mdm2 phosphorylation on Ser166. Thus, statins and TCDD induces Mdm2 Ser166 phosphorylation and attenuates p53 stabilization in response to DNA damage in HepG2 cells. TCDD also attenuated p53 stabilization in response to DNA damage in rats. As statin-induced Mdm2 Ser166 phosphorylation was attenuated by rapamycin (an inhibitor of mTOR) and Mdm2 Ser166 phosphorylation occurred in parallel to mTOR phosphorylation, it is likely that mTOR induced Ser166 phosphorylation. Our findings thus suggests that mTOR is one of the kinases inducing Mdm2 Ser166 phosphorylation. TCDD- and statin-induced attenuation of p53 response might interfere with the cells ability to handle genotoxic agents. TCDD has been associated with cancer, especially liver cancer in rodents. Statins, on the other hand, rather seems to have anticarcinogenic properties. However, if statins are used together with genotoxic substances, attenuation of p53 response could affect its capacity to kill cells.

Mdm2 phosphorylation within the epitopes of antibody 2A10 occurred at much lower concentrations of genotoxic substances benzo[*a*]pyrene, BPDE, mitomycinC and etoposide than those inducing p53 stabilization. This suggests that Mdm2 can be used as a marker for certain types of DNA damage. Mdm2 2A10 phosphorylation induced by lower doses than those inducing p53 stabilization did not occur after exposure to dibenzo[*a,l*]pyrene and its metabolite DBPDE. We suggest that Mdm2 phosphorylated within the 2A10 epitopes might be involved in DNA repair of BPDE adducts, since BPDE induces 2A10 phosphorylation and DNA repair while DBPDE do not. Mdm2 has been shown to interact with proteins involved in DNA repair such as DNA polymerase epsilon and Nbs1. Mdm2 also partly localize to double stranded DNA breaks in response to IR irradiation. Mdm2 2A10- and p53 phosphorylation in response to DNA damage were shown to be induced by different signalling pathways.

LIST OF PUBLICATIONS

I. Pääjärvi, G., Viluksela, M., Pohjanvirta, M., Stenius, U. and Högberg, J. (2005) TCDD activates Mdm2 and attenuates the p53 response to DNA damaging agents. *Carcinogenesis*, **26**, 201-208.

II. Pääjärvi, G., Roudier, E., Crisby, M., Högberg, J. and Stenius, U. (2005) HMG-CoA reductase inhibitors, statins, induce phosphorylation of Mdm2 and attenuate the p53 response to DNA damage. *FASEB J.*, **19**, 476-478.

III. Pääjärvi, G., Jernström, B., Stenius, U. and Seidel, A. (2004) Exposure of mammalian cells to diol epoxides from benzo[*a*]pyrene and dibenzo[*a,l*]pyrene and effects on Mdm2. *Polycyclic Aromatic Compounds*, **24**, 537-548.

IV. Pääjärvi, G., Jernström, B., Seidel, A. and Stenius, U. *Anti*-diol epoxide from benzo[*a*]pyrene induces transient Mdm2 phosphorylation/chromatin binding, while *anti*-diol epoxide from dibenzo[*a,l*]pyrene induces a persistent p53 Ser15 phosphorylation. Submitted.

V. Malmlöf, M., Pääjärvi, G., Högberg, J. and Stenius, U. Benzo[*a*]pyrene and other DNA damaging compounds, but not dibenzo[*a,l*]-pyrene, induce Mdm2 alterations and chromatin binding; Mdm2 as a sensitive marker for genotoxicity. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

2A10-epitopes	Epitopes for Mdm2 antibody 2A10
AhR	Aryl hydrocarbon receptor
BP	Benzo[*a*]pyrene
BPDE	The carcinogen metabolite of benzo[*a*]pyrene
DBP	Dibenzo[*a,l*]pyrene
DE	Diol epoxide
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
NER	Nucleotide excision repair
PAH	Polycyclic aromatic hydrocarbons
TCDD	2,3,7,8-Tetrachlorodibenzo-p-Dioxin

1 GENERAL BACKGROUND

In this thesis the tumor suppressor protein p53 and its regulator protein Mdm2 have been studied. Thus, the effects of DNA damaging agents on Mdm2 and p53 and how TCDD (dioxin, a carcinogenic environmental contaminant) and statins (cholesterol lowering drugs) modifies Mdm2 and p53 response to DNA damaging agents have been studied in some detail.

1.1 Mdm2 and p53

1.1.1 p53 and cancer

p53 controls the levels of several proteins important for the cell's ability to respond to DNA damage and other types of stress (Meek 2004). p53 can regulate cell cycle arrest and apoptosis (programmed cell death) (Vousden and Lu 2002). These responses are important, since they may stop cells with damaged DNA from proliferating. Cells with DNA alterations might otherwise be transformed to tumor cells. A few important examples of the many targets of p53 transcriptional activities mediating the cell cycle arrest and apoptosis responses are p21, Bax and PUMA (Meek 2004, Vousden and Lu 2002). Direct mutational inactivation of the p53 gene has been found to be one of the most frequent single genetic alterations associated with human cancer (Harris 1996). If p53 is not functional, cells with damaged DNA might be able to proliferate and transform to tumor cells.

1.1.2 The Mdm2-p53 feedback loop

During control conditions p53 is continuously produced and degraded and cellular levels of p53 are low. This is regulated by Mdm2 and Mdm2 and p53 forms an autoregulatory loop (Levine 1997, Oren et al 2002), see Figure 1. p53 increases Mdm2 level through transcriptional activation on the Mdm2 P2 promoter (Zauberman et al 1995). Mdm2 decreases p53 levels by increasing ubiquitination and proteasomal degradation of p53. Furthermore, binding of Mdm2 to the transactivation domain of p53 may inhibit p53 from regulating cellular levels of important proteins (Meek 2004). Mdm2 can also mediate translocation of p53 to the cytoplasm, thereby removing it from its site of action (Meek 2004). Basal levels of Mdm2 are also regulated p53 independent by the Mdm2 P1 promoter (Zaubermann et al 1995). Deletion of Mdm2 results in embryonic lethality due to apoptosis. If p53 is deleted simultaneously, Mdm2 knockout is not lethal (Chavez-Reyes et al 2003). This show the importance of Mdm2 in regulating p53 levels during development.

When DNA damage occurs, the autoregulatory loop is perturbed and p53 accumulates in the cell (Meek 2004). This p53 accumulation stops damaged cells from proliferating by inducing cell cycle arrest or apoptosis (Vousden and Lu 2002).

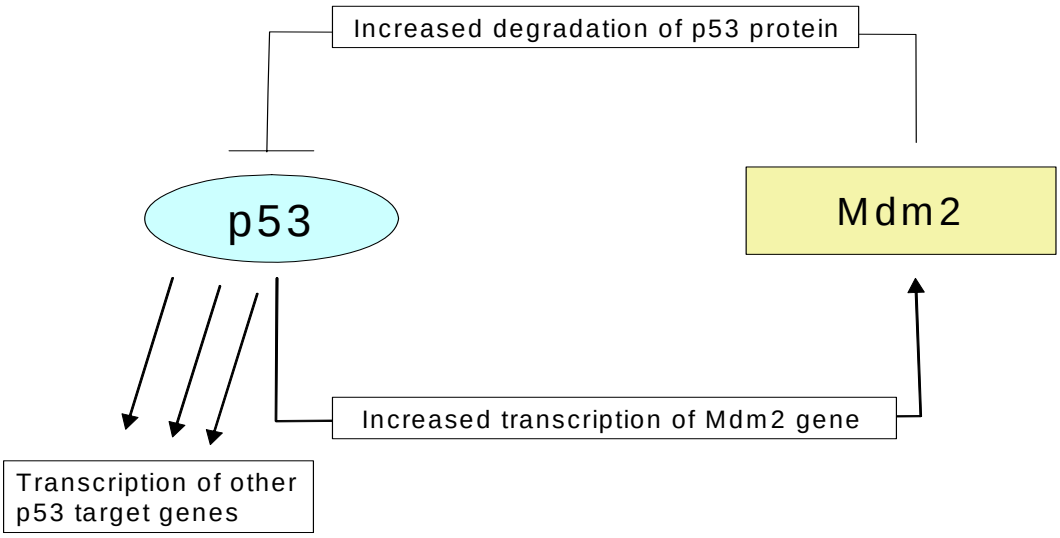


Figure 1. The Mdm2-p53 autoregulatory feedback loop
Mdm2 maintains low cellular level of p53 through ubiquitination and degradation of p53. p53 increases transcription of Mdm2 and this increases the Mdm2 level in the cell. Through this loop p53 is kept at a low level despite continuous synthesis.

1.1.3 Posttranslational modifications of Mdm2 and p53

When DNA is damaged, the Mdm2-p53 feedback loop is perturbed, and the cellular levels of p53 increase so that p53 can regulate the transcriptional activity of proteins mediating responses such as apoptosis or cell cycle arrest (Meek 2004). DNA damage may induce posttranslational modifications on p53 and Mdm2, such as phosphorylations or dephosphorylations induced by different kinases. These posttranslational modifications may modify the functions of Mdm2 and p53 (Meek 2004, Meek and Knippschild 2003). This study focuses on Mdm2 phosphorylation within the epitope of antibody 2A10, and phosphorylation of Mdm2 on Ser166.

Phosphorylations of Mdm2 within the 2A10 antibody epitopes.

