Effects of nitrosubstituted polycyclic aromatic hydrocarbons on DNA, cell signalling and apoptosis in BEAS-2B and Hepa1c1c7 cells.

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SUMMARY

Several epidemiological studies indicate that there is a correlation between air pollution and diesel exhaust, and increased risk of lung cancer. In this study we have evaluated and compared the effects from different nitro-polycyclic aromatic hydrocarbons (nitro-PAHs), which are mutagenic and carcinogenic. In diesel exhaust we find both PAHs and nitro-PAHs, of which some are classified as mutagenic and carcinogenic. The mouse hepatoma cell line Hepa1c1c7 and human bronchial cell line BEAS-2B were exposed to 1-nitropyrene (1-NP), 1.3-dinitropyrene (1.3-DNP) and 1.8-dinitropyrene (1.8-DNP). The compounds lead to both apoptosis and necrosis cell death in Hepa1c1c7, however, no major effect where found in BEAS-2B besides that 1-NP caused a small increase in release of the cytokine CXCL8 (IL8). After examination in the Hepa1c1c7 cells it was clear that 1.3-DNP was the most cytotoxic, followed by 1-NP. 1.8-DNP caused a delayed cell death as determined by fluorescence microscopy after longer exposure. 1.3-DNP did show, after western blotting, a cleavage of caspase 3 and PARP indicating that cells die from an apoptotic process, in addition the inhibitor zVAD-FMK did show a partly inhibition of the cell death, indicating that there is need for caspase activation, but also that there is a parallel pathway. Flow cytometric analysis showed a shift in cell cycle. Exposure to 1-NP and 1.3-DNP caused the cells to accumulate in G_2-phase, whereas 1.8-DNP caused an accumulation of cells in S-phase. A reason for the shift in cell cycle can be that the DNA is damaged. Little DNA single strand breaks (SSB) were seen after analysing comet assay using standard condition. With modification to detect oxidized purines and alkali labile sites, however, there were a great increase in the response. Further investigation of the DNA damage response (DDR) shows that 1.8-DNP activates both H2AX, p53 and Chk1, however, NOXA did not show any upregulation. Activation of p53 by 1.8-DNP showed a translocation, which is in contradiction to our last study. p53 inhibition after 1.3-DNP exposure by pifithrin-α, did fully reduce the amount of cell death, indicating that signalling and activation of p53 is important in the cell death. In contrast, pifithrin-μ gave no reduction in cell death, supporting that it is the transcriptional activity of p53 that is needed to execute the cell death. The study illustrates that a change in conformation by nitro-group gives a dramatic change in cytotoxicity. Although a p53 transcriptional activity is needed, possibly also other signalling pathways are involved in the triggering of cell death.

ABBREVATIONS

AhR, aromatic hydrocarbon receptor; BEAS-2B, Human bronchial epithelia cell line; CXCL8, IL-8 (interleukin 8); DMSO, dimethyl sulfoxide; ELISA, *Enzyme-Linked ImmunoSorbent Assay*; 1-NP, 1-nitropyren; 1,3-dNP, 1,3-dinitropyren; 1,8-dNP, 1,8-dinitropyren; Chk, checkpoints; DDR, DNA damage response; nitro-PAH, nitro substituted-polycyclic aromatic hydrocarbons; γH2AX, phosphorylated *H2A.X*; Hoechst 33258, 2(2-(4-hydroxyphenyl)-6-benzimidazole-6-(1-methyl-4-piperazyl)benzimidazole hydrochloride); Hoechst 33342, 2´-(4-ethoxyphenyl)-2´,5´-bis-1*H*-benzimidazole hydrochloride); PAH, polycyclic aromatic hydrocarbon; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PFT, pifithrin; SSB, single strand breaks; zVAD-FMK, benzyolcarbonayl-Val-Ala-Asp-fluoromethyl ketone.

1. BACKGROUND

1.1 Introduction

Sources and health effects of air pollutants

A number of natural (volcanoes, fire, etc.) and/or anthropogenic activities can result in environmental pollutants. By definition, an air pollutant is any substance which may harm humans, animals, vegetation or material. Particulate matter (PM) is the generic term used for a type of air pollutants, consisting of complex and varying mixtures of particles suspended in the breathing air, which vary in size and composition, and are produced by a wide variety of natural and anthropogenic activities (Kampa and Castanas 2008; Air quality guidelines for Europe 2000).

Engine exhausts contain thousands of gaseous and particulate substances. Internal combustion engines have been used in cars, trucks, locomotives and other motorized machinery for about 100 years. Particles emitted from engines operating with diesel are different from gasoline, in terms of their size distribution and surface properties. Diesel engines produce two to 40 times more particulate emissions and 20-30 times more nitroarenes than gasoline engines with a catalytic converter in the exhaust system, when the engines have similar power output (IARC monographs on the evaluation of carcinogenic risks to humans. Diesel and gasoline engine exhausts and some nitroarenes. International Agency for Research on Cancer 1989). As more knowledge has been discovered regarding emissions and the hazardous effects it can give several measures has been taken to reduce the emission. Various techniques can be used to minimize emission like engine maintenance, exhaust filtration and use of reformulated fuels (Volkwein et al. 2008). Today there are advanced three-way catalysts systems in engines resulting in exceptionally low emissions (Twigg 2005).

Exposure to diesel exhaust and urban air pollution have shown association with an increased risk of lung cancer in epidemiological studies (Arlt 2005). However negative association with air pollution is not a new phenomenon. Percival Pott saw a connection between cancer of the scrotum and testicles and exposure to soot already in 1775 (Brown and Thornton 1957). It was proven, in 1875 by von Volkman, that industrial tar, coal tar, and crude paraffin caused

skin cancer (Hajdu 2006; Guengerich 2005). The first episode that leads to scientific proof of the potential for atmospheric pollution to cause deaths and disease was not the London fog of December 1952, but the fog that affected the Meuse Valley in Belgium in December 1930. Between December 1 and December 5 a thick fog covered Belgium. From December 3 hundreds of people in the villages in the narrow part of the valley started to have severe respiratory signs and symptoms, and more than 60 people died during the next three days (Nemery, Hoet, and Nemmar 2001). The Great Smog of December 1952, in London, that lasted 5 executive days, from December 5 to December 9, were caused by atmospheric conditions, with a lack of wind and a layer of cold air overlaid by warm air. This smog, that was particulate based, were estimated to have killed about ca 4000 lives, and an additional estimate of 8000 during the 10 weeks that followed (Black 2003; Schwartz 1994; Bell and Davis 2001). Smog is made up of the words smoke and fog and is a mixture of possibly hundreds of pollutants, some in their original state and some converted as a result of chemical reactions occurring with sunlight, ozone, oxides of nitrogen and other factors in the atmosphere (Bryan 1954). This phenomenon can be seen in many major cities like New York, Los Angeles, Beijing and Tokyo (Senn 1948).

The primary components to air pollution are sulphurdioxide (SO_2), nitrogen oxides (NO_x), carbonmomonoxide (CO), ozon (O_3) and suspended dust (Brook et al. 2004). Usually particle matter (PM) is described as mass (weight) per volume unit air, for example $\mu g/m^3$, and the particles are described by their aerodynamic diameter. The aerodynamic diameter is somewhat different than geometric diameter and is the theoretical diameter to a spherical particle with net weight 1 and will fall as fast in air as the real particle. Different net weight give different aerodynamic diameter to particles that are geometric identical (Ormstad and Lovik 2002). PM_{10} (AD < 10 µm) thoracic particles, $PM_{10\ to\ 2.5}$ coarse particles, $PM_{2.5}$ (AD < 2.5 µm) fine particles and $PM_{0.1}$ (AD < 0.1 µm) ultrafine particles, this is one way to categorize particulate matter regarding size. Greater particles deposit more often in the upper trachobronchial regions, whereas smaller particles show greater deposition in the deep lung (Brook et al. 2004).

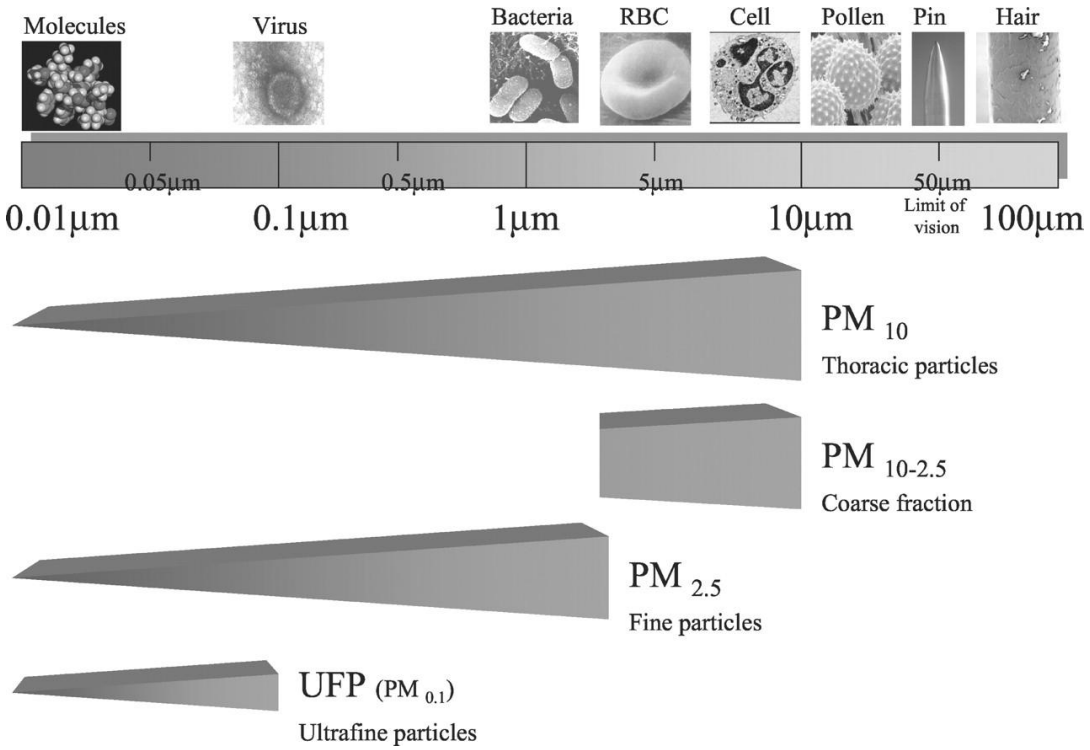


Figure: Illustration of the different sizes of the particle fractions. The coarse fraction has the same size as a cell, the fine particles as bacteria and the ultrafine particles like virus and molecules. The illustration is from Brook and colleagues. (Brook et al. 2004)

Urban outdoor air pollution is described in an article in Lancet in 2002 as major cause of mortality due to combined respiratory and selected cardiovascular causes (Ezzati et al. 2002). Several epidemiological studies have shown negative health effects related to air pollution. The most important sources of particulate matter is diesel exhaust and particles from road dust and wood fuel (Ormstad and Lovik 2002). Inhalation of airborne particles has been recognized and documented as a high health risk. And epidemiological studies have shown associations between exposure to particulate matter in the air and increases in morbidity and mortality. Size has also been highlighted as nanoparticles have increased specific toxicity relative to larger particles composed of the same materials (Gorbunov et al. 2009).

1.2 Diesel exhaust

Diesel exhaust particles are mainly composed of nano-sized particles (Hesterberg et al. 2010). The largest single source of airborne PM from vehicles is derived from diesel exhaust (Riedl and Diaz-Sanchez 2005). The particles in diesel motor emissions are composed of a center core of elemental carbon and adsorbed organic compounds as well as small amounts of

sulfate, nitrate, metals, and other trace elements. Diesel particulate matter consists of fine particles and a high number of ultrafine particles. Their small size makes them highly respirable and they have the potential to reach the deep lung. Diesel motor emission include both polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs (Wichmann 2007). Diesel exhaust particles are the major component of $PM_{2.5}$ in urban areas (Kawasaki et al. 2001). 1-NP has been recognized as an environmental carcinogen of concern to humans and one of the major genotoxic components of diesel exhaust particles (Hatanaka et al. 2001).

Since the 1970s, there has been concern that inhalation of diesel exhaust may cause lung cancer in humans (Garshick et al. 2004). Now, epidemiological studies have found an elevated risk of lung cancer among truck drivers when they were regularly exposed to diesel exhaust, for example among short distant lorry drivers in the urban areas of Sweden (Garshick et al. 2008; Jakobsson, Gustavsson, and Lundberg 1997).

1.3 Polycyclic aromatic hydrocarbons and nitro PAHs.

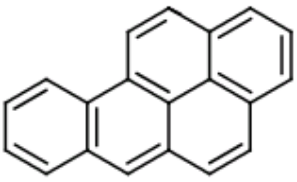
PAHs, polycyclic aromatic hydrocarbons, are organic compounds with at least two aromatic rings fused together. They are formed primarily as a result of incomplete combustion of fossil fuels and other organic materials. Other sources contributing to production of PAHs are smoking, burning wood in indoor stoves, car- and air traffic and barbecuing. PAH compounds were one of the first pollutants to be identified as human carcinogens (Zhu et al. 2011; Overall evaluations of carcinogenicity: an updating of IARC Monographs volumes 1 to 42 1987). The best studied of the various PAHs is benzo[*a*]pyrene (B[*a*]P), it is categorized as a group 2B carcinogen by IARC (IARC monographs on the evaluation of carcinogenic risks to humans. Diesel and gasoline engine exhausts and some nitroarenes. International Agency for Research on Cancer 1989).

Nitro-polycyclic aromatic hydrocarbons (nitro-PAH)

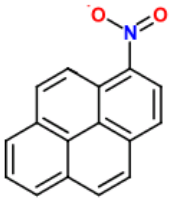
A nitro-PAH is build up one or more nitro groups ($-NO_2$) attached to a polycyclic aromatic hydrocarbon. Nitroaromatic compounds can form naturally in both atmospheric and aqueous environments, it is shown that polycyclic aromatic hydrocarbons react with oxides of nitrogen to form nitroaromatics under conditions that might be expected in polluted air and in combustion processes (Purohit and Basu 2000). In urban settings, hydrocarbons released from

natural combustion processes and the incomplete combustion of fossil fuels serve as substrates for nitration with atmospheric nitrogen dioxide (Ju and Parales 2010). The nitropyrenes are common environmental pollutants. 1-NP is one of the major mutagenic components of diesel particulates, whereas the dinitropyrenes (DNPs) are present in much lower concentrations. 1-NP accounts for approximately 25% of the mutagenicity of diesel emissions (Purohit and Basu 2000). By undergoing substitutions reactions PAHs can rapidly form nitro-PAHs. Nitro-PAHs are of concern because they can direct acting as mutagens and carcinogens (Yaffe et al. 2001). Nitro-PAHs require metabolic activation in order to exert their genotoxic activities (Fu et al. 1994). Oxidation and reduction products of nitroaromatic compounds can damage DNA directly or cause the formation of adducts that induce mutagenesis by misincorporation of nucleotides during DNA synthesis. Structural and spectroscopic studies have found that the position of the nitro group on the aromatic ring and the presence of other functional groups can influence the mutagenicity and carcinogenicity of these chemicals. Factors that can contribute to covalent binding to DNA include hydrophobicity, metabolism, ability to intercalate, orientation prior to binding and DNA sequence specificity of binding. Additional, factors that contribute to the mutagenicity of the adduct formation induced by the activated compounds are DNA-bound confirmation, interactions with DNA polymerases and repair proteins, and effect of DNA sequence. (Purohit and Basu 2000; Ju and Parales 2010).

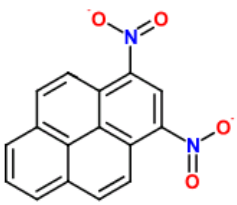
The different PAHs used in this study are illustrated in this figure:



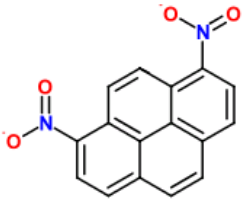
Benzo[*a*]pyrene (B[*a*]P)



1-nitropyrene (1-NP)



1.3-dinitropyrene (1.3-DNP)



1.8-dinitropyrene (1.8-DNP)

1.4 Metabolism

The biological activities of PAHs rely on different properties. PAHs can metabolically be converteted to reactive electrophilic intermediates that can covalently bind nucleophilic

targets in DNA, RNA and proteins. This is why adducts will be formed in DNA and mutations will be induced that can eventually lead to tumors. Reactive metabolites can also be formed; these can react with other cellular targets and interfere with transcription, DNA replication, and protein synthesis. In addition, some PAHs can, after metabolism induce inflammatory processes (Bostrom et al. 2002). Nitro-PAHs can be biotransformed by CYP enzymes, nitroreductases and phase II biotransformation enzymes. The location of the nitro group on the aromatic system is crucial since it affects metabolism and DNA binding properties (Chae et al. 1999; Purohit and Basu 2000). Nitro-PAHs require metabolism to form reactive electrophilic species in order to exert their genotoxic activity (Arlt 2005). 1-NP is classified as possibly carcinogenic to humans (class 2B) by The International Agency for Research on Cancer (Hatanaka et al. 2001). Nitro-PAHs are metabolized via nitroreduction and P450-mediated C-oxidation (Yamazaki et al. 2000). Reduction of nitroaromatic compounds occurs by one- or two-electron reductive pathways. This can lead to the corresponding amine (R-NH_2) and generates electrophilic reactive intermediates (nitroso and hydroxyamino) or reactive oxygen species depending of the nitroreductase (NR) involved in the process and the availability of oxygen. There are two types of NR, type I (oxygen insensitive) proceeds with two-electron reduction. In contrast, type II (oxygen-sensitive) catalyzes one-electron reduction, generating the corresponding nitro anion radical intermediate that can be oxidized back to the parent nitro compound in the presence of molecular oxygen (Ask et al. 2004). When the redox cycle take place and regeneration of the nitro compound and production of superoxide anion take place, then dismutation yields hydrogen peroxide. Therefore, reduction of some nitroaromatic compounds can lead to the formation of reactive intermediates and/or potentially toxic reactive oxygen species (Ask et al. 2003).

When nitroarenes bind covalent to macromolecules nitro reduction is the first important step that takes place, this is an *N*-directed metabolic activation. The nitro group can either be partially reduced to form a nitroso or an *N*-hydroxyl intermediate or be completely reduced to an amine (Scheepers et al. 1994).

Cancer – inflammation, cell death and DNA-damage. Chronic inflammatory processes can induce oxidative stress. This makes a continuous and self-perpetuating production of excess ROS among other. Cellular DNA, RNA and proteins can then be damaged by reactions, such as oxidations, nitration, nitrosation, halogenation, and exocyclic ring formation in nucleic acid

bases. Inhibition of DNA repair enzymes and blockage of proapoptotic pathways may occur. This leads to increased genetic changes and altered functions of important proteins with ensuing deregulation of cell homeostasis, facilitating the initiation normal cells, their growth, and progression to malignancy (Bartsch and Nair 2006).

1.5 Cell death

Cell death and survival is essential for cell differentiation, and to maintain homeostasis (Nicotera et al. 2007). Cell death, and especially apoptosis act as a barrier to cancer, this was first raised in 1972 by Kerr, Wyllie and Currie. Loss of the tumor suppressor gene p53 is seen in greater than 50% of human cancers, and is a key component of the DNA damage sensor that can induce apoptosis (Hanahan and Weinberg 2000). Also in development we encounter cell death, apoptosis is a major form of cell death that is used to remove excess, damaged or infected cells throughout life (Bratton and Cohen 2001). Cell death is important in many other parts of life, but also in disease we encounter cell death. Both too much cell death, like in neurodegenerative diseases e.g. Parkinson and Alzheimer, and too little, e.g. development of cancers can be hazardous but for different reasons.

1.5.1 Permanent cell cycle arrest (senescence)

The term senescence is derived from the Latin word *senex*, meaning ”old age” or ”advanced in age”. Senescence at the cellular level is a physiological program of cellular growth arrest that is triggered by the shortening of telomers or by stress (Singh, George, and Shukla 2010). Senescent cells display phenotypic alterations that include a permanent growth arrest. This arrest typically occurs in the G_1 phase of the cell cycle and is often accompanied by expression of senescence associated β-galactosidase (SA-β-gal) activity. Senescent cells undergo gene expression alterations, including increased expression of cell cycle inhibitors such as p21 and p16 and down regulation of cell cycle proteins. Despite lack of proliferation these cells are metabolic active and may secrete proteins with tumor-promoting activities. Cellular senescence is a potent anti-cancer mechanism controlled by tumor suppressor genes, particularly p53 and pRb. P53 gets activated upon genotoxic and non genotoxic stresses like

oxidative damage and activated p21 and ultimately culminates the cell to senescence (Singh, George, and Shukla 2010) (Pazolli and Stewart 2008).

1.5.2 Autophagy

In Greek autophagy means self-eating (Gozuacik and Kimchi 2004). Autophagy is a tightly regulated pathway involving the lysosomal degradation of cytoplasmic organelles or cytosolic components. This catabolism enables recycling to maintain nutrient and energy homeostasis. This pathway can be stimulated by multiple form of cellular stress, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens. (Kroemer, Marino, and Levine 2010). Autophagy as also been linked to cell death, innate immunity, adaptive immunity, and tumor genesis. Morphologically, autophagic cell death is defined by the massive cytoplasmic accumulation of autophagocomes in dying cells without chromatin condensation, the hallmark of apoptosis (Malhi, Guicciardi, and Gores 2010).

1.5.3 Mitotic catastrophe

Mitotic catastrophe (MC) is a type of cell death that results from premature or inappropriate entry of cells into mitosis and can be caused by chemical and physical stresses. Mitotic catastrophe can be looked at as a process (prestage) preceding cell death, which can occur through necrosis or apoptosis. The final outcome of MC depends of the molecular profile of the cell.