The 2A10 antibody detects two epitopes on the Mdm2 protein (Meek and Knippschild 2003). One of the epitopes is located within the central acidic domain of Mdm2. There are several phosphorylation sites within this epitope. The kinases mediating the phosphorylations are not thoroughly known, but there are some kinases known to be able to phosphorylate these sites. ATM can phosphorylate Mdm2 within this epitope (Meek and Knippschild 2003, Maya et al 2001, Balass et al 2002). CK2 and CK1δ can also phosphorylate Mdm2 on multiple sites *in vitro* (Allende-Vega et al 2005, Winter et al 2004). Mdm2 has been reported to be phosphorylated in the central acidic domain in cells without DNA damage (Meek and Knippschild 2003). Recently, it has been shown that phosphorylation of Mdm2 in the central domain (within the 2A10 epitope) enhances binding between p53 and Mdm2 (Kulikov et al 2006). Phosphorylation within the central acidic domain of Mdm2 has been reported to be

essential for p53 degradation (Ma et al 2006). A study has shown that Mdm2 is dephosphorylated in the central acidic domain in response to ionizing radiation thereby possibly hindering p53 degradation (Blattner et al 2002).

There is also another epitope, including Tyr394 and Ser395, that antibody 2A10 recognises. Phosphorylation sites within this epitope are Ser395 which have been shown to be mediated by ATM, and Tyr394 mediated by c-Abl (Maya et al 2001, Balass et al 2002, Goldberg et al 2002). DNA damage activated ATM and c-Abl might mediate Ser395 and Tyr394 phosphorylation. These phosphorylations seems to mediate a proapoptotic response since they decrease interaction between Mdm2 and p53, resulting in p53 stabilization (Meek and Knippschild 2003).

The detectability of Mdm2 with the 2A10 antibody is decreased if Mdm2 is phosphorylated within the epitopes of the antibody. If Western blot membranes are treated with alkaline phosphatase, the detectability is increased (see Figure 2), and the increased detectability might be interpreted as reflecting the fraction of phosphorylated Mdm2 within the epitope of the 2A10 antibody (Maya and Oren 2000). It has been suggested that since the 2A10 antibody recognise two binding sites on Mdm2, different Mdm2 interacting proteins might recognise both of the 2A10 binding sites (Maya et al 2001)

Several studies have indicated that ATM may phosphorylate Mdm2 in response to γ-irradiation and X-irradiation. It seems not to occur in response to UV-irradiation. Phosphorylation of Mdm2 by ATM occurs both within the epitope of antibody 2A10 and outside the epitopes of this antibody. Phosphorylation by ATM reduces detectability both with antibodies 2A10 and SMP- 14 (Maya et al 2001, de Toledo et al 2000, Khosravi et al 1999).

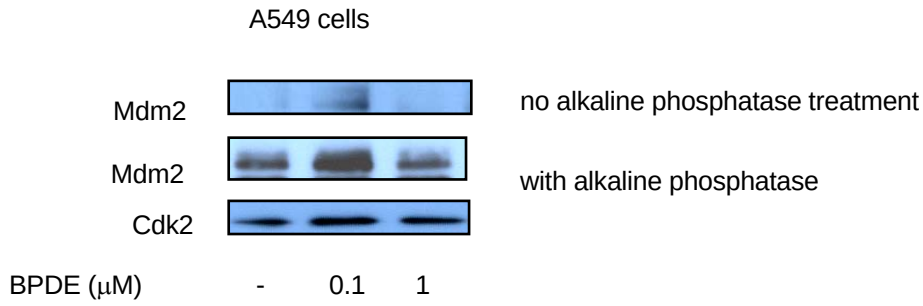


Figure 2. The detectability of Mdm2 with the 2A10 antibody is increased if Western blot membranes are treated with alkaline phosphatase. This indicates that phosphorylations on Mdm2 which hinders recognition by the antibody has been removed by the treatment.

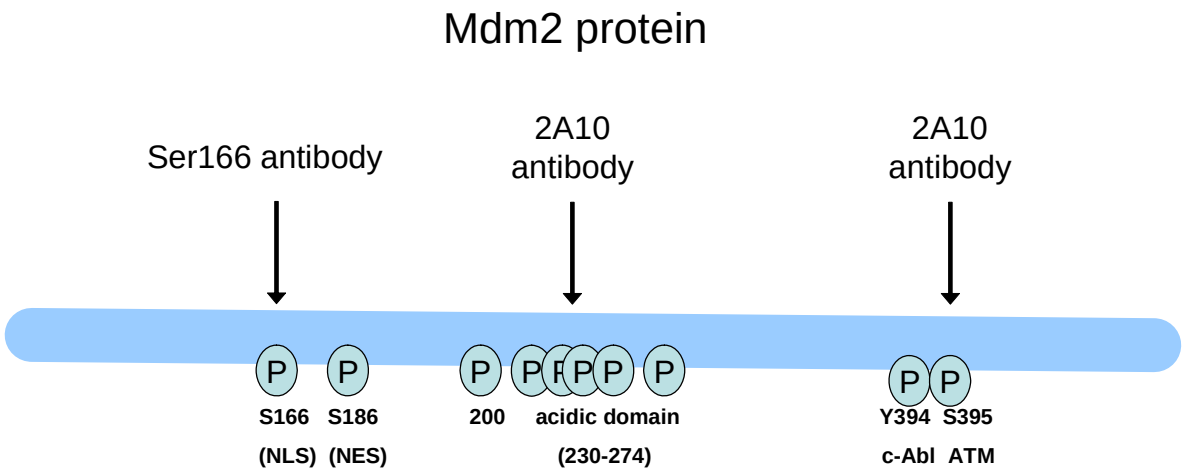


Figure 3. Posttranslational phosphorylations of Mdm2
Schematic view of the Mdm2 protein showing the two epitopes of antibody 2A10. Phosphorylations within the epitopes are shown. The identities of the kinases mediating phosphorylations within the central acidic domain are not thoroughly known. Also shown is the Ser166 phosphorylation at the NLS (nuclear localization signal).

Phosphorylation of Mdm2 on Serine 166

Mdm2 Ser166 phoshorylation occurs at a nuclear localization signal on Mdm2 (Meek and Knippschild 2003). Several studies have shown that Mdm2 might be phosphorylated on Ser166 by Akt, and this has been associated with an enhanced ability of Mdm2 to degrade p53. Some studies also show that Ser166 phosphorylation increase nuclear localization of Mdm2 (Mayo and Donner 2001, Ashcroft et al 2002, Feng et al 2004). Later studies have shown that other kinases may also mediate Mdm2 phosphorylation on Ser166 (Figure 4). There might also be organ or cell type specific Ser166 phosphorylations of Mdm2. In a recent paper by our group it is shown that hepatocytes respond differently as compared to e.g. lung cells. In hepatocytes the Raf-MEK-ERK signalling pathway phosphorylates Mdm2 on Ser166. Surprisingly, PI3-kinase inhibitors activates Mdm2 in hepatocytes. This effect can be explained by a cross talk between Akt and Raf-1 (Malmlöf et al 2006).

Thus as shown in Figure 4, Akt can induce Mdm2 phosphorylation and nuclear localization of Mdm2 (Mayo & Donner 2001). Another study shows that Mdm2 binds to Akt in response to growth factor stimulation of normal cells (human and mouse fibroblasts). This effect was not thoroughly inhibited by mutation of amino acids Ser166 and Ser186 of Mdm2 (the sites known to be phosphorylated by Akt). This suggests that Akt might mediate phosphorylation on an

additional site of Mdm2 (Ashcroft et al. 2002). Akt activation has also been shown to increase Mdm2 mediated degradation of the androgen receptor (Lin et al 2002). The Akt-induced phosphorylation of Mdm2 protects from self-ubiquitination and proteasomal degradation. This may lead to increased degradation of p53 by Mdm2. IGF-1 induces Akt-dependent *in vivo* phosphorylation of Mdm2 on Ser166 and Ser 186 (Feng et al 2004).

Anisomycin (an antibiotic inhibiting protein synthesis) and UV-irradiation activates MAPKAP2. MAPKAP2 can phosphorylate Mdm2 on Ser166 *in vitro* (Weber et al 2005). DAP kinase (also called ZIP-kinase) has also been shown to be able to mediate Mdm2 Ser166 phosphorylation (Burch et al 2004). Recently, S6K1 (a downstream target of mTOR) was shown to be able to phosphorylate Mdm2 on Ser166. Akt was not involved in S6K1 mediated Ser166 phosphorylation of Mdm2. Overexpression of S6K1 was shown to increase Mdm2 expression and Ser166 phosphorylation. Inhibition of S6K1 with siRNA inhibited these effects (Fang et al 2006).