The term ”mitotic catastrophe” is used to explain a mechanism of a delayed mitotic-linked cell death. The G_2 checkpoint of the cell cycle is responsible for blocking mitosis when DNA of cells is exposed to sustained insults. DNA damage activates a number of molecules that promote cellular activities such as cell-cycle arrest, DNA repair or apoptosis, if the damage is too harsh to be repaired. If the G_2 checkpoint is defective a cell can enter mitosis prematurely, before DNA replication of complete or DNA damage is repaired. This aberrant mitosis causes the cells to undergo death by mitotic catastrophe. The final step of MC is almost always characterized by the formation of nuclear envelopes around individual clusters of missegregated chromosomes. MC is also correlated with incomplete DNA synthesis and

premature chromosome condensation (Vakifahmetoglu, Olsson, and Zhivotovsky 2008; Jin and El-Deiry 2005).

1.5.4 Necrosis

Necrosis is thought to represent an accidental from of cell death with simultaneous disruption of multiple pathways. However, extensive failure of normal physiological pathways that are essential for maintaining cellular homeostasis, such as regulation of ion transport, energy production and pH balance can lead to necrosis .Necrosis is derived from the Greek “necros” for corpse and is characterized by oncosis (Greek for swelling) and the formation of plasma membrane blebs. There is also a lack of apoptotic markers like chromatin condensation or nucleosomal-sized DNA fragmentation. A cellular feature of necrosis is rupture of the plasma membrane, which leads to the release of cellular contents that can stimulate inflammation and damage surrounding cells. Other markers for necrosis are ATP depletion, excessive formation of reactive oxygen species and sustained increases in intracellular calcium. Necrosis can be triggered as a direct result of “extrinsic” stimulation by death cytokines in the TNF superfamily or by “intrinsic” signals such as DNA damage. Recently a new way of categorizing necrosis have also been used, necroptosis, as it too seems to have its own set of pathways steps (Challa and Chan 2010; Malhi, Guicciardi, and Gores 2010; Jin and El-Deiry 2005).

1.5.5 Apoptosis

Apoptosis is derived from a Greek word that describes “leaves falling from a tree”; it is used to define a specific morphological aspect of cell death characterized by membrane blebbing, shrinkage of the cell, chromatin condensation, and nuclear fragmentation, followed by scission of the cell into membrane defined bodies termed apoptotic bodies. Apoptosis is usually initiated and executed by activation of intracellular enzymes termed caspases as a contraction for cysteine-dependent aspartate specific protease. Caspases are synthesized as zymogens that must undergo proteolytic cleavage to exert proteolytic activity. Executioner caspases such as caspases 3, 7 and 6 are activated by cleavage by initiator caspases at aspartate residues and can be activated by either death receptor or mitochondrial pathways of apoptosis. These caspases may activate caspase-activated DNase (CAD) by cleaving ICAD, an inhibitor of this enzyme. CAD activation results in DNA cleavage at internucleosomal

linker regions of DNA resulting in the ladder pattern of DNA cleavage thought to be characteristic of apoptosis (Malhi, Guicciardi, and Gores 2010).

Apoptosis is a non-inflammatory form of cell death because apoptotic cells display "eat-me" signals such as phosphatidylserine on the cell surface early during the cell death process. This enable fast clearance of apoptotic cells by phagocytes before they can release cellular adjuvants to the environment and trigger an inflammatory response (Challa and Chan 2010).

Apoptosis is an essential part of life for multicellular organisms that plays an important role in development and tissue homeostasis. During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing organs and tissues (Jin and El-Deiry 2005). The term *programmed cell death* is often used in the literature, this refers to a genetically controlled process (Malhi, Guicciardi, and Gores 2010). There are to major apoptotic pathways; the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway) within a cell. The extrinsic pathway is activated by apoptotic stimuli comprising extrinsic signals such as the binding of death inducing ligands to cell surface receptors. Or, apoptosis is initiated following intrinsic signals including DNA damage induced by irradiation or chemicals, growth factor deprivation or oxidative stress (Jin and El-Deiry 2005; Clarke and Allan 2009).

The extrinsic pathway is driven by signals from the outside of the cell that binds as a ligand to so-called death receptors (DSs) on the cell surface. The fas receptor is as such a death receptor and is a part of the tumor necrosis factor (TNF) family. Multimerization of trimeric receptors leads to direct or indirect recruitment of the adaptor protein FADD allowing for activation of initiator pro-caspase 8 and subsequent activation of effector caspase 3, 6a and 7 (Tischner et al. 2010).

The intrinsic pathway, which leads to mitochondrial outer membrane permeabilization (MOMP) gives the release of apoptogenic factors from the inner-membrane space, most importantly cytochrome c, triggering oligomerization of the adapter molecule Apap-1, leading to recruitment and activation of initiator pro-caspase 9 molecules (Tischner et al. 2010). Caspase activation leads to the cleavage of cellular substrates and apoptosis. Bcl-2 family of proteins includes both pro- (Bax, Bak) as well as anti-apoptotic (Bcl-2, Bcl-x_L) proteins (Gross, McDonnell, and Korsmeyer 1999). Bax has been shown to homodimerize as well as

heterodimerize with Bcl-2 to repress cell death. Bad heterodimerizes with Bcl-x_L it displaces Bax from Bcl-x_L and promotes cell death (Yang et al. 1995).

1.6 Inflammation

Inflammation is the response of an organism's immune system to damage done to its cells and vascular tissue. It is usually a healing response, but can also proceed and become a chronic state (Weiss 2008). The inflammatory process includes a tissue-based reaction to trauma, go/no-go decision based on integration of molecular clue for tissue penetration by microbes, the killing of microbes and host cells they infect. Persistent inflammation can oxidize DNA severe enough to promote neoplastic transformation. The transformation from tissue damage to tissue repair begins as complement, neutrophils and macrophages kill microbes, and macrophages secrete more SLPI, a serine protease inhibitor expressed late after exposure to microbial products or cytokines (Nathan 2002).

1.7 Cytokines

An infection or injury will most likely cause an inflammation in the body. The recruitment of netrophiles to the site of injury is directed by chemotactic polypeptides, chemokines (Hoffmann et al. 2002). The chemokine family consists of approximately 50 small, basic proteins that are expressed and released by a wide range of normal and malignant cells. Based on their molecular structure these cytokines are divided into the two major subgroups CCL and CXCL chemokines that bind CCR or CXCR receptors respectively (Singh et al. 2010; Olsnes, Hatfield, and Bruserud 2009). There is increasing evidence that many proinflammatory and anti-inflammatory cytokines may promote tumor progression and affect the antitumor response (Tsujimoto et al. 2010). Interleukin-8 (IL-8) is a multifunctional protein that acts as intercellular regulatory factor. It is produced by immunocompetent cells such as T lymphocytes and monocytes in local inflammatory tissue infiltrates. Perionontal tissue components such as fibroblasts and epithelial and endothelial cells also participate in cytokine formation during inflammatory responses. IL-8 has a proinflammatory function. In noninduced cells, IL-8 is barely secreted, but its production is rapidly induced by a wide range of stimuli. Some stimuli can up-regulate IL-8 by more than 100-fold (Hoffmann et al. 2002; Candel-Marti et al. 2011).

1.8 DNA-damage

DNA lesions can be caused by either endogenous (reactive oxygen species (ROS) resulting from metabolic processes) or exogenous (ionizing radiation (IR), UV) agents, this happens frequently, approximately 10^4 per cell per day. The repair pathway activated is dependent of the type of lesion created. Base excision repair (BER) and nucleotide excision repair (NER) pathways are typically activated in response to damage to individual DNA bases (Seviour and Lin 2010).

1.9 DNA-damage response

To overcome all the lesions formed on DNA, cells have complex damage detection and repair system. This system is termed the DNA damage response (DDR), and encompasses the recognition of DNA damage, the transduction of signals through appropriate pathways and the activation of cellular responses ranging from DNA repair and chromatin remodeling to the activation of cell death if the damage is irreparable (Seviour and Lin 2010).

H2AX is a member of histone H2A family, which is one of the five types of histones that package and organize eukaryotic DNA into chromatin. The basic composition of chromatin is the nucleosome. Each nucleosome consists of eight histone molecules, two from each of the four core histones (H2A, H2B, H3 and H4) to form an octamer (Yuan, Adamski, and Chen 2010). Upon induction of a DNA double-strand break (DSB), the H2AX omega-4 serine residue becomes rapidly phosphorylated to from gamma-H2AX (γ-H2AX). Phosphorylation of H2AX is carried out by one of three phospho-inositide-3-kinase-related protein kinases – ATM ATR or DNA-PK (Coster and Goldberg 2010). H2AX has a role as a tumor suppressor (Dickey et al. 2009). H2AX is one of the most conserved H2A-variants and is present in chromatin at levels that vary between 2 and 25% of the H2A pool (Kinner et al. 2008).

Chk1 and Chk2 are serine-threonine checkpoint kinases, and are considered to be the major effectors of the DDR in regulating cell cycle checkpoints. Chk1 and Chk2 are phosphorylated by ATR and ATM, respectively. They are effector kinases that regulate DNA-damage-induced arrest at specific stages of the cell cycle by targeting key cell-cycle regulators. Chk1 is essential for early embryonic development and the G_2 checkpoint response to DNA damage

and replication block (Smits, Reaper, and Jackson 2006; Smith et al. 2010; Clarke and Allan 2009).

p53 is one of the best studied tumor suppressors. In more than 50% of human tumors it is mutated. It is a nuclear transcription factor that transactivate a variety of target genes implicated in the induction of cell cycle arrest (e.g. $p21^{waf}$), regulatory functions (e.g.Mdm2), DNA repair, and apoptotic cell death (e.g. IGF-BP3, BAX). The activation domain is located at the N-terminus of the protein. This domain can interact with components of the basal transcription machinery and promote transcription of genes with p53 binding sites. When cells are unstressed the p53 level is kept low by the negative regulator of p53, Mdm2 (Zhang, Liu, and Wang 2011). In response to cellular stress, like DNA damage, it is activated, by phosphorylation, and promotes cell cycle arrest followed by the replacement of DNA lesions and/or apoptotic cell death (Ozaki and Nakagawara 2011; Mak 2011; Unger et al. 1999).

2. AIMS

The objective with this study was to characterize the toxicity of the different nitro-PAHs in both the human bronchial epithelial cell line BEAS-2B and in the mouse hepatoma cell line Hepa1c1c7. From the first screening the focus were set to Hepa1c1c7 since the BEAS-2B gave little effect.

Sub-goals:

- To investigate the toxicity of the different nitro-PAH compounds, by studying the level of cell death (apoptosis and necrosis).
- To investigate the compounds induce survival and death signals.
- To study the compounds effect on cell cycle.
- To investigate the compounds effect on DNA-damage and DNA-damage signaling.