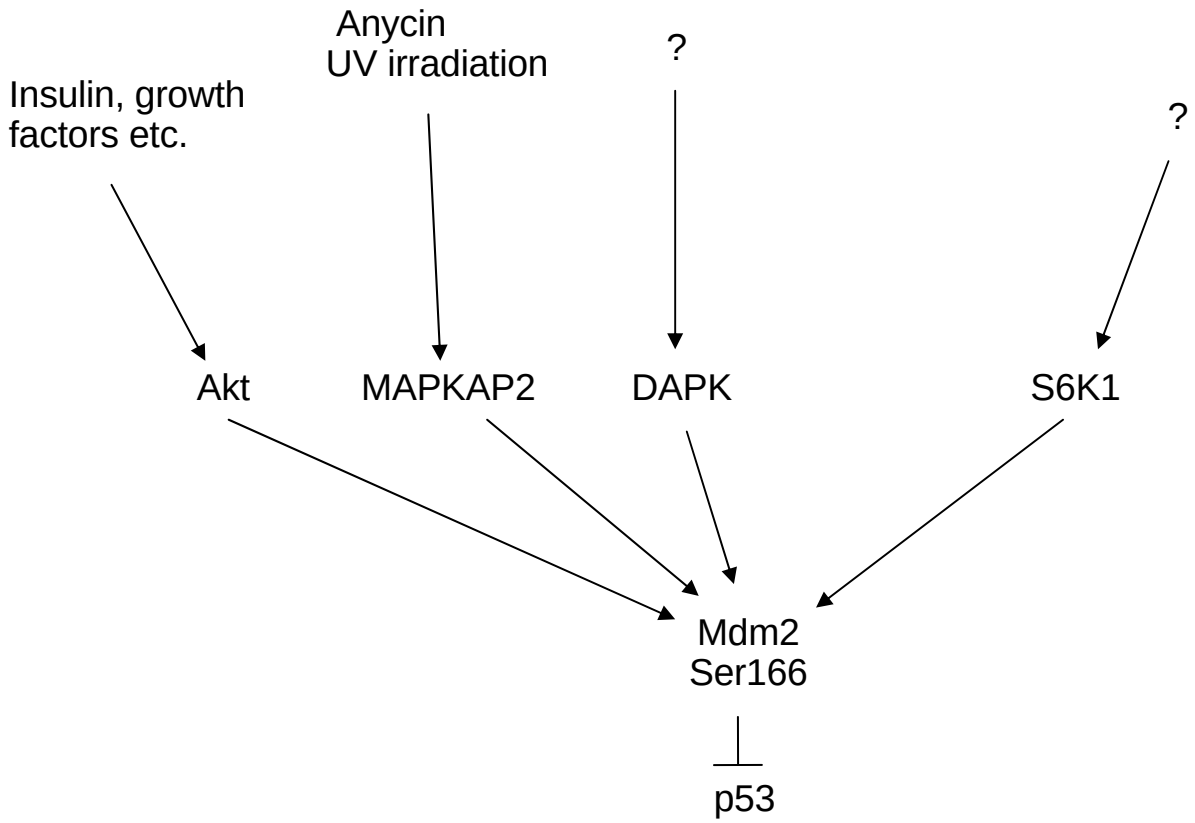


Figure 4. Pathways shown to induce Mdm2 Ser166 phosphorylation.

Phosphorylation of p53 on Serine 15 and Serine 46

Phosphorylation of p53 on Ser15 often occurs in response to DNA damage and leads e.g. to p53 stabilization. Several kinases can phosphorylate p53 on Ser15, including ATM, ATR and DNA-PK (Bode and Dong 2004).

p53 Ser46 phosphorylation is associated with apoptosis. Cisplatin and UV irradiation may induce HIPK2 and subsequent p53 Ser46 phosphorylation and activation of apoptotic pathways (DiStefano et al 2004, D´Oraci et al 2001). PKC1δ has also been reported to induce p53 Ser46

phosphorylation after exposure to adriamycin (Yoshida et al 2006). WOX1 is another kinase that may induce p53 Ser46 phosphorylation after exposure to staurosporine, UV, anisomycin, etoposide and hypoxia (Chang et al 2005). p38 has been suggested to induce p53 Ser46 phosphorylation *in vitro* (Yoshida et al 2006). In contrast, IR irradiation does not induce p53 Ser46 phosphorylation (Yoshida et al 2006). High doses of etoposide induce p53 Ser46 phosphorylation while lower doses, which induce Mdm2, do not. p53 Ser46 phosphorylation may modify p53 transcription factor function by changing promoter selection of p53 (Mayo et al 2005).

1.2 TCDD

1.2.1 TCDD and cancer

TCDD is a persistent, bioaccumulating pollutant. It is a carcinogen without being genotoxic. However, the detailed mechanisms for cancer induction are unknown (IARC 1997, Huff et al 1994). In this thesis we have studied on how TCDD induces modifications of Mdm2 and p53 and how it affects the response to DNA damaging agents.

TCDD (Figure 5A) is classified as a group 1 carcinogen to humans by the International Agency for Research on Cancer (IARC 1997). TCDD has been convincingly shown to be a carcinogen in laboratory animals (IARC 1977, Huff et al 1994). However, it still seems to be controversial if TCDD is carcinogen in humans (Cole et al 2003, Steenland et al 2004). TCDD has been shown to act mainly through a mechanism involving the aryl hydrocarbon receptor (AhR). Both humans and laboratory animals have functional AhR pathways, and this supports a role for TCDD in human carcinogenicity. This was used to classify TCDD as a known human carcinogen (IARC 1997, Connor et al 2006).

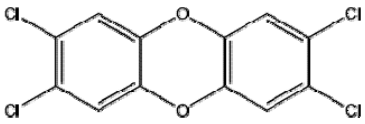
1.2.2 Sources and exposure to TCDD

TCDD mainly comes from unintentional production, such as combustion of organic matters or as a by-product of industrial processes. The greatest unintentional production of TCDD occurs from waste incineration, metal production, and fossil fuel and wood combustion (ATSDR 1998). Foods such as meat, fish and dairy products are the major source (> 90 %) of human exposure. TCDD occurred as a contaminant in herbicides that were widely used in 1960s and 1970s to control weeds, and as a defoliant during the Vietnam war (ATSDR 1998). There have also been several accidental exposures, for example in Seveso, Italy, when an industrial accident exposed several thousand people to substantial quantities of TCDD. The accident took place in the trichlorophenol production department of a chemical plant. Given the concomitant failure of a safety device, the contents of the reactor were vented directly into the atmosphere (Baccarelli et al 2004). TCDD is very persistent in the environment and bioaccumulates in the food chain (ATSDR 1998).

1.2.3 Metabolism and action of TCDD

Some studies have suggested that the dose-response for TCDD-induced cancer is non-linear and that there is a biological threshold value for TCDD carcinogenic effects (Popp et al 2006), while other studies suggests a linear dose response (Huff 1994). It is well established that the binding of TCDD to the AhR as a ligand, and AhR signalling are the first steps of the major toxic actions of TCDD (Mandal 2005).

A.



B.

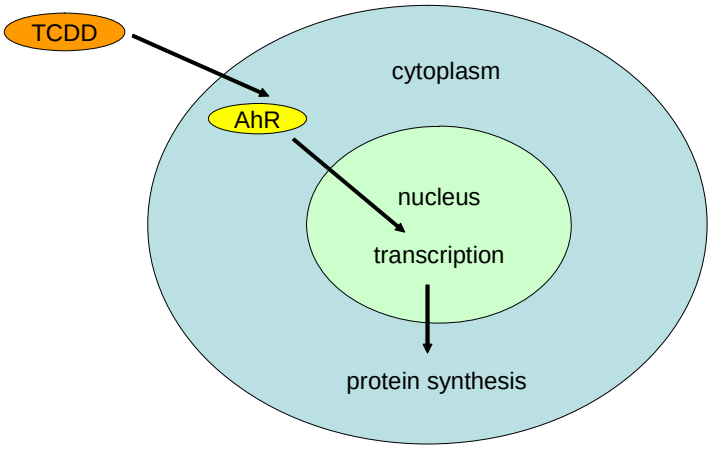


Figure 5. **A**. Structure of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)
B. TCDD binds to AhR and translocates it to the nucleus where transcription occurs.

As shown in Figure 5B, in the absence of a ligand AhR is present in the cytosol of the cell in a complex with other proteins. Upon TCDD binding, the complex dissociates, the AhR subunit translocates into the nucleus (to form a heterodimer with its partner molecule, Arnt), and eventually acts as a nuclear transcription factor by binding to dioxin-responsive elements and regulate transcription and thereby cellular levels of different proteins. Among target proteins are enzymes mediating biotransformation of xenobiotics (Mandal 2005).

1.2.4 Toxic effects of TCDD

Human exposure to TCDD has been associated with chloracne and alterations in liver enzyme levels. High exposure to TCDD may be lethal and the lethal doses may vary more than 5000-fold between different animal species and strains. Lethal doses of TCDD result in a delayed death preceded by excessive body weight loss (called wasting). Other signs of TCDD intoxication include thymic atrophy, hypertrophy/hyperplasia of hepatic, gastrointestinal, urogenital and cutaneous epithelia, atrophy of the gonads, subcutaneous oedema and systemic haemorrhage (IARC 1997). TCDD induces biological responses such as induction of cytochrome P-450 1A1, disruption of normal hormone signalling pathways, immunotoxicity, reproduction and development defects, liver damage and neurotoxicity (Mandal 2005).

1.2.5 Other studies with TCDD and p53

Recently it was suggested that TCDD inhibits p53 transcriptional activity (Tijet et al 2006). Another recent study has shown that repression of p53 transcriptional activity by TCDD

absolutely requires AhR and is accompanied by promoter methylation, and that TCDD alone is sufficient to immortalize human keratinocytes (Ray and Swanson 2004). A study has shown that TCDD attenuates the p53 response to UV-irradiation, and inhibited apoptosis in rat hepatocytes. The study also indicated that TCDD induced hyperphosphorylation of p53 (Schrenk et al 2004).

1.3 STATINS

1.3.1 Statins and cancer

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are drugs used to treat hypercholesterolemia (Stamm and Ornstein 2005). Statins have also been suggested for treatment and prevention of other conditions, such as stroke, osteoporosis, progression of multiple sclerosis and Alzheimer's disease (Stamm and Ornstein 2005, Graaf et al 2004). Lovastatin, pravastatin (Pravachol) and simvastatin (Zocor) are fungal-derived statins. Fluvastatin (Lescol), atorvastatin (Lipitor), cerivastatin and rusovastatin (Crestor) are synthetic statins (Stamm and Ornstein 2005).