This master-thesis is a part of a larger project. Western analysis of Chk1, pp53 and NOXA was done by Leni Ekerheim at the National Health Institute, Division of environmental medicine,

3. EXPERIMENTAL CONSIDERATIONS

The experimental system BEAS-2B cells

A transformed continuous cell line, BEAS-2B cells, derived from normal human bronchial epithelial cells after transfection with an adenovirus 12-SV40 hybrid virus (Xatzipsalti and Papadopoulos 2007). A change in codon 47 of one allele to p53 was found in this line. But this change was also present in another sample, and later it was established that this change is

present in 3% of individuals in the population, and it is most likely a polymorphism (Reddel et al. 1995; Hussain et al. 2001).

The experimental system Hepa1c1c7 cells

A cancer cell line (Hepa1c1c7), derived from liver from mice. Several studies have shown that B[*a*]P and other PAHs can induce apoptosis in Hepa1c1c7 cells. The metabolism of these cells represent the situation *in* vivo better than many ofther cells closer to exposure sites, like cells from the lung. This cell line expresses aromatic hydrocarbon (Ah) receptor and is shown that it can induce cytochrome P450A1, which is an important enzyme in metabolism of PAHs. Several mutants of this cell line exist, among others an AhR-defective mutant.

Determination of cell death by fluorescence.

The sensitivity (the lower detection limit) of fluorescence microscopy is good since the signal-to-noise ratio is so high. By using fluorochromes no enzymes are needed that can be influenced by temperature, pH or other limiting conditions. The output of data is also large, as many cells can be stained at the same time, increasing the throughput of an experiment. This procedure is fairly easy and has a low cost, compared to other techniques like ELISA. By adding more than one flourochrome at the same time, more than one parameter can be investigated.

Cytokine release

Enzyme-Linked ImmuoSorbent Assay (ELISA) is an immulologic method that gives exact and sensitive detection, meanwhile it quantified the level of antigens in the supernatant. *Sandwich-ELISA*, were an antibody is attached to the bottom of a well gives both antigen binding and immunospesificity, while a secondary antibody that is conjugated to an enzyme gives detection and is an amplification factor. This can only be the case if the antigen has two epitopes and lead to double sensitivity, while Western blotting and flow cytometry only have simple sensitivity. In this study, the CXCL8 cytokine was investigated (Leng et al. 2008).

Flow cytometry

Flow cytometry is a very objective method, since it is automated and do not require visual inspection of slides. The physical properties of cells are measured by flow cytometry and also the fluorescence characteristics. Size and internal complexity of cells are detected by light scatter signals. By using fluorochromes that attach to proteins or DNA the emitted signal from a laser exciting the flouorochromes to a higher state can be gathered with optics and by running it through filters and dichronic mirrors the wavelength of interest can be isolated. The number of cells that are scored in this technique are $\geq$ 5000 cells, that will strengthen the statistically powers (Avlasevich et al. 2011; Wedemeyer and Potter 2001). Analysis of DNA content can display information of cell ploidy, position in the cell cycle and the frequency of apoptotic cells. Distribution of cells in the major phases of the cell cycle is based on differences in DNA content between cells in prereplicative phase ($G_{0/1}$) versus the cells that replicate DNA (S phase) versus the postreplicative plus mitotic (G_2 + M) phase cells. This method rely on staining of DNA by a fluorochrome (Darzynkiewicz, Halicka, and Zhao 2010).

Cell cycle

The cell cycle consist of phases were various events occur. They are called G_0, G_1, S, G_2 and M phase. Cells in G_0 are said to be quiescent, they are not dividing. G_1 is the phase before a cell start to divide, the cell will increase in size. DNA is replicated in the following S phase. G_2 ensures that the cell is ready to enter the M phase and divide. M phase, mitosis, is where the cell divides into two daughter cells (Clarke and Allan 2009).

Western blotting

In short, Western blotting is the transfer of proteins that are separated by electrophoresis to a nitrocellulose membrane. The first consideration that needs to be made is the creation of protein lysate. The Western blot analysis will only be as good as the protein lysates prepared for polyacrylamide gel electrophoresis (PAGE). The proteins in the sample should be collected and lysed quickly, preferably while chilled. SDS-PAGE is the most common strategy to electrophoretically separate proteins, and it also the method used in this study. SDS is a strongly anionic detergent, that binds denatured proteins within their hydrophobic regions, as a result becomes negatively charged. The amount of SDS that binds to the

denaturized proteins is approximately proportional to the molecular mass of the polypeptides facilitating directional migration based on the polypeptide size. After electrophoresis is done, a replica of the separated proteins can be made on a membrane, in this study a nitrocellulose membrane was used, this is called electro blotting. After blots are prepared they need to be blocked. This is because it can help mask non-specific binding sites on the membrane itself and it can promote renaturation of antigenic sites. After blocking the membrane is probed with a primary antibody that was created for the antigen at target. The membrane is then washed and incubated with an enzyme-conjugated secondary antibody that is reactive to the primary antibody. The membrane is washed again incubated with an appropriate enzyme substrate. The signal is then detected either with film or imaging for chemiluminescence (MacPhee 2010; Alegria-Schaffer, Lodge, and Vattem 2009).

DNA damage

The comet assay (single cell electrophoresis) is a popular method to measure low levels of damage in cellular DNA. Cells are embedded in agarose on a film and lysed. Supercoiling will be released by strand breaks, and loops can expand. These will move when exposed to electrophoresis and move against the anode, giving the appearance of a comet tail. The comet assay measures DNA strand breaks (SSBs) and alkali-labile sites (ALS). With a modification it can also measure oxidized bases such as 7.8-dihydro-8-oxoguanine (8-oxoG) and various ring-opened purines, this is enabled by including formamidopyrimidine DNA N-glycosylase/AP-lyase (Fpg). The % of DNA in the tail reflects the break frequency. (Hansen et al. 2010; Shaposhnikov, Frengen, and Collins 2009). Fpg is a multifunctional protein (from *Escherichia coli*) that excises damaged purine bases from DNA to generate aldehydic abasic sites and then catalyzes the successive cleavage of the phosphodieste bonds first at the 3'-side and then on the 5'-side of the abasic site to generate 5'- and 3'-phosphate ends, thereby exciting the deoxyribose residue (Bhagwat and Gerlt 1996). To allow comparison between different replicates of an experiment it is important that the films get the same treatment, but also for the same length of time. As prolonged electrophoresis for instance would give longer tails and hence influence the results, as they would appear as more damage on DNA.

Statistic analysis

ANOVA, analysis of variance, can compare two or more means at the same time. To use ANOVA the data have to be independent of each other, and normally distributed and have similar varians for each sample.

4. CONCLUSION

DNA is at all times subject to damage, mutations and other types of harm. Both caused by the cell itself and from external sources like PAHs. This is the reason why cells have developed an intricate resonse system to detect and repair damage, or if the damage is too great to manage, trigger apoptosis. In this study we have showed that different PAHs induce cell death in both BEAS-2B and Hepa1c1c7 cells, however the effect was greatest in the hepatoma cell line. We therefore investigated the signals given after exposure to PAHs in the Hepa1c1c7 cell line.

- BEAS-2B cells exposed to 1-NP showed increased cell death, in contrast to 1.3-DNP and 1.8-DNP. There was also an increase in CXCL8 from the cells after exposure to 1-NP.

- Hepa1c1c7 cells exposed to the nitro-PAHs showed both apoptotic and necrotic cell death. 1.3-DNP proved to be the most cytotoxic compound of the three nitro-PAHs tested. An increase in cell death could be seen between 12 and 20 hrs. 1.8-DNP gave no marked cell death until at a later time. The increased cell death found in Hepa1c1c7 cells when compared to BEAS-2B cells were likely due to a higher degree of metabolic activation often found in Hepa1c1c7 cells.

- 1.3-DNP induced cleavage of both caspase 3 and PARP indicating an apoptotic response. 1.8-DNP, however, gave no response to these two apoptotic markers. Indicating that there is no signals telling the cell to undergo apoptosis and die, which can lead to the compound being more genotoxic and mutagenic. The toxicity of 1.3-DNP was partly inhibited by zVAD-FMK indicating that caspases take part in the activation of the apoptotic process, but also suggesting that there could be an alternative pathway bypassing the caspase activation.

- All the compounds lead to alterations in cell cycle. 1.8-DNP lead to an accumulation of cells in S-phase, in contrast 1–NP and 1.3-DNP gave an accumulation of cells in

G_2.phase . 1.8-DNP gave also a marked increase in p53 phosohorylation together with oxidized damage.

- 1-NP, 1.3-DNP and 1.8-DNP showed a remarkable increase in oxidized damage. Suggesting that there can be ROS formation during metabolism of the compounds contributing to DNA damage.

- In addition to phosphorylation of p53 by 1.8-DNP we saw an activation of H2AX and Chk1 which supports a DNA damage response even if the cells do not die. We find p53 to be translocated to nuclei, however, observed no marked increase of NOXA nor p21 (data not shown). Thus, as previously suggested, the DNA damage response are possibly not fully triggered/inhibited; alternatively, there could be another damage signalling pathway in the cell that is needed to be triggered for execution of apoptosis.

- PFT-α fully inhibited 1.3-DNP induced cell death indicating that the p53 nuclear translocation and the following transcription is playing a central role in the apoptosis pathway. In contrast, PFT-μ did not inhibit apoptosis, thus suggesting that in this case a p-p53 translocation to the nucleus is not needed for the apoptotic process

- 1.3-DNP seemed to demand a smaller p53 response in order to trigger apoptosis. Immunocytochemical studies revealed that the p53 did accumulate somewhat in the nucleus in apoptotic cells. In addition, we saw some DNA damage response. 1.8-DNP did induce activation of Chk1 and p53. In contrast to the previous study, p53 was found to translocate to the nucleus, however, no effect was seen on NOXA. These findings suggest that other parallel cell death pathways may be lacking.

FURTHER WORK

- Comparative analysis of cytokines in Hepa1c1c7 cells by investigating levels of MIP-2 to compare these to the CXCL8 in BEAS-2B.

- The partly inhibition of cell death after 1.3-DNP exposure zVAD-FMK it can be caused by use to low concentration, studies with higher concentration could rule out if there really is a secondary pathway if a higher concentration led to fully inhibition of cell death.

- A further clarification and characterization of the DNA damage response, as well as any role of other cellular death signaling pathways are highly needed..

- Inhibition of aldo-ketoreductase 1C2 by UA (ursodeoxycholic acid). Aldo-ketoreductase 1C2 has been essential for 1-NP induction of p53, so a further investigation if the same would regard 1.3-DNP would be of great interest.

- Investigation of apoptosis by flow cytometri would be interesting, as that is a totally different methology and would support our finding.

REFERENCES

Air quality guidelines for Europe. 2000. *WHO Reg Publ Eur Ser* (91):V-X, 1-273.

Alegria-Schaffer, A., A. Lodge, and K. Vattem. 2009. Performing and optimizing Western blots with an emphasis on chemiluminescent detection. *Methods Enzymol* 463:573-99.

Arlt, V. M. 2005. 3-Nitrobenzanthrone, a potential human cancer hazard in diesel exhaust and urban air pollution: a review of the evidence. *Mutagenesis* 20 (6):399-410.

Ask, K., N. Decologne, N. Asare, J. A. Holme, Y. Artur, H. Pelczar, and P. Camus. 2004. Distribution of nitroreductive activity toward nilutamide in rat. *Toxicol Appl Pharmacol* 201 (1):1-9.

Ask, K., S. Dijols, C. Giroud, L. Casse, Y. M. Frapart, M. A. Sari, K. S. Kim, D. J. Stuehr, D. Mansuy, P. Camus, and J. L. Boucher. 2003. Reduction of nilutamide by NO synthases: implications for the adverse effects of this nitroaromatic antiandrogen drug. *Chem Res Toxicol* 16 (12):1547-54.

Avlasevich, S., S. Bryce, M. De Boeck, A. Elhajouji, F. Van Goethem, A. Lynch, J. Nicolette, J. Shi, and S. Dertinger. 2011. Flow cytometric analysis of micronuclei in mammalian cell cultures: past, present and future. *Mutagenesis* 26 (1):147-52.

Bartsch, H., and J. Nair. 2006. Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbecks Arch Surg* 391 (5):499-510.

Bell, M. L., and D. L. Davis. 2001. Reassessment of the lethal London fog of 1952: novel indicators of acute and chronic consequences of acute exposure to air pollution. *Environ Health Perspect* 109 Suppl 3:389-94.

Bhagwat, M., and J. A. Gerlt. 1996. 3'- and 5'-strand cleavage reactions catalyzed by the Fpg protein from Escherichia coli occur via successive beta- and delta-elimination mechanisms, respectively. *Biochemistry* 35 (2):659-65.

Black, J. 2003. Intussusception and the great smog of London, December 1952. *Arch Dis Child* 88 (12):1040-2.

Bostrom, C. E., P. Gerde, A. Hanberg, B. Jernstrom, C. Johansson, T. Kyrklund, A. Rannug, M. Tornqvist, K. Victorin, and R. Westerholm. 2002. Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. *Environ Health Perspect* 110 Suppl 3:451-88.

Bratton, S. B., and G. M. Cohen. 2001. Apoptotic death sensor: an organelle's alter ego? *Trends Pharmacol Sci* 22 (6):306-15.

Brook, R. D., B. Franklin, W. Cascio, Y. Hong, G. Howard, M. Lipsett, R. Luepker, M. Mittleman, J. Samet, S. C. Smith, Jr., and I. Tager. 2004. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation* 109 (21):2655-71.

Brown, J. R., and J. L. Thornton. 1957. Percivall Pott (1714-1788) and chimney sweepers' cancer of the scrotum. *Br J Ind Med* 14 (1):68-70.

Bryan, F. A. 1954. Problems of Research on Smog. *Calif Med* 81 (5):353-4.

Candel-Marti, M. E., A. J. Flichy-Fernandez, T. Alegre-Domingo, J. Ata-Ali, and M. A. Penarrocha-Diago. 2011. Interleukins IL-6, IL-8, IL-10, IL-12 and periimplant disease. An update. *Med Oral Patol Oral Cir Bucal*.

Chae, Y. H., T. Thomas, F. P. Guengerich, P. P. Fu, and K. El-Bayoumy. 1999. Comparative metabolism of 1-, 2-, and 4-nitropyrene by human hepatic and pulmonary microsomes. *Cancer Res* 59 (7):1473-80.

Challa, S., and F. K. Chan. 2010. Going up in flames: necrotic cell injury and inflammatory diseases. *Cell Mol Life Sci* 67 (19):3241-53.

Clarke, P. R., and L. A. Allan. 2009. Cell-cycle control in the face of damage--a matter of life or death. *Trends Cell Biol* 19 (3):89-98.

Coster, G., and M. Goldberg. 2010. The cellular response to DNA damage: A focus on MDC1 and its interacting proteins. *Nucleus* 1 (2):166-178.

Darzynkiewicz, Z., H. D. Halicka, and H. Zhao. 2010. Analysis of cellular DNA content by flow and laser scanning cytometry. *Adv Exp Med Biol* 676:137-47.

Dickey, J. S., C. E. Redon, A. J. Nakamura, B. J. Baird, O. A. Sedelnikova, and W. M. Bonner. 2009. H2AX: functional roles and potential applications. *Chromosoma* 118 (6):683-92.

Ezzati, M., A. D. Lopez, A. Rodgers, S. Vander Hoorn, and C. J. Murray. 2002. Selected major risk factors and global and regional burden of disease. *Lancet* 360 (9343):1347-60.

Fu, P. P., D. Herreno-Saenz, L. S. Von Tungeln, J. O. Lay, Y. S. Wu, J. S. Lai, and F. E. Evans. 1994. DNA adducts and carcinogenicity of nitro-polycyclic aromatic hydrocarbons. *Environ Health Perspect* 102 Suppl 6:177-83.

Garshick, E., F. Laden, J. E. Hart, B. Rosner, M. E. Davis, E. A. Eisen, and T. J. Smith. 2008. Lung cancer and vehicle exhaust in trucking industry workers. *Environ Health Perspect* 116 (10):1327-32.

Garshick, E., F. Laden, J. E. Hart, B. Rosner, T. J. Smith, D. W. Dockery, and F. E. Speizer. 2004. Lung cancer in railroad workers exposed to diesel exhaust. *Environ Health Perspect* 112 (15):1539-43.

Gorbunov, B., N. D. Priest, R. B. Muir, P. R. Jackson, and H. Gnewuch. 2009. A novel size-selective airborne particle size fractionating instrument for health risk evaluation. *Ann Occup Hyg* 53 (3):225-37.

Gozuacik, D., and A. Kimchi. 2004. Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* 23 (16):2891-906.

Gross, A., J. M. McDonnell, and S. J. Korsmeyer. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 13 (15):1899-911.

Guengerich, F. P. 2005. Life and times in biochemical toxicology. *Int J Toxicol* 24 (1):5-21.

Hajdu, S. I. 2006. Thoughts about the cause of cancer. *Cancer* 106 (8):1643-9.

Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100 (1):57-70.

Hansen, S. H., A. K. Olsen, E. J. Soderlund, and G. Brunborg. 2010. In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes. *Mutat Res* 696 (1):55-61.

Hatanaka, N., H. Yamazaki, Y. Oda, F. P. Guengerich, M. Nakajima, and T. Yokoi. 2001. Metabolic activation of carcinogenic 1-nitropyrene by human cytochrome P450 1B1 in Salmonella typhimurium strain expressing an O-acetyltransferase in SOS/umu assay. *Mutat Res* 497 (1-2):223-33.

Hesterberg, T. W., C. M. Long, C. A. Lapin, A. K. Hamade, and P. A. Valberg. 2010. Diesel exhaust particulate (DEP) and nanoparticle exposures: what do DEP human clinical studies tell us about potential human health hazards of nanoparticles? *Inhal Toxicol* 22 (8):679-94.

Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *J Leukoc Biol* 72 (5):847-55.

Hussain, S. P., P. Amstad, K. Raja, M. Sawyer, L. Hofseth, P. G. Shields, A. Hewer, D. H. Phillips, D. Ryberg, A. Haugen, and C. C. Harris. 2001. Mutability of p53 hotspot codons to benzo(a)pyrene diol epoxide (BPDE) and the frequency of p53 mutations in nontumorous human lung. *Cancer Res* 61 (17):6350-5.

IARC monographs on the evaluation of carcinogenic risks to humans. Diesel and gasoline engine exhausts and some nitroarenes. International Agency for Research on Cancer. 1989. *IARC Monogr Eval Carcinog Risks Hum* 46:1-458.

Jakobsson, R., P. Gustavsson, and I. Lundberg. 1997. Increased risk of lung cancer among male professional drivers in urban but not rural areas of Sweden. *Occup Environ Med* 54 (3):189-93.

Jin, Z., and W. S. El-Deiry. 2005. Overview of cell death signaling pathways. *Cancer Biol Ther* 4 (2):139-63.

Ju, K. S., and R. E. Parales. 2010. Nitroaromatic compounds, from synthesis to biodegradation. *Microbiol Mol Biol Rev* 74 (2):250-72.

Kampa, M., and E. Castanas. 2008. Human health effects of air pollution. *Environ Pollut* 151 (2):362-7.

Kawasaki, S., H. Takizawa, K. Takami, M. Desaki, H. Okazaki, T. Kasama, K. Kobayashi, K. Yamamoto, K. Nakahara, M. Tanaka, M. Sagai, and T. Ohtoshi. 2001. Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 24 (4):419-26.

Kinner, A., W. Wu, C. Staudt, and G. Iliakis. 2008. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res* 36 (17):5678-94.

Kroemer, G., G. Marino, and B. Levine. 2010. Autophagy and the integrated stress response. *Mol Cell* 40 (2):280-93.

Leng, S. X., J. E. McElhaney, J. D. Walston, D. Xie, N. S. Fedarko, and G. A. Kuchel. 2008. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. *J Gerontol A Biol Sci Med Sci* 63 (8):879-84.

MacPhee, D. J. 2010. Methodological considerations for improving Western blot analysis. *J Pharmacol Toxicol Methods* 61 (2):171-7.

Mak, A. S. 2011. p53 regulation of podosome formation and cellular invasion in vascular smooth muscle cells. *Cell Adh Migr* 5 (2):144-9.

Malhi, H., M. E. Guicciardi, and G. J. Gores. 2010. Hepatocyte death: a clear and present danger. *Physiol Rev* 90 (3):1165-94.

Nathan, C. 2002. Points of control in inflammation. *Nature* 420 (6917):846-52.

Nemery, B., P. H. Hoet, and A. Nemmar. 2001. The Meuse Valley fog of 1930: an air pollution disaster. *Lancet* 357 (9257):704-8.

Nicotera, P., O. H. Petersen, G. Melino, and A. Verkhratsky. 2007. Janus a god with two faces: death and survival utilise same mechanisms conserved by evolution. *Cell Death Differ* 14 (7):1235-6.

Olsnes, A. M., K. J. Hatfield, and O. Bruserud. 2009. The chemokine system and its contribution to leukemogenesis and treatment responsiveness in patients with acute myeloid leukemia. *J BUON* 14 Suppl 1:S131-40.

Ormstad, H., and M. Lovik. 2002. [Air pollution, asthma and allergy--the importance of different types of particles]. *Tidsskr Nor Laegeforen* 122 (18):1777-82.

Overall evaluations of carcinogenicity: an updating of IARC Monographs volumes 1 to 42. 1987. *IARC Monogr Eval Carcinog Risks Hum Suppl* 7:1-440.

Ozaki, T., and A. Nakagawara. 2011. p53: the attractive tumor suppressor in the cancer research field. *J Biomed Biotechnol* 2011:603925.

Pazolli, E., and S. A. Stewart. 2008. Senescence: the good the bad and the dysfunctional. *Curr Opin Genet Dev* 18 (1):42-7.