Animal studies have raised concern that statin might cause cancer. There is no persuasive evidence linking statins to the development of cancer in humans. On the contrary, currently available data suggests that statins may prevent cancer. Phase I/II studies suggests that lovastatin may have modest anticancer activity in treatment of high-grade brain tumors. In contrast, a phase II study of lovastatin in advanced gastric cancer demonstrated no response among 16 patients (Larner et al 1998, Kim et al 2001). Pravastatin had anticancer activity when administered continuously to patients with advanced hepatocellular cancer (Kawata et al 2001). A recent study concerning breast cancer growth prevention by statins suggests that only lipophilic statins have anticancer activity *in vitro* (Campbell et al 2006). There are many studies ongoing of statin interactions with other cancer therapies. The results are often promising, but so far it is not yet clear how statins are best used in cancer therapy.

1.3.2 Metabolism of statins

All statins are competitive inhibitors of HMG-CoA reductase and prevent the conversion of HMG-CoA to mevalonate (Figure 6). This results in an overall decrease in the amount of cholesterol produced by the liver. Several intermediate products in cholesterol synthesis are also inhibited by statins and this might account for effects of statins other than cholesterol reduction.

Examples of proteins which need intermediates in the cholesterol synthesis pathway for their function are for example Ras, which require the addition of a farnesyl moiety for activity, and Rho GTP-binding proteins which require geranylgeranylation for activity in cell signalling (Stamm and Ornstein 2005). Other examples are transducin γ and rhodopsin kinase (Graaf et al 2004). Lamin A, a constituent of the nuclear membrane, requires farnesylation for its localization and function, and its degradation seems to be important in apoptosis (Jakobisiak and Golab 2003).

Statins concentrate in the liver, little drug circulates in plasma and is highly protein bound. All statins except rosuvastatin are metabolized in the liver. Pravastatin and rosuvastatin are hydrophilic, whereas the remaining members of the class are lipophilic. Pravastatin is not

metabolized by the cytochrome P450 system, while all the other statins are. Differences in hydro/lipophilicity may affect distribution and effect of different statins (Stamm and Ornstein 2005).

1.3.3 Other effects of statins

Statins have few side effects, but cerivastatin has been voluntarily removed from the US market because of its association with higher than expected number of fatal cases of rhabdomyolysis (breakdown of skeletal muscles, huge amounts of degradation products from the muscle cells may also further damage the body). Rhabdomyolysis is a rare but potentially fatal complication of statin use (Stamm and Ornstein 2005).

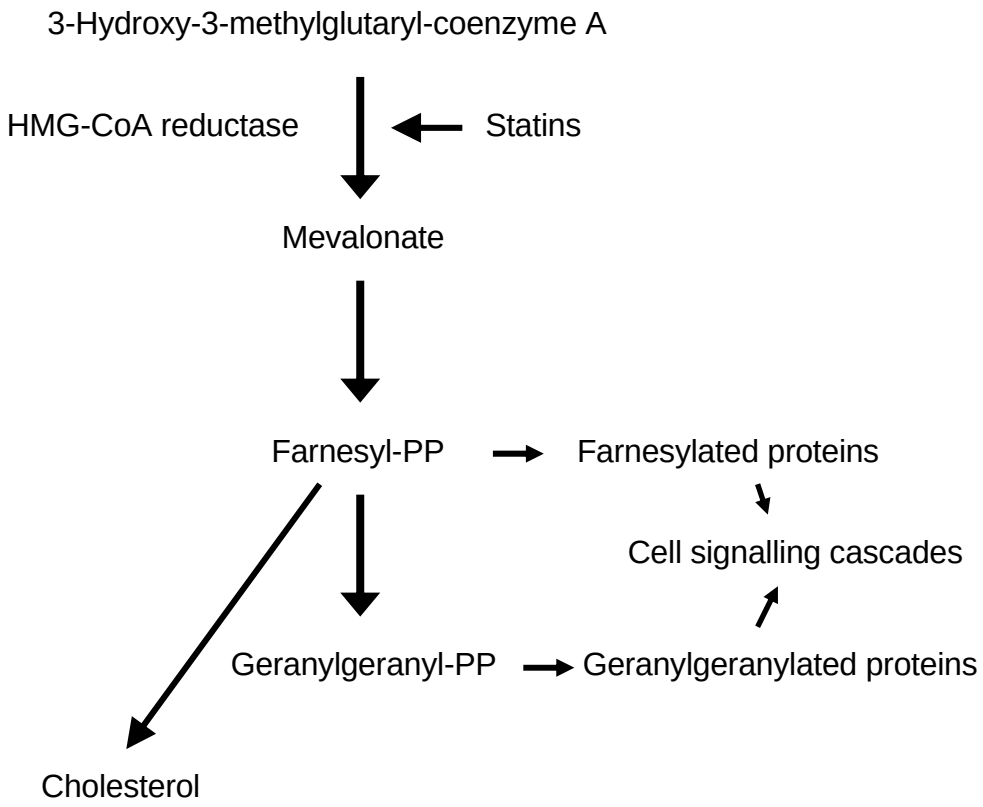


Figure 6. The synthesis pathway of cholesterol, showing statin inhibition of cholesterol and its intermediates. (Adapted from Graaf et al 2004)

Statins trigger apoptosis in numerous experimental cancer models (Wong et al 2002). In general, tumor cells can be rescued from statin-induced apoptosis by addition of mevalonate or geranylgeranylpyrophosphate (GGPP) but only partially (if at all) rescued by farnesylpyrophosphate (FPP) and not at all rescued by intermediates downstream of GGPP and FPP, including cholesterol (Stamm and Ornstein 2005). Many studies have indicated that Ras is not explaining antiproliferative and proapoptotic effects of statins (Graaf et al 2004). Different studies have reported both pro- and antiangiogenic effects of statins. It might be possible that low statin concentrations are pro- and high statin concentrations are antiangiogenic (Stamm and Ornstein 2005). Statins may have antiproliferative effects, and may regulate cyclin-dependent kinases (CDKs) and CDK inhibitors (Jakobisiak and Golab 2003). Statins may inhibit tumor

growth, have antimetastatic effects, and potentiate antitumor effects of some other treatment regimens (Jakobisiak and Golab 2003).

1.4 POLYCYCLIC AROMATIC HYDROCARBONS (PAH)

Polycyclic aromatic hydrocarbons (PAH) are a group of chemicals that are formed during incomplete combustion of organic material. PAHs generally occur as complex mixtures. They are found throughout the environment in the air, water and soil. The primary sources for human exposure to PAHs are consumption of PAH in foods, inhalation of the compounds in tobacco smoke, wood smoke and ambient air. PAHs can also be absorbed trough the skin. PAH exposure occurs in the workplace (coal tar production, aluminium production, asphalt production, waste incineration etc). People living near industries and incineration sites may be exposed through contact with contaminated air, water and soil (ATSDR, 1995).

1.4.1 Metabolism

Biotransformation of PAHs makes some PAHs more toxic, other PAHs less toxic (ATSDR 1995). The PAHs studied in this thesis are benzo[*a*]pyrene (BP), dibenzo[*a,l*]pyrene (DBP) and their ultimate carcinogen diolepoxide (DE) metabolites (+)-*anti*-BPDE and (—)-*anti*-DBPDE. In order to be biological active both BP and DBP need to be biotransformed by the cytochrome P450 system to form their ultimate carcinogen metabolites, which forms adducts on DNA (Thakker et al 1985, Dipple 1985). Due to biotransformation, PAHs in general do not bioaccumulate (ATSDR 1995).

1.4.2 Structure of BP and DBP and their metabolites

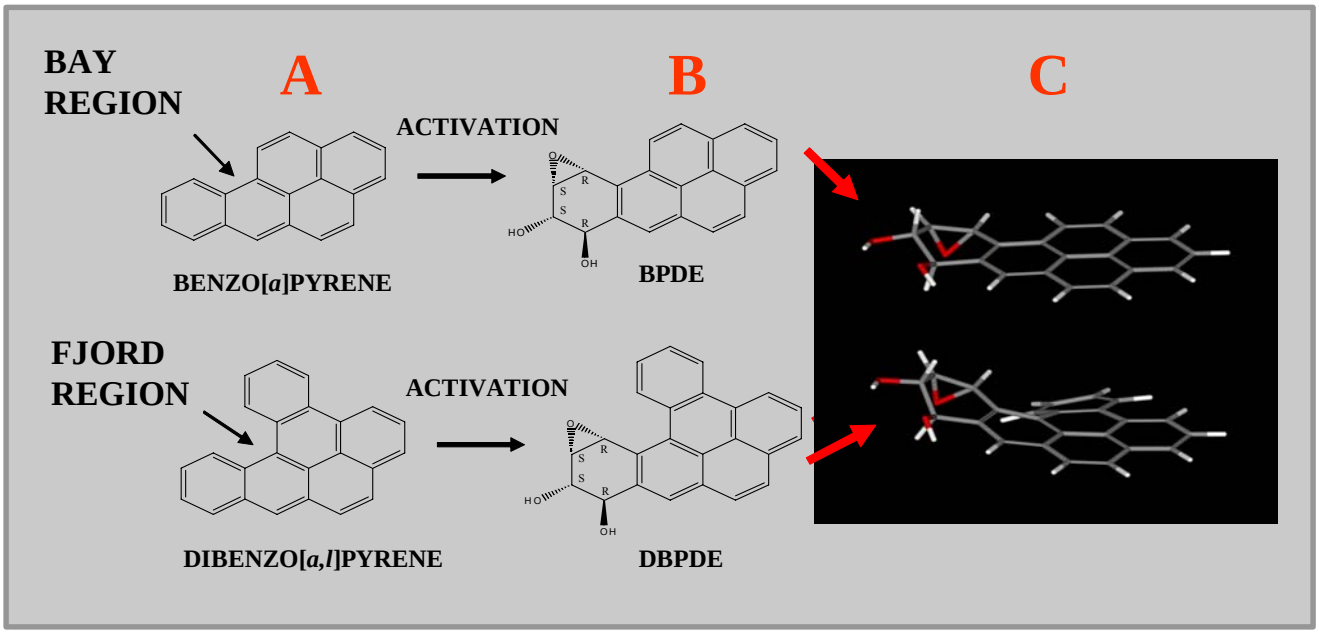


Figure 7. Benzo[*a*] pyrene and dibenzo[*a,l*] pyrene

- **A.** Structure of benzo[*a*]pyrene and dibenzo[*a,l*]pyrene, showing bay- and fjord regions.
- **B.** The diolepoxides are the ultimate mutagen/carcinogen metabolites.
- **C.** The aromatic residue of BPDE is rigid and planar, while that of DBPDE is distorted and more flexible.

1.4.3 DNA adduct formation, and removal of DNA adducts

BP is a bay-region while DBP is a fjord-region PAH, see Figure 7A. Fjord-region DE generally binds more extensively to DNA than the bay-region analogous and predominantly reacts with adenine residues rather than guanine in DNA (Dipple et al 1987, Ralston et al 1995). These facts seems to be reflected in DNA adduct recognition and the subsequent handling by nucleotide excision repair (NER) and may contribute to the great difference in DNA adduct removal and carcinogenic potency of fjord- and bay-region DEs, respectively (Lloyd and Hanawalt 2002, Luch et al 1999). A recent study show that DNA adducts of (—)-*anti*-DBPDE in A549 cells are much more refractory to removal than adducts of (+)-*anti*-BPDE (Dreij et al 2005).

1.4.4 PAHs and cancer

Many PAHs are carcinogens and it is clearly established that both BP and DBP cause tumors in experimental animals (IARC, 1983). Effects of BP have been much more studied than effects of DBP. BP has been used as a surrogate marker to measure exposure to carcinogenic PAH mixtures. This role has been questioned by new findings on the presence of more carcinogenic PAHs than BP, such as DBP (Okona-Mensah et al 2005). DBP is several orders of magnitude more active as a carcinogen in mouse skin and rat mammary gland than BP and a potent transplacental carcinogen (Seidel et al 2004, Cavalieri et al 1991, Higginbotham et al 1993, Yu et al 2006). It is likely that the carcinogenic potency might possibly vary depending on route of exposure and biotransformation- and DNA repair efficiency and capacity in exposed tissue (Okona-Mensah et al 2005).

1.4.5 Repair of diol epoxide DNA adducts, translesion synthesis.

Both BP and DBP need to be metabolized to be able to produce diol epoxides which forms adducts with DNA (Thakker et al 1985, Dipple 1985). BPDE, the diol epoxide metabolite of benzo[*a*]pyrene, forms DNA adducts which are thought to be effectively repaired mainly through nucleotide excision repair (NER) (Gunz et al 1996, Wood 1999, Braithwaite et al 1999, Mitchell et al 2003). DBPDE, the diolepoxide metabolite of dibenzo[*a,l*]pyrene, produces DNA adducts which are not effectively repaired. The reason for this difference between the diol epoxides might be that BPDE produces DNA adducts that are more easily recognised by NER (Dreij et al 2005). The bay-region DE demonstrates a high preference for the exocyclic amino group of dG and the rigid pyrenyl residue of the major *trans*-adducts is externally localised and located in the minor grove of DNA (Geacintov et al 1997). The fjord-region DE, on the other hand, demonstrates a preference for the exocyclic amino group of dA, accessible via the major grove of DNA. The benzo[*e*]pyrenyl residue is in this case most likely intercalated. Recent studies on site-specifically modified oligonucleotides indicate that adducts derived from fjord-region DEs, being more flexible and twisted, distort DNA less than the more rigid bay-region DEs (Geacintov et al 1997, Geacintov et al 2005). These facts may contribute to the great difference in carcinogenic potency of fjord- and bay-region DEs, respectively.

A study has shown that repair of BPDE DNA-adducts is dependent on functional p53 at low adduct levels caused by exposure to 100 nM or 600 nM racemic BPDE. In contrast, repair of BPDE adducts is p53 independent after a higher dose of (1.2 μM) racemic BPDE. This study indicate that DNA repair at low DNA adduct levels might be mediated by a different mechanism than repair at high adduct levels (Lloyd and Hanawalt 2000). In p53-negative H1299 cells, 100 nM BPDE has been shown to induce transient translesion synthesis (DNA synthesis of unrepaired DNA lesions, see 1.5.4 on page 16) mediated by DNA polymerase κ, while 600 nM BPDE induced DNA polymerase κ foci which persisted for at least 24 hours (Bi et al. 2005). There are studies showing that DNA polymerase κ may bypass BPDE adducts relatively error free in *in vitro* systems without p53 (Bavoux et al 2005). The human DNA polymerase κ promoter is negatively regulated by p53 in human cells (Wang et al. 2004, Velasco-Miguel et al. 2003, Bavoux et al 2005), suggesting that translesion synthesis does not occur to the same extent in p53 positive human cells.

1.5 MDM2 AND P53 IN DNA REPAIR

It is essential that DNA remain intact in cells. Cells with damaged DNA can respond to the damage with DNA repair, cell cycle arrest, apoptosis, senescence or translesion synthesis resulting in mutations. In the following sections the roles of Mdm2 and p53 in DNA repair and translesion synthesis will be discussed.

1.5.1 p53 in DNA repair

The five main DNA repair processes in mammalian systems are nucleotide-excision repair (NER), base-excision repair, mismatch repair, and non-homologous and homologous end-joining. p53 modulates most of these DNA repair processes by both transactivation-dependent and -independent pathways. Thus, p53 play a role in almost all DNA repair processes (Sengupta and Harris 2005). NER is thought to be the most important process for removing DNA damage caused by benzo[*a*]pyrene and (+)-*anti*- BPDE (Gunz et al 1996, Wood 1999, Braithwaite et al 1999, Mitchell et al 2003).

p53 in NER

There are two NER subpathways, global genome repair (GGR) and transcription- coupled repair (TCR, mediating repair of actively transcribed DNA strands). GGR and TCR differ in initial lesion recognition, but the subsequent steps in DNA repair might be identical (Mitchell et al 2003, Hanawalt et al 1994). p53 has a role in GGR, since p53 can regulate protein levels of XPC and DDB2 (p48) which are necessary proteins mediating GGR (Sengupta and Harris 2005). p53 might also function as a chromatin accessibility factor in NER, possibly through recruiting histone acetyltransferases to NER foci (Rubbi and Milner 2003). Most rodent tissues are deficient in GGR (Hanawalt et al 2003). This might possibly relate to findings that p53 regulates translesion synthesis differently in rodents than in humans (Velasco-Miguel et al. 2003, Bavoux et al 2005). p53 also can affect NER by binding to XPB and XPD (TFIIH helicase subunits), and thereby modulating their helicase activity. XPB and XPD are also components of the p53-mediated apoptotic pathway (Sengupta and Harris 2005).

1.5.2 Mdm2 in DNA repair

Accumulating literature data indirectly indicate that Mdm2 is involved in DNA-repair (Vlatkovic et al 2000, Kurki et al 2003, Alt et al 2005). Mdm2 has been shown to interact with proteins involved in DNA repair such as DNA polymerase ε and Nbs1 (Asahara et al 2003, Alt et al. 2005). Mdm2 also partly colocalize with the MNR complex to double stranded DNA breaks in response to IR irradiation (Alt et al 2005). The MNR complex consists of the proteins Mre11, Rad50 and Nbs1 and the complex has been shown to localize to double strand DNA breaks. Mdm2 can be associated in promyelotic leukemia (PML) foci in response to UV irradiation (Kurki et al 2003). PML nuclear bodies are closely associated with chromatin and changes in chromatin structure might also affect functions of PML nuclear bodies. PML nuclear bodies has been suggested to sense double-strand DNA breaks (Dellaire et al 2006). Mdm2 has also been shown to be able to ubiquitylate histones (Minsky and Oren 2006). It is unclear what function ubiquitylation of histones have. However, ubiquitylation of histones has been suggested to regulate transcription and some histone ubiquitylations have been connected to DNA repair (Bergink et al 2006).

Nbs1

A study has shown that Mdm2 and Nbs1 partly colocalize to DNA damage foci (double strand breaks caused by γ-irradiation) (Alt et al 2005). There was not colocalization in all foci, and it was interpreted that the colocalization was transient. This study also documented how overexpression of Mdm2 affected repair of double strand breaks. Overexpression resulted in slower repair of double strand breaks. If Nbs1 binding domain of Mdm2 was deleted overexpression of Mdm2 did not slow down repair of double strand breaks, indicating that this effects is dependent on interaction between Mdm2 and Nbs1. The site in Mdm2 involved in binding of Nbs1 is located within the epitope of antibody 2A10 (amino acids 198-314 of Mdm2). Nbs1 has been shown to regulate how PML nuclear bodies responds to double strand DNA breaks (Dellaire et al 2006). Thus both Nbs1 and Mdm2 interacts with PML.