Purohit, V., and A. K. Basu. 2000. Mutagenicity of nitroaromatic compounds. *Chem Res Toxicol* 13 (8):673-92.

Reddel, R. R., R. De Silva, E. L. Duncan, E. M. Rogan, N. J. Whitaker, D. G. Zahra, Y. Ke, M. G. McMenamin, B. I. Gerwin, and C. C. Harris. 1995. SV40-induced immortalization and ras-transformation of human bronchial epithelial cells. *Int J Cancer* 61 (2):199-205.

Riedl, M., and D. Diaz-Sanchez. 2005. Biology of diesel exhaust effects on respiratory function. *J Allergy Clin Immunol* 115 (2):221-8; quiz 229.

Scheepers, P. T., M. M. Straetemans, J. P. Koopman, and R. P. Bos. 1994. Nitroreduction and formation of hemoglobin adducts in rats with a human intestinal microflora. *Environ Health Perspect* 102 Suppl 6:39-41.

Schwartz, J. 1994. Air pollution and daily mortality: a review and meta analysis. *Environ Res* 64 (1):36-52.

Senn, C. L. 1948. General atmospheric pollution; Los Angeles smog. *Am J Public Health Nations Health* 38 (7):962-5.

Seviour, E. G., and S. Y. Lin. 2010. The DNA damage response: Balancing the scale between cancer and ageing. *Aging (Albany NY)* 2 (12):900-7.

Shaposhnikov, S., E. Frengen, and A. R. Collins. 2009. Increasing the resolution of the comet assay using fluorescent in situ hybridization--a review. *Mutagenesis* 24 (5):383-9.

Singh, R., J. George, and Y. Shukla. 2010. Role of senescence and mitotic catastrophe in cancer therapy. *Cell Div* 5:4.

Singh, S., A. P. Singh, B. Sharma, L. B. Owen, and R. K. Singh. 2010. CXCL8 and its cognate receptors in melanoma progression and metastasis. *Future Oncol* 6 (1):111-6.

Smith, J., L. M. Tho, N. Xu, and D. A. Gillespie. 2010. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res* 108:73-112.

Smits, V. A., P. M. Reaper, and S. P. Jackson. 2006. Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Curr Biol* 16 (2):150-9.

Tischner, D., C. Woess, E. Ottina, and A. Villunger. 2010. Bcl-2-regulated cell death signalling in the prevention of autoimmunity. *Cell Death Dis* 1 (6):e48.

Tsujimoto, H., S. Ono, T. Ichikura, Y. Matsumoto, J. Yamamoto, and K. Hase. 2010. Roles of inflammatory cytokines in the progression of gastric cancer: friends or foes? *Gastric Cancer* 13 (4):212-21.

Twigg, M. V. 2005. Controlling automotive exhaust emissions: successes and underlying science. *Philos Transact A Math Phys Eng Sci* 363 (1829):1013-33; discussion 1035-40.

Unger, T., R. V. Sionov, E. Moallem, C. L. Yee, P. M. Howley, M. Oren, and Y. Haupt. 1999. Mutations in serines 15 and 20 of human p53 impair its apoptotic activity. *Oncogene* 18 (21):3205-12.

Vakifahmetoglu, H., M. Olsson, and B. Zhivotovsky. 2008. Death through a tragedy: mitotic catastrophe. *Cell Death Differ* 15 (7):1153-62.

Volkwein, J. C., S. E. Mischler, B. Davies, and C. Ellis. 2008. Field measurement of diesel particulate matter emissions. *Ann Occup Hyg* 52 (2):99-105.

Wedemeyer, N., and T. Potter. 2001. Flow cytometry: an 'old' tool for novel applications in medical genetics. *Clin Genet* 60 (1):1-8.

Weiss, U. 2008. Inflammation. *Nature* 454 (7203):427.

Wichmann, H. E. 2007. Diesel exhaust particles. *Inhal Toxicol* 19 Suppl 1:241-4.

Xatzipsalti, M., and N. G. Papadopoulos. 2007. Cellular and animals models for rhinovirus infection in asthma. *Contrib Microbiol* 14:33-41.

Yaffe, D., Y. Cohen, J. Arey, and A. J. Grosovsky. 2001. Multimedia analysis of PAHs and nitro-PAH daughter products in the Los Angeles Basin. *Risk Anal* 21 (2):275-94.

Yamazaki, H., N. Hatanaka, R. Kizu, K. Hayakawa, N. Shimada, F. P. Guengerich, M. Nakajima, and T. Yokoi. 2000. Bioactivation of diesel exhaust particle extracts and

their major nitrated polycyclic aromatic hydrocarbon components, 1-nitropyrene and dinitropyrenes, by human cytochromes P450 1A1, 1A2, and 1B1. *Mutat Res* 472 (1-2):129-38.

Yang, E., J. Zha, J. Jockel, L. H. Boise, C. B. Thompson, and S. J. Korsmeyer. 1995. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80 (2):285-91.

Yuan, J., R. Adamski, and J. Chen. 2010. Focus on histone variant H2AX: to be or not to be. *FEBS Lett* 584 (17):3717-24.

Zhang, X. P., F. Liu, and W. Wang. 2011. Two-phase dynamics of p53 in the DNA damage response. *Proc Natl Acad Sci U S A*.

Zhu, X., Z. T. Fan, X. Wu, K. H. Jung, P. Ohman-Strickland, L. J. Bonanno, and P. J. Lioy. 2011. Ambient concentrations and personal exposure to polycyclic aromatic hydrocarbons (PAH) in an urban community with mixed sources of air pollution. *J Expo Sci Environ Epidemiol*.

ARTICLE MANUSCRIPT

Effects of nitrosubstituted polycyclic aromatic hydrocarbons on DNA, cell signalling and apoptosis in BEAS-2B and Hepa1c1c7 cells.

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ABBREVIATIONS

AhR, aromatic hydrocarbon receptor; BEAS-2B, Human bronchial epithelia cell line; CXCL8, IL-8 (interleukin 8); DMSO, dimethyl sulfoxide; ELISA, *Enzyme-Linked ImmunoSorbent Assay*; 1-NP, 1-nitropyren; 1,3-dNP, 1,3-dinitropyren; 1,8-dNP, 1,8-dinitropyren; Chk, checkpoints; DDR, DNA damage response; nitro-PAH, nitro substituted-polycyclic aromatic hydrocarbons; γH2AX, phosphorylated *H2A.X*; Hoechst 33258, 2(2-(4-hydroxyphenyl)-6-benzimidazole-6-(1-methyl-4-piperazyl)benzimidazole hydrochloride); Hoechst 33342, 2´-(4-ethoxyphenyl)-2´,5´-bis-1*H*-benzimidazole hydrochloride); PAH, polycyclic aromatic hydrocarbon; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PFT, pifithrin; SSB, single strand breaks; zVAD-FMK, benzyolcarbonayl-Val-Ala-Asp-fluoromethyl ketone.

ABSTRACT

We show that nitrated-polycyclic aromatic hydrocarbons (nitro-PAHs) induce cell death in Hepa1c1c7 cells, as measured by fluorescence microscopy. BEAS-2B cells, however did not show any relevant effects. 1.3-DNP induce formation of the active form of both caspase 3 and its intracellular substrate, poly(ADP-ribose)polymerase (PARP). The most important finding was that the most mutagenic and carcinogenic compound, 1.8-DNP, induced a delayed cell death, when compared to 1.3-DNP, despite that this compound seemed to give the same amount of oxidative damage, judged by comet assay, increased phosohorylation of p53 and accumulation of cells in S-phase. 1-NP and 1.3-DNP gave an accumulation of cells in G_2-phase. 1.3-DNP seemed to demand a smaller p53 response in order to trigger apoptosis. Immunocytochemical studies revealed that the p53 did accumulate in the nucleus. In addition, we saw some DNA damage response. 1.8-DNP did induce activation of p53 and Chk1, however, no effect was seen on NOXA. These findings suggest that other parallel cell death pathways may be lacking.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants formed during incomplete combustion of organic material. An important subgroup of PAHs is the nitro-PAHs. They are formed when organic material is burned. PAHs are found on diesel exhaust particles. Epidemiological studies have shown that exposure to diesel exhaust and urban air pollution is associated with an increased risk of lung cancer (Garshick et al. 2004; Arlt 2005). Nitro-PAHs have shown in studies, that they are highly mutagenic in bacterial systems and that they can cause tumours in animal models (Takayama et al. 1983). Nitroarenes are common environmental pollutants (Purohit and Basu 2000).

Metabolism of nitro-PAHs is usually done by nitroreductases, CYP enzymes and phase II biotransformation enzymes (Purohit and Basu 2000). Cytochrome P450 (CYP) enzymes can activate PAHs to epoxide intermediates that can be further converted to more reactive dipole-epoxides. Nitro-PAH can also be converted to *N*-hydroxylated metabolites that subsequently are modified by phase II enzymes, such as acetyltransferase and sulfotransferase, to ultimate carcinogens (Shimada and Fujii-Kuriyama 2004). However, the most important metabolism of nitro-PAHs is the reductive pathways for nitroreduction that can lead to the corresponding amine with the production of electrophilic reactive intermediates or reactive oxygen species, depending on the nitroreductase (NR) and the availability of oxygen. One NR is oxygen insensitive, and have a two electron reduction and the other is oxygen sensitive and catalyses a one-electron reduction, which generates the corresponding nitro anion radical that can be oxidized back to the parent nitro compound in the presence of molecular oxygen (Ask et al. 2004). Due to the redox-cycle that takes place during the regeneration of the nitro compound and production of superoxide anion, there is a dismutation that yields hydrogen peroxide. This is the reason why metabolism of some nitroaromatic compounds can lead to reactive intermediates and potentially reactive oxygen species (Ask et al. 2003).

Nitro-PAH can induce different types of DNA damage. Formation of DNA adducts has been investigated in a numbers of articles (Ma and Ma 2002; Landvik et al. 2007). Oxidative damage can also be caused by nitro-PAHs, this can be due to metabolism that forms reactive metabolites. Metabolism by nitroreductases can also cause an ATP depletion in the cell.