DNA polymerase epsilon (DNA pol ε)

DNA pol ε interacting domain of Mdm2 is located within amino acids 50-166 (Vlatkovic et al 2000). This encompasses the p53 binding domain of Mdm2 (amino acids 26-108) (Meek and Knippschild 2003). Mdm2 binds to the C-terminal part of DNA polymerase epsilon. It is currently believed that DNA pol ε is both a component of the replication machinery and a sensor of stalled replication forks. Presumably, when DNA polymerase epsilon encounters damage or when replication machinery is otherwise blocked, the replication complex would be reorganized with recruitment of recombination/repair proteins (Asahara et al 2003). Mdm2 is speculated to take part in this process, possibly displacing other proteins from DNA polymerase epsilon to allow reconfiguration from a replication complex to a repair complex (Asahara et al 2003). DNA polymerase epsilon also has a function in nucleotide excision repair (NER). In NER, repair factors are recruited to the damage and the damage and several nucleotides on each side of the damage is excised. The excised oligomer and most of the repair factors dissociate from the duplex, but at least one repair factor (presumably RPA) remains in the gap. Then repair synthesis proteins RFC/PCNA and DNA polymerase delta and epsilon fill in the gap and the repair patch is sealed by DNA ligase 1 (Reardon and Sancar 2005).

1.5.3 Mdm2 association with chromatin

Mdm2 has been shown to associate with chromatin (White et al 2006, Alt et al 2005). White et al (2006) suggests that Mdm2 is bound to p53 on chromatin in cells not exposed to genotoxic substances. The association of Mdm2 with p53 on chromatin might prevent p53 from mediating transcription of some p53 targets, such as Mdm2. When cells are exposed to genotoxic agent (etoposide in Whites model), Mdm2 might dissociate from p53 on chromatin, and this may increases the transcriptional activity of p53. Thus the protein level of Mdm2 not associated to p53/chromatin may increase (White et al 2006).

1.5.4 Translesion synthesis

Translesion polymerases have been shown to be able to bypass DNA adducts induced by BPDE in cell free systems (Huang et al 2003). It has also been shown that exposure of p53 negative cells to BPDE transiently induces DNA polymerase kappa (Bi et al). Translesion synthesis is a way to handle irreparable DNA damage and allows cells to tolerate genomic lesions. Translesion polymerases, which belong to Y-family polymerases, are DNA polymerase eta, DNA polymerase iota, DNA polymerase kappa and Rev1 in humans and rats. They have no sequence homology with ordinary error free DNA polymerases. Translesion polymerases can copy damaged DNA without stalling of replication forks at irrepairable lesions. However, translesion polymerases might produce much more replication errors than ordinary polymerases (Bavoux et al 2005). Translesion synthesis might account for characteristic mutation spectras seen after exposure to certain genotoxic substances (Xie et al 2003). Monoubiquitylation of PCNA has been shown to activate translesion synthesis (Kannouche and Lehmann 2004), and all translesion polymerases has ubiquitin binding domains (Bienko et al 2005). p53 has also been shown to have a role in regulating translesion synthesis (Liu and Chen 2006, Wang et al. 2004, Velasco-Miguel et al. 2003, Bavoux et al 2005).

2 PRESENT STUDY

2.1 AIM OF THE STUDY

The aim of the present study was to characterize the Mdm2 and p53 responses induced by DNA damaging xenobiotics and how these responses can be modified by non-genotoxic xenobiotics.

Specific aims

1. To determine if the persistent pollutant TCDD modifies the Mdm2 and p53 DNA damage response. TCDD is a carcinogen without being genotoxic. The mechanism for carcinogenicity is not clear but the aryl hydrocarbon receptor is of importance.

2. To determine if cholesterol lowering drugs, statins, which have been suggested to have anticarcinogenic effects, affects the Mdm2 and p53 DNA damage response. This is important for understanding how statins may be best used in cancer therapy.

3. To characterize the Mdm2 and p53 DNA damage responses to polycyclic aromatic hydrocarbons inducing DNA damages exhibiting different repair characteristics.

A long term goal is to find mechanistically informative markers for exposure to DNA damaging substances. It is anticipated that an understanding of signalling pathways elicited by DNA damages can improve cancer risk assessment.

2.2 MATERIAL AND METHODS

In vivo studies

The Han/Wistar (Kuopio) and Long-Evans (Turku/AB) rats used in Paper I were available via collaboration with the Department of Environmental Health at the National Public Health Institute, Kuopio, Finland. These rats differ in the sensitivity to TCDD. Long-Evans rats are very sensitive to the acute lethal effects of TCDD (oral LD_{50} 10 µg/kg bodyweight), while Han/Wistar rats are more resistant (oral LD_{50} >9600 µg/kg bodyweight). Han/Wistar rats have a point mutation in the AhR gene that leads to an abnormal C terminus transactivation domain (Pohjanvirta et al 1998). There is no substantial difference between the strains in hepatic AhR levels or binding affinity of TCDD to the AhR. Both strains show similar sensitivity to induction of CYP1A1 activity, thymic atrophy, and embryotoxicity. Thus, there are TCDD induced effects that are similar in both strains (e.g. CYP1A1 induction) and other effects that differ between the strains (e.g. lethality) (Pastorelli et al 2006).

In Paper I the adult female rats were divided into six groups for each rat strain. Each group consisted of four rats. Group 1 was exposed to corn oil (vehicle for TCDD) 3 days before death, Group 2 to 1 µg TCDD/kg bodyweight p.o. 3 days before death, Group 3 to 10 µg TCDD/kg bodyweight 8 days before death, Group 4 to 0.6 mmol diethyl nitrosamine (DEN) 24 h before death, Group 5 to 1 µg TCDD/kg bodyweight p.o. 3 days before death and 0.6 mmol diethylnitrosamine (DEN) 24 h before death, Group 6 to 10 µg TCDD/kg bodyweight 8 days before death and 0.6 mmol diethylnitrosamine (DEN) 24 h before death.

In Paper I adult AHRKO (AhR knock out) mice and their congenic wild-type littermates (obtained from Jackson Laboratories, USA) were treated p.o. as follows: Group 1: corn oil 3 days before death, Group 2: 300 µg TCDD/ kg bodyweight 3 days before death. Group 3: Corn oil and 0.6 mmol DEN kg bodyweight 24 h. before death. Group 4: 300 µg TCDD/ kg bodyweight and 0.6 mmol DEN/kg bodyweight 24 h. before death 3 days before death. Each group consisted of two animals per gender and genotype.

In Paper II female Sprague-Dawley rats were treated with pravastatin (4 mg/kg body weight p.o.) two times 48 and 25 h before death. Some rats were challenged with DEN (0.99 mmol/kg body weight i.p.) 24 h before death.

Primary rat hepatocytes

Primary rat hepatocytes were used in Paper II. Primary hepatocytes were isolated from female and Sprague-Dawley rats employing collagenase perfusion and then seeded on collagen-coated plates. These cells were cultured in complete medium for 1.5 h and thereafter in serum free RPMI 1640 medium (Life Technologies).

A549 and HepG2 cells

HepG2 and A549 cell lines were used. A549 cells are of lung origin. HepG2 cells of liver origin. HepG2 cells have higher ability to metabolize benzo[*a*]pyrene and dibenzo[*a,l*]pyrene to their ultimate carcinogen metabolites than A549 cells. This might be due to more effective induction of enzymes of the CYP1 family (Iwanari et al 2002). HepG2 cells have a higher Akt expression than A549 cells.

2.3 RESULTS

Paper I

Pre-treatment of rats with TCDD was found to attenuate the p53 response to diethylnitrosamine (DEN) in the liver. We also detected an increase in phosphorylated Mdm2 on 2A10 specific epitopes. Simultaneous exposure to DEN and TCDD increased levels of slowly migrating p53 species, which could be interpreted as increased ubiquination of p53. TUNEL staining indicated decreased apoptosis in these rats. Studies on AhR knockout mice indicate that the effect may be AhR-mediated. These *in vivo* data were confirmed *in vitro*. Thus pre-treatment of HepG2 cells with TCDD attenuated the p53 response to different genotoxic agents. Stabilization of the p53 downstream target p21 was also decreased. Furthermore, TCDD was shown to accelerate p53 and Mdm2 degradation. In HepG2 cells, TCDD induced Ser166 phosphorylation of Mdm2. Ser166 phosphorylation has been shown to enhance the ubiquitination-promoting function of Mdm2 and is associated with active Mdm2. These data indicate that TCDD has the capacity to attenuate the p53 response to DNA damaging agents. There appear to be a threshold level for p53 attenuation by TCDD. The threshold level seems to be higher after exposure with benzo[*a*]pyrene, an AhR substrate, than after exposure to non-AhR-substrates.

Paper II

We found that a cholesterol lowering drug, pravastatin, attenuated the p53 stabilization in response to DNA damaging substances in HepG2 cells. Pravastatin also increased Ser166 phoshorylation of Mdm2 in HepG2 cells and this phosphorylation was not inhibited by PI3K inhibitors. The Ser166 phosphorylation of Mdm2 was inhibited by the mTOR inhibitor rapamycin. We also found that statins induced phosphorylation on mTOR at Ser2448. These effects were associated with an attenuated p21 response and less apoptosis. We also show that the p53 response to diethylnitrosamine was attenuated in rat liver in pravastatin pre-treated rats. Taken together, we have shown that statins induce a Ser166 phosphorylation of Mdm2 and that this effect can attenuate the duration and intensity of the p53 response to DNA damage. A changed localization of Mdm2 occurred in rat liver after exposure to statins. This might be due to the fact that the Mdm2 Ser166 phosphorylation occurs within a nuclear localization sequence in Mdm2 (Meek and Knippschild 2003)

Paper III

We found that very low concentrations (< 1 pM) of the ultimate carcinogen benzo[*a*]pyrene metabolite (+)-*anti*-BPDE increased Mdm2 levels in HepG2 cells. The detectability was increased by alkaline phosphatase treatment, suggesting that unmasking of a phosphospecific epitope in the Mdm2 protein was needed. p53 was not affected at these low concentrations and higher concentrations of the more carcinogenic dibenzo[*a,l*]pyrene metabolite (-)-*anti*-DBPDE was needed to elicit the same response. This indicates the involvement of different signalling pathways in the Mdm2/p53 response to DNA damage induced by these two carcinogens.

Paper IV

We extended our study of effects of diolepoxides to A549 human non small cell lung cancer cells, and found that very low concentrations of (+)-*anti*-BPDE increased Mdm2 levels also in these cells. BPDE-induced DNA adducts are efficiently repaired, which is not the case with DNA adducts from the more carcinogenic dibenzo[*a,l*]pyrene metabolite (-)-*anti*-DBPDE. We

found that BPDE induced a transient Mdm2 and p53 phosphorylation and binding of Mdm2 to chromatin which correlated with the time course of repair of DNA adducts. DBPDE in concentration inducing similar number of DNA adducts induced no Mdm2 phosphorylation, a persistent p53 Ser15 phosphorylation and phosphorylation of p53 at Ser46, an apoptosis related phosphorylation site. γH2AX, p53 Ser15 phosphorylation and p21 induction was more pronounced after exposure to DBPDE than to BPDE. Kinase inhibitors indicated that Mdm2 and p53 phosphorylations occurred via non-identical pathways. These findings indicate that BPDE and DBPDE induce structurally different DNA-adducts and activate different DNA-damage signaling pathways. Thus, Mdm2 and p53 phosphorylations and chromatin binding pattern differentiate the effect of these metabolites. To conclude, in this study we report that (+)-*anti*-BPDE-induced Mdm2 phosphorylation at a 2A10 specific site correlated with the transient p53 Ser15 response and removal of DNA adducts suggesting that Mdm2 may have an essential function in DNA repair. The fjord-region (–)-*anti*-DBPDE induce DNA adducts obviously refractory to the NER system, associated with persistent H2AX phosphorylation, p53 binding to chromatin and increased phosphorylation of p53 at Ser46, a phosphorylation site associated with apoptosis. Data also show that 2A10 specific phosphorylation of Mdm2 can be used as a sensitive marker for BPDE-induced genotoxicity.

Paper V

We investigated the effect of different genotoxic compounds on the phosphorylation of Mdm2. It was found that Mdm2 was phosphorylated at the 2A10 specific epitope when HepG2 cells were exposed to nanomolar and micromolar concentrations of genotoxic compounds such as 5-flourouracil, benzo[*a*]pyrene, mitomycinC and etoposide. We show that Mdm2 phosphorylation was induced by lower concentrations of genotoxic compounds than those inducing detectable p53 accumulation and that this Mdm2 phosphorylation was independent of p53. The lowest concentrations which induced Mdm2 phosphorylation did not induce detectable p53 stabilization or H2AX phosphorylation, as measured in chromatin enriched fraction. It was also found that Mdm2 phosphorylations could be detected in lysed cells or in chromatin at earlier time points than p53 stabilization. UV-irradiation and dibenzo[*a,l*]pyrene did not induce phosphorylated Mdm2. Surprisingly the Mdm2 phosphorylation induced by benzo[*a*]pyrene was amplified in cells transfected with siRNA for ATM. These data indicate that Mdm2 phosphorylation at 2A10 specific epitope can be a very sensitive marker for certain types of genotoxicity.

2.4 DISCUSSION

2.4.1 Paper I and II

Attenuation of p53 response to DNA damaging substances and phosphorylation of Mdm2 Ser166 by TCDD and statins.

Both TCDD (Paper I) and statins (Paper II) attenuated the p53 stabilization in response to DNA damaging agents. This attenuation of p53 DNA damage response was associated with Mdm2 phosphorylation on Ser 166. Thus, statins and TCDD induces Mdm2 Ser166 phosphorylation and attenuates p53 stabilization in response to DNA damage in HepG2 cells. TCDD also attenuated p53 stabilization in response to DNA damage in the Han/Wistar and Long-Evans rats. The response was similar in both rat strains. We have thus encountered several situations when Mdm2 Ser166 phosphorylation parallels an attenuation of p53 stabilization in response to DNA damage. It could not be excluded that the mechanism mediating attenuation of p53 DNA damage response by TCDD is identical to the mechanism mediating similar response in statin-treated cells. Further studies are needed to elucidate the pathways involved.

Statins induced Mdm2 Ser166 phosphorylation in HepG2 cells, and we found (Paper II) that this phosphorylation was not Akt-mediated. As statin-induced Mdm2 Ser166 phosphorylation was attenuated by rapamycin (an inhibitor of mTOR) and Mdm2 Ser166 phosphorylation occurred simultaneously as phosphorylation of mTOR it is likely that mTOR induced Ser166 phosphorylation. So our findings suggest that mTOR is one of the kinases inducing Mdm2 Ser166 phosphorylation. In addition to Akt and mTOR, MAPKAP2, DAPk and S6K1 have also been shown be able to phosphorylate Mdm2 (Mayo and Donner 2001, Ashcroft et al 2002, Feng et al 2004, Weber et al 2005, Burch et al 2004, Fang et al 2006) (Figure 8).

In rare cases statins have been reported to cause memory loss, sometimes the memory loss appeared to resolve after discontinuation of the statins (Wagstaff et al 2003). This is interesting since we found that statins induces Ser166 phosphorylation of Mdm2 and another substance called anisomycin also induces Ser166 phosphorylation of Mdm2 and memory loss (Weber et al 2005, Rudy et al 2006). In this case the memory loss is thought to be due to inhibition of protein synthesis, but an apoptotic response as a cause of memory loss induced by anisomycin cannot be excluded (Rudy et al 2006). Additional studies are needed to further explore this observation.

Neither TCDD nor statins alone increased Mdm2 mRNA levels, even though protein expression increased (Paper I and II).

TCDD- and statin-induced attenuation of p53 response might interfere with the cells ability to handle genotoxic agents. TCDD has been shown to cause cancer, especially liver cancer in rodents. Statins, on the other hand, rather have anticarcinogenic properties. However, the possibility that pravastatin selectively induce cancer in elderly is a remaining controversy, and has not been adequately studied. Thus, a report from the PROSPER randomised trail indicated an unexpected increase ($p= 0.02$) of new cancer cases among elderly (70 – 82 years). Gastrointestinal cancers dominated, but according to the authors their observation was best explained by chance (Shepherd et al 2002). Others disputed this conclusion. They showed that differences in cancer incidences between the pravastatin group and the placebo group increased

with treatment time (Devroey et al 2003). Furthermore, in an earlier trail, in which younger and older users of pravastatin were analysed separately, there was a non-significantly increased cancer incidence in the group of elderly and a non-significant decrease in the group of young people (Hunt et al 2001).

TCDD belongs to the group of compounds often termed tumor promoters. These agents interact with genotoxic carcinogens and may potentiate their carcinogenic potential. It may thus be speculated that Ser166 on Mdm2 is a marker for interactions that may lead to cancer.

It would be interesting to determine if the TCDD- and statin-mediated attenuated p53 response to DNA damage correlates with changes in repair of DNA damage caused by genotoxic agents. A recent study in HUVEC-cells has shown that lovastatin at therapeutically relevant concentrations attenuated p53 and p21 stabilization in response to IR-irradiation. The study found, however, that IR-induced repair of double strand and single stand DNA breaks was not changed by lovastatin. H2AX phosphorylation by IR-irradiation was not attenuated by lovastatin. The study concluded that lovastatin protects HUVEC against radiation-induced cell death possibly in part due to inhibition of proapoptotic signalling by attenuation of p53 response (Nübel et al 2006). An even more recent paper by the same authors suggests that lovastatin protects HUVEC cells from the cytotoxicity of topoisomerase II inhibitors doxorubicin and etoposide by reducing susceptibility of topoisomrease II to these inhibitors. This results in lower level of double-strand DNA breaks and a reduction in stress responses triggered by DNA damage including activation of p53 (Damrot et al 2006). A recent study from our group show that statins induce mTOR-mediated inhibition of Akt phosphorylation and nuclear translocation and sensitizes cells to cytostatic drugs. This effect was counteracted in p53 expressing cells by statin induced effects on p53 (Roudier et al 2006).