DNA damage resulting from different sources produces a complex protein kinase signalling cascade that promotes repair of the damaged DNA and activates cell cycle checkpoints or apoptosis (E et al. 2011). DNA damage response is the detection system for lesions formed on DNA, DNA adducts as well as single- (SSB) and double- strand breaks (DSB). ATM ataxia telangiectasia mutated) is the major response to DSBs. This kinase is autophosphorylated when breaks occur, and will activate a number of downstream signals by phosphorylation resulting in a halt of cell cycle (Chk2) and initiating DNA repair (Ghosh et al. 2011). ATR (ATM and Rad3-related) is activated by DNA replication intermediates and thus monitor the DNA replication fork (Hurley and Bunz 2007; Kang et al. 2011). It does activate Chk1 by phosphorylation. Chk1 and Chk2 are checkpoint kinases and induce arrest at specific stages of the cell cycle, hence delaying cell division to let repair of DNA to take place. More specifically, they interact with the p53 protein, known as "the guardian of the genome". p53 is a tumour suppressor which controls genome stability by inducing growth arrest or apoptosis. It responds to DNA damage in various ways, depending on cell and stress types including DNA damage including that caused by gentotoxic pollutants such as PAHs, hypoxia and ultraviolet radiation irradiation. In response to DNA damage it is phosphorylated via Chk1/2, which leads to activation of the protein and dissociation from its repressor Mdm2. p53 directly up-regulates the expression of p21 resulting in cell cycle arrest, as well as cell surface receptors like Fas, mitochondrial pro-apoptotic proteins like Bax, Bak, PUMA and NOXA (Strom et al. 2006; Zhang, Liu, and Wang 2011). If the damage is too severe induction of the latter proteins will induce cell death often via apoptosis (Smits, Reaper, and Jackson 2006; Smith et al. 2010; Clarke and Allan 2009). H2AX is a member of these proteins, detecting the often lethal double strand breaks (DSB), and is than phosphorylated into γH2AX and function as a tumour suppressor protein. (Seviour and Lin 2010; Coster and Goldberg 2010).

Cell death is traditionally classified as apoptosis or necrosis. Apoptosis is an active process that requires energy to be executed. The programmed cell death, as it often is referred to, involvement of caspases and is characterized by shrinkage of the cell, nuclear fragmentation and chromatin condensation followed by the formation of apoptotic bodies. Necrosis, however, is characterized by cellular swelling and rupture of the plasma membrane, and it is often looked at as a passive type of cell death. Of other types of cell death does include senescence, autophagy and mitotic catastrophe (Malhi, Guicciardi, and Gores 2010),.

In previous studies Hepa1c1c7, liver epithelia cells, have been used as a model system to look at PAHs as they are competent to metabolic activate these chemicals into reactive metabolites. They have proven to be an interesting model to study PAH/nitro-PAHs-induced cell death (Solhaug et al. 2004; Solhaug, Refsnes, and Holme 2004; Solhaug et al. 2005; Landvik et al. 2007). The most important finding is that pro- and anti-apoptotic signals often occur simultaneously. In this context, it is important to further elucidate cellular signaling pathways modulating the survival/ death balance, which is of crucial importance for final carcinogenic. In a more recent study by Landvik 1.8-DNP induced little cell death despite the fact that it gave the most DNA damage, judged by DNA adducts, p53 activation and S-phase accumulation of cells, suggesting that the mutagenic and carcinogenic potential might be increased as the cells survive.

In the present study we have extended these findings by measuring single stand brakes and oxidative DNA damage by comet assay, and as well as the DNA-damage response has been investigated.

MATERIALS AND METHODS

Chemicals

LHC-9 cell culture medium was provided by Invitrogen (Carlsbad, CA, USA). Alexa Fluor 388 was purchased from Invitrogen (Eugene, Oregon, USA). Sterile HBS and purified collagen, PureCol™ was from Inamed Biomateriamls (Freemont, CA 94538 USA). 1-nitropyrene (1-NP), 1,3-dinitropyrene (1,3-DNP), 1,8-dinitropyrene (1,8-DNP), Benzo[a]pyrene (B[a]P), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylenediamineteraacetic acid (EDTA), Hoechst 33258, Hoechst 33342, aprotinin, Pifithrin-μ (PFT-μ), Ponceau S, phenylmethylsulfonyl fluoride (PMSF), propidium iodide (PI), polyoxyethylene octyl phenyl ether (Triton X-100) and zVAD-FMK were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Pifithrin-α (PFT- α) and Pepstatin A were from Calbiochem (Cambridge, MA, CA, USA). Leupeptin was from Amersham Biosciences (Uppsala, Sweden). Bio-Rad DC protein assay from Bio-Rad Laboratories, Inc (Hercules, CA, USA). Foetal calf serum (FCS), gentamycin, MEM alpha medium with L-glutamine, without ribonucleosides and deoxyribonucleosides were from Gibco BRL (Paisley, Scotland, UK). UltraPure™ Low Melting-Point Agarose was purchased from Invitrogen (Paisley, UK). Cytokine ELISA assay for CXCL8 (Human IL-8 Cytoset) was purchased from Biosource International (Camarillo, CA, USA). FPG enzymes were kindly donated by Prof. Andrew Collins (Department of Nutrition, Universtiy of Oslo, Norway). All other chemicals were of analytical grade and purchased from commercial sources.

Antibodies

Antibodies against: cleaved caspase 3, phospho-p53 (Ser15), p53, phospho-Chk1 (Ser345), cleaved PARP (Asp214), β-actin, phospho-H2A.X (Ser139) were obtained from Cell Signaling (Beverly, MA, USA); NOXA were purchased from Santa Cruz Biotechnology, Inc, (Santa Cruz, CA, USA), As secondary antibodies horseradish peroxidase-conjugated goat-anti-rabbit (Sigma Chemical Company, St. Louis, MO, USA), horseradish peroxidase-

conjugated rabbit anti-goat or rabbit anti-mouse IgG from Dako (Glostrup, Denmark) were used.

Cell culture

The mouse hepatoma Hepa1c1c7 cell line was purchased from European Collection of Cell Culture (ECACC). Earlier studies have shown that this cell line is a good substitution to bronchial epithelial cells as the metabolism resembles the *in* vivo situation (Landvik et al. 2007). Maintenance of the cells was done according to ECACC's guidelines and they were grown in alpha MEM medium with 2mM L-glutamine, without ribonucleotides and deoxyribonucleotides. Supplemented to the media were 10% heat-inactivated foetal calf serum (FCS, 10%) and 0,1mg/mL of the fungicide gentamycin. The cells were incubated in 5% CO_2 humified air at 37^{o}C, where they were kept in a logarithmic growth 1-9x10^{9} cells/75 cm^{2} flasks and split twice a week, Monday and Friday respectively. When splitting the cells, they were washed with phosphate-buffered saline (without Ca^{+} and Mg^{2+}; PBS) and added 0.0025% trypsin, which was removed shortly afterwards. The cells were then incubated for 1 min at 37^{o}C. The trypsin was inactivated by adding culture medium with FCS, and 1/10-1/20 of the cells was transferred into new flasks for further growth. On Wednesday fresh medium was added.

BEAS-2B cells, an immortalized SV40-adenovirus-hybrid (Ad12SV40) transformed human bronchial epithelial cell line were purchased from the American Tissue Type Culture Collection (ATTCC, Rockville, MD, USA). The p53 of this cell line is mutated in codon 47 but this does not change its functional properties (Hussain et al. 2001; Reddel et al. 1988). Cells were grown in LHC-9 medium on collagen (PureCol™)-coated culture flasks and dishes. Cells were incubated in 5% CO_2 humidified air at 37^{o}C, were they where kept in a logarithmic growth (1-9x10^{6} cells/75 cm^{2} flasks), and split twice a week, Monday and Thursday respectively. When splitting the cells, they were washed with phosphate-buffered saline (without Ca^{+} and Mg^{2+}; PBS) and added 0.0025% trypsin, which was removed shortly afterwards. The cells were then incubated for 3 min at 37^{o}C. The trypsin was inactivated by adding LHC-9 medium with fatty acid-free albumin, and 1.2x10^{6} or 1.0x10^{6} (Mondays and Thursday respectively) of the cells were transferred into new coated flasks for further growth. The day after the splitting procedure, fresh medium was added.

Exposure

Hepa1c1c7 cells were seeded in dishes (35mm 6-well culture dishes or 90mm culture dishes) or trays at a concentration of 70 000 per cm^2 the day before exposure. Fresh medium was added before exposure. When inhibitors were used, the cells were pre-incubated with the inhibitor for one h before adding test substances. Cells were treated with 1-NP, 1.3-DNP, 1.8-DNP or DMSO only as a control. All the compounds were dissolved in DMSO. The amount of DMSO added to the culture medium was ≤ 0,5%. After exposure cells were analyzed by light microscopy, fluorescence microscopy, flow cytomerty, comet assay, and western blotting.

BEAS-2B cells were plated in 35mm 6-well dishes ($8x10^4$ or $10x10^4$ cells/well). Fresh medium was added the day after seeding and right before exposure. Cells were treated with 1-NP, 1.3-DNP or 1.8-DNP, all the compounds were dissolved in DMSO or DMSO only as a control. The amount of DMSO in culture medium was ≤ 0,5%. After exposure cells were analyzed by light microscopy, fluorescence microscopy or ELISA.

Light microscopy

After exposure to the polycyclic aromatic hydrocarbons (PAHs) for 24hrs, 48hrs and 72hrs, the culture morphology was determined using light microscopy. To judge the relative amount of cytotoxicity in the cultures, the floating (dead) cells were compared to the attached (mostly living) cells. To record the living cultures, a digital camera (Nikon D40) coupled to the microscopy was used.

Fluorescence microscopy

To further characterize cytotoxicity, the cells were analyzed using fluorescence microscopy after staining to view changes in nuclear morphology and plasma membrane damage associated with apoptosis and necrosis. The cells were exposed to various concentrations (1-

30 μM) of different PAHs for 24 and 72 hrs. After trypsinating and staining of the cells with Hoechst 33342 (5 μg/mL) and propidium iodide (PI) (10 μg/mL), the cells were incubated in the dark for 30 min at room temperature. Followed by centrifugation, the pellet was resuspended with foetal calf serum (FCS) and prepared on a microscopy slide and air dried quickly. Both dye stains DNA, but only PI cannot penetrate an intact cell membrane. Cell morphology was evaluated using a Nikon Eclipse E 400 fluorescent microscope, with an UV-2A excitation filter 330-380 nm (magnification x 1000). At least 300 cells were counted per slide and classified as either viable, apoptotic or necrotic. Cells with clearly condensed and/or fragmented nuclei (both PI-negative and PI-positive) as well as PI-negative cells with partial chromatin condensation were counted as apoptotic and determined as a fraction of the total number of cells. PI-stained cells exhibiting a rounded morphology and homogenously stained nucleus (typical necrotic) or partially condensed chromatin with less flourescent intensities were termed PI positive. Non apoptotic cells, excluding PI, were considered as viable cells. (Asare et al. 2009; Solhaug, Refsnes, and Holme 2004)

Cytokine measurements

Enzyme-Linked Immuno-Sorbant Assay (ELISA), the biochemical technique for cytokine measurements that is the most widely used and best validated. This method enables high sensitivity and specificity to measure cytokines in a sample.(Leng et al. 2008)

BEAS-2B cells were grown in 35 mm, six-well dishes, exposed to selected compounds and incubated for 24 hrs at 37^{o}C. After exposure the medium was harvested and centrifuged at 250x*g* to remove floating cells. The final supernatants were stored at -70^{o}C. Cytokine protein levels were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's guidelines. Absorbance was measured and quantified using a plate reader (TECAN Sunrise Phoenix Research Products, Hayward, CA, USA) complete with software (Magellan V 1.10).