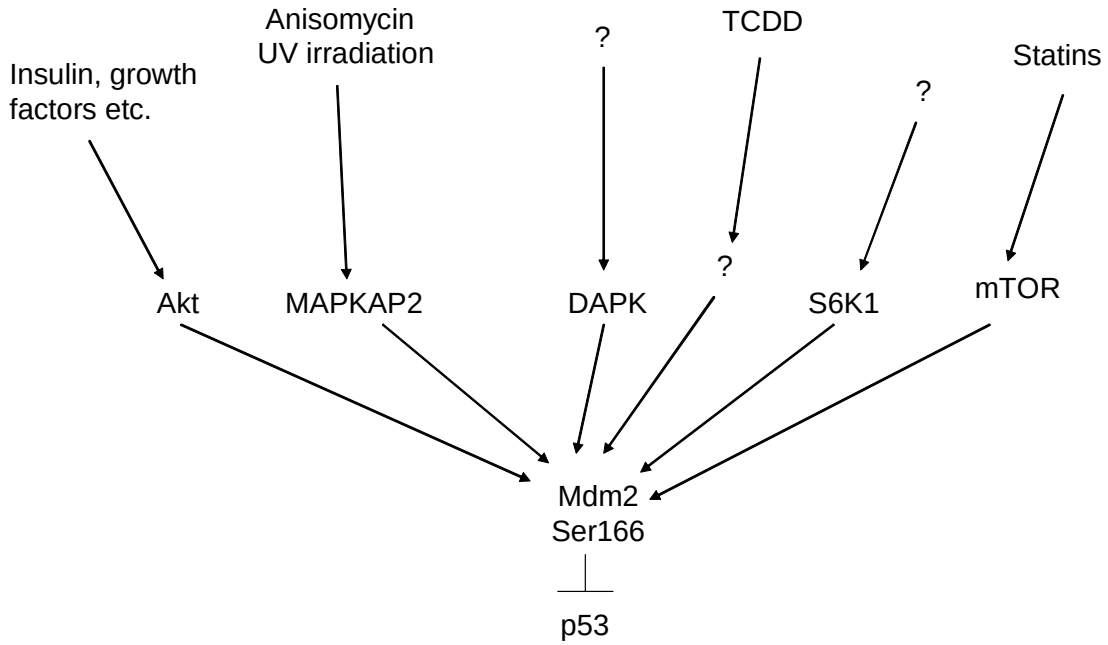


Figure 8. Pathways shown to induce Mdm2 Ser166 phosphorylation. Now statins and TCDD have been added.

2.4.2 Paper III, IV and V

Induction and phosphorylation of Mdm2 (2A10 antibody-specific) in response to some types of DNA damage. A role for Mdm2 in DNA repair and as a marker for some types of DNA damage.

Mdm2 phosphorylation within the epitopes of antibody 2A10 occurred at much lower doses of genotoxic substances than those inducing p53 stabilization after exposure to benzo[*a*]pyrene, BPDE, mitomycinC and etoposide. This suggests that Mdm2 can be used as a marker for certain types of DNA damage. Mdm2 2A10 phosphorylation by lower doses than those inducing p53 stabilization did not occur after exposure to dibenzo[*a,l*]pyrene, DBPDE and UV-irradiation (Paper III-V).

The detectability of Mdm2 with the 2A10 antibody is decreased if Mdm2 is phosphorylated within the epitopes of the antibody. If Western blot membranes are treated with alkaline phosphatase, the detectability is increased (see Figure 2), and the increased detectability might be interpreted to reflecting the fraction of phosphorylated Mdm2 within the epitopes of the 2A10 antibody (Maya and Oren 2000).

The 2A10 antibody detects two epitopes on the Mdm2 protein (Meek and Knippschild 2003). With the alkaline phosphatase treatment method it is not possible to discriminate whether phosphorylations occurs on the epitope within the central acidic domain or within the epitope containing Tyr394 and Ser395. Phosphorylations within the central acidic domain seem to have opposite effect on p53 turnover as compared to phosphorylation at Tyr394 and Ser395.

Mdm2 has been reported to be phosphorylated in the central acidic domain in cells without DNA damage (Meek and Knippschild 2003). Recently, it has been shown that phosphorylation of Mdm2 in the central domain (within the 2A10 epitope) enhances binding between p53 and Mdm2 (Kulikov et al 2006). Phosphorylation within the central acidic domain of Mdm2 has been reported to be essential for p53 degradation (Ma et al 2006). On the other hand phosphorylation sites within the other epitope, Ser395 and Tyr394, seems to mediate a proapoptotic response since they decrease interaction between Mdm2 and p53, resulting in p53 stabilization (Meek and Knippschild 2003, Maya et al 2001, Balass et al 2002, Goldberg et al 2002). If antibodies recognising Mdm2 phosphorylated at Ser395 were available, they could be used to determine whether phosphorylation occurs at this site.

Exposure to BPDE induced a transient increase in Mdm2 phosphorylation within 2A10-epitopes that correlated with removal of BPDE DNA adducts (Paper IV). This together with literature data showing that Mdm2 interacts with proteins involved in DNA repair suggests that Mdm2 might have a role in repair of BPDE adducts. Mdm2 expression was never seen at the same time as p53 Ser46 phosphorylation occurred, while p21 expression only occurred when p53 was phosphorylated at Ser46 (Paper IV). This supports the idea that p53 Ser46 phosphorylation switches p53 promoter selection so transcription switches from Mdm2 to p21 (Mayo et al 2005). p53 Ser46 phosphorylation occurred only at high doses (1 μM) of BPDE and when Mdm2 was transcriptionally inactivated (Paper III and IV). Thus if Mdm2 has a role in repair of adducts this would thus only occur at low adduct levels when Mdm2 is expressed. This reasoning is supported by data showing that repair of BPDE adducts is mediated by another

mechanism at low adduct levels (lower than after exposure to 1.2 μM racemic BPDE) than at higher adduct levels (Hanawalt and Lloyd 2000).

A remaining question is why the Mdm2 endpoint is so sensitive. Several explanations are possible. To our surprise we found that siRNA for ATM increased the sensitivity to BP. The cells became almost equally sensitive to the parent compound as to the metabolite BPDE (Paper V). This may be explained by a release of Mdm2 from chromatin. This reasoning is supported by a recent study which suggests that Mdm2 is bound to chromatin in undamaged cells (White 2006).

The finding that Mdm2 alterations induced by low concentrations of DNA damaging xenobiotics can be detected by using the 2A10 antibody suggests that that this endpoint can be used for monitoring purposes. We show that certain types of DNA damaging agents can be detected by monitoring this signalling pathway. Future studies on e.g. lymphocytes taken from carcinogen exposed people, such as smokers, may thus be a way to evaluate this endpoint for monitoring purposes. It can be expected that the endpoint should be very sensitive and our data suggest that it can be selective for certain types of DNA damages includind those caused by BP. Our data thus predict that smokers should exhibit increased levels of Mdm2 in their lymphocytes, as detected by the 2A10 antibody.

An alternative to lymphocytes in studying PAH exposure on the respiratory tract could be nasal mucosa cells. Nasal tissue contains cells that make first contact with ambient air and are therefore likely to be more susceptible to the DNA damaging effects of air pollution than other cells from the systemic circulation. The procedure used to obtain nasal mucosa (nasal brushing) is considered to be minimally invasive, yielding approximately 1 million cells (which comprise mainly of mucociliary epithelium, goblet cells and neutrophils) with good cell viability (Okana-Mensah et al 2005).

In previous studies it was indicated that using the 2A10 antibody can facilitate the identification targeted cells in histological sections. Risk assessment of carcinogens is a complex endeavor which includes several steps. Some are based on analyzing correlations such as dose response relationships in animal or epidemiological studies. Other steps include mechanistic reasoning. One such step is to pinpoint cells that are targeted by carcinogenic effects by a certain carcinogen and to compare identified target cells to the cell type of origin of induced tumors. As it is reasonable to assume that the endpoint used here reflects rate limiting damages leading to tumor development, the use of this endpoint may greatly facilitate the identification of target cells for carcinogens. This reasoning can be easily tested in animals by studying a panel of carcinogens with well defined target cells.

2.5 CONCLUSIONS

- Both statins and TCDD attenuates p53 stabilization in response to DNA damaging agents. This was shown in HepG2 cells and in rat liver.

- Both statins and TCDD induces Mdm2 phosphorylation at Ser166 in HepG2 cells. It is plausible that Mdm2 Ser166 phosphorylation is necessary for the attenuated p53 response.

- Mdm2 Ser166 phosphorylation induced by statins might be mediated by mTOR independently of Akt activation in HepG2 cells.

- Mdm2 phosphorylation within the epitope of antibody 2A10 occurred at much lower doses of genotoxic substances than doses inducing p53 stabilization. The substances were benzo[*a*]pyrene, BPDE, mitomycinC and etoposide. The same pattern was not seen after exposure to dibenzo[*a,l*]pyrene, DBPDE or UV-irradiation.

- Our findings suggests that 2A10 phosphorylated Mdm2 can be used as a sensitive marker for some types of DNA damage.

- Our findings also suggest that Mdm2 phosphorylated within the 2A10 epitopes might be involved in some types of DNA repair.

2.6 FUTURE PERSPECTIVES

It would be interesting to determine if p53 attenuation caused by statin and TCDD occurs via the same mechanism, in particular if statins and TCDD can interact. Another question is whether the attenuated p53 response correlates with defects in repair of DNA damage caused by genotoxic agents.

Mdm2 might be used as a marker for some types of DNA damage in the future. In case site specific antibodies for involved phosphorylations cannot be developed mass spectrometric analysis can perhaps be used instead. Further studies will be needed to assess the specificity for Mdm2 as a marker. Persistent H2AX and p53 phosphorylation might possibly be used as markers for irrepairable DNA damage.

It would also be interesting to further investigate the role of Mdm2 in DNA damage response and in DNA repair, and which signalling pathways are involved.

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