Flow cytomerty and cell cycle analysis

In flow cytometry, a suspension of cells is passed through a highly focused beam of laser light. Flow cytometry measures both the deviation of light (light scattering) as a function of size and optical properties of the cells as well as the absorption/emission of fluorescence from cell components that were stained with a fluorescent dye. This enables multiple parameters from each cell to be recorded at the same time; e.g. granulation and size. (Otsuki, Li, and Shibata 2003)

After treatment, cells were trypsinated and prepared for flow cytometry. The DNA of the cells were stained by incubating approximately $0.5x10^6$ cells with Hoechst 33258 (1.0 μg/mL) and Triton X-100 (0.1%) in the dark for 15 min. Triton X-100 makes the cells permeable and Hoechst 33258 stains DNA. To measure the blue fluorescence an Argus 100 Flow cytometer (Skatron, Lier, Norway) were used. Percentages of cells in the different phases of cell cycle were distinguished on the basis of their DNA content (Hoechst fluorescence) and cell size (forward light scatter). Percentages of cells in the different phases of cell cycle were estimated using the Multicycle Program (Phoenix Flow system, San Diego, CA, USA) (Darzynkiewicz and Huang 2004).

Western blotting immunoassay

Western blotting is a method to separate and to identify proteins. This is done by SDS-PAGE (sodium dodecyl sulphate polyachrylamide gel electrophoresis) which denaturates an equal amount of protein and separate them according to size. These are then transferred to a nitrocellulose (used in our study) support membrane by electrophoresis. Primary monoclonal or polyclonal antibodies are added to interact with a specific antigen epitope, which is presented by the target protein attached to the support membrane. Additionally a secondary antibody, conjugated with HRO (horse radish peroxidase) is added and binds to the primary antibody-antigen complex. Luminescent substrates are then used to visualize the bound components.

Hepa1c1c7 cells were grown in 90 mm Culture dishes ($7x10^5$ cells/dish) and exposed to various test substances for 24 hrs as described earlier. Cells were frozen at -20^{o}C for one h

and then at -80oC for at least 24 hrs, thawed and lysed in 20 mM Tris buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerol phosphate, 1mM Na3VO4, 1 mM NaF, 10 mg/mL leupeptin, 1 mM PMSF, 10 mg/mL aprotinin and 10 mg/mL pepstatin A. After cell lysis, solution were sonicated, centrifuged (290x*g*), and supernatant containing cellular proteins were collected. Protein concentration was measured by using a Bio-Rad DC protein assay kit. The samples were adjusted to contain equal amount of protein by adjusting with lysis buffer. 5 x SDS-PAGE sample buffer (0.312 M Tris-HCl, pH 6.8, 10% DSD, 25% β-mercaptoethanol, 0.005% bromophenol blue) and 10% glycerol was added. Samples were mixed and boiled for 5 min.

A sample of 12.5 µg protein in each well was subjected to 12% or 15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane by electro blotting. Staining with Ponceau S revealed equal protein loading. The blots were blocked with 3% fat-free dry milk, and then incubated with primary antibodies overnight at 4^{o}C or 2 hrs at room temperature in 3% fat-free dry milk or 5% bovine serum albumin (BSA), according to the manufacturer's recommendations. Then blots were incubated with horseradish peroxidase-conjugated secondary anti-rabbit, anti-mouse or anti-goat antibodies 1:5000 for 2 hrs at room temperature. Between each step the membranes were washed three times in cold 1xTBS (Tris-buffered saline). The western blots where developed using either the ECL chemiluminescence system according to the manufacturer's instructions or digitally. The results are from one out of three individual experiments.

Immunocytochemistery

Immunocytochemistery is a technique, like Western blotting, detecting proteins by specific antibody binding. The major difference to Western blotting is that the antibody-antigen interaction is within the cells, which enables visualization of the protein in question relative too for example the nucleus of the cell.

Hepa1c1c7 were exposed to 30 µM of 1.3-DNP and 1.8-DNP or DMSO only as a control, for 24 hrs as described earlier and seeded in 60 mm dishes ($7x10^5$ cells/well). Cells were washed in phosphate-buffered saline (PBS), and adherent cells were fixed with absolute methanol for 5 min in room temperature. To divide each dish in 3 different areas to be treated individually,

a PAP Pen was used. It is a special marking pen that provides a thin, film-like, green-tinged, hydrophobic barrier when a circle is drawn around a specimen on a slide, and prevents mixing of reagents when differently staining two or more sections on the same slide. Cells were incubated in humidified atmosphere, at room temperature overnight in the dark with primary antibody diluted in PBS with 1% BSA. One area of the dish was the control area without any primary antibody, serving the purpose to see if there were any unspecific binding that would cause auto fluorescence later. Finally the cells were incubated with secondary anybody Alexa Flour 488 (1:50) for 2 hrs under the same conditions as the primary antibodies. Between each step the dishes were washed three times with PBS. The last step in the procedure included adding a drop of DAPI GOLD, which stains the nuclei, as a mounting medium, and sealing with a cover glass to prevent drying and movement under the microscope. Data is from one individual experiment.

Single cell gel electrophoresis (comet assay)

The comet assay (single cell gel electrophoresis or SCGE) is a quick, simple, sensitive, reliable and fairly inexpensive way of measuring DNA damage. The alkaline comet assay resolves break frequencies up to a few thousand per cell, so the distances between breaks are of the order of 10^9 Da. The DNA is digested with a lesion-specific repair endonuclease, formamidopyrimidine-DNA N-glycosylase (Fpg, active on 8-OH-gua), which introduces breaks at sites of damage. (Collins, Dobson, et al. 1997)

The comet assay was performed as described previously (Hansen et al. 2010). In short, cells were exposed to 1-NP, 1.3-DNP, 1.8-DNP (1, 3, 10 or 30 µM) or B[a]P (10 µM) for 24 hrs. Media were removed and cells were trypsinated and resuspended at 10^6 cells/ mL in medium containing 10% FCS. Cells were dissolved in 0.75% low melting point agarose dissolved in PBS with EDTA and molded as 48 (7 µL) gels onto GelBond films attached to plastic frames to facilitate subsequent treatment steps. After lysis over night at 4^{o}C (2.5 M sodium chloride, 0.1 M Na_2EDTA, 10 mM Trizma base, 1% lauroylsarcocine sodium salt (≥94%) pH 10, with 1% Triton X-100 and 10% DMSO freshly added), films were washed in destilled water and then treated with a bacterial formamidopyrimidine-DNA glycosylase (FPG) enzymatic extract (1.0 µg/mL) or in the same buffer but without the FPG enzyme extract (1 h, 37^{o}C), and DNA

strand breaks and alkali-labile sites were detected. DNA unwinding was performed in electrophoresis buffer in the dark (5 + 35 min, 4^{o}C). After electrophoresis at 8-10^{o}C (0.8 V/cm, 300 mA, 20 min, pH 13.2) and neutralization (0.4 M Trizma base buffer pH 7.5 for 2 x 5 min), films were fixed in ethanol and dried. Rehydrated films were stained with SyrbGold (0.08 µl/ml in TE buffer, pH 7.4, 20 min in the dark) and scored with a Comet IV capture system (version 4.11; Perceptive Instruments, UK) counting 30 nuclei per gel window. The level of DNA damage is expressed as tail intensity, i.e. per cent fluorescence in the comet tail, relative to the total fluorescence of the comet.

Statistical analysis

All data were representatives of three or more independent experiments with identical conditions. Data is presented as mean ± SEM. Statistical significance was evaluated using analysis of variance (ANOVA) with the Dunnet post-test (two way ANOVA). This enables the possibility to obtain p-values to determine statistically significant differences between controls and treated sets. $P<0.05$ was considered significant. All calculations were executed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cell death

Morphology changes

BEAS-2B cells and Hepa1c1c7 cells were treated with different nitro-PAHs for 24 and 72 hrs, or DMSO only as a negative control. The morphology of the cultures was examined by using a reverted light microscopy. To judge the relative amount of cytotoxicity in the cultures, the floating (dead) cells were compared to the attached (mostly living) cells. As can be seen in figure 2, BEAS-2B did show little cytotoxicity, only 1-NP gave a marked effect. Seen in figure 3, Hepa1c1c7 showed increased cytotoxicity with treatment to 1-NP and 1.3-DNP, but gave no marked effect with treatment to 1.8-DNP. In addition, treatment to 1-NP resulted in formation of vacuoles.

Induction of apoptosis and necrosis

BEAS-2B and Hepa1c1c7 cells were treated with the different nitro-PAHs and DMSO for 24 hrs, then to further characterize the effects cells were stained with Hoechst 33342 and PI. This staining enables differentiation between apoptotic, necrotic and viable cells. Pictures in figure 4 shows cell death; typical living, apoptotic, necrotic, as well as apoptotic-necrotic cells induced by the compound that were tested. Some PI-positive cells showed partial chromatin condensation, indicating apoptosis, but with damage to the plasma membrane (apoptotic-necrotic cells). These cells were categorized and counted as apoptotic.

After exposure, 1-NP increased cell death in BEAS-2B cells. Increasing from 6% in control to 245% in 30 µM (Fig. 5A). No effect was seen on 1.3-DNP and 1.8-DNP as seen in figure 5.

Hepa1c1c7 cells however, did show increased cell death following 24 hrs of treatment with 1.3-DNP after 24 hrs of exposure (Fig. 6); increasing from 4 % in control to 31 % and 50 % at 10 and 30 µM, respectively. In contrast 1.8-DNP did not induce any significant cell death

after 24 hrs. However, after 72 hrs a significant effect was seen at 30 µM, with cell death increasing from 6.2 % in control to 25 % in the highest concentration, in details by (Fig. 7B). The time course of cell death induced by 1.3-DNP was further investigated by harvesting cells after 0, 8, 12, 20 and 24 hrs. A significant increase in apoptosis and necrosis were first seen after 12 hrs, from 1% to 3% in control and 30 µM, respectively. After 20 hrs the combined cell death was increased to 37%, and after 24 hrs it was at 36%.

Cytokine release

CXCL8

To measure the cytokine response from the cells, the ELISA technique was used. CXCL8 may contribute to tumor growth. This study showed a small increase of IL-8 after 1-NP exposure, in contrast 1.3-DNP and 1.8-DNP showed no increase (Fig 8).

Cleavage of Caspase 3 and PARP

Caspases are normally activated when apoptosis is executed, and can of that reason be used as a marker for apoptosis. Caspase 3 is a effector caspase, that can cleave several structural and regulatory proteins in apoptotic processes (Bratton and Cohen 2001). Cleavage of poly (ADP-ribose) polymerase (PARP) by caspase-3 is known to be a hallmark of apoptosis (Diefenbach and Burkle 2005). After exposure for 24 hrs to various concentrations of 1.3-DNP, Hepa1c1c7 were sampled and the lysate was analyzed with the western blotting method, The analysis showed an reduction in the level of protein with a similar molecule weight corresponding pro-caspase 3, and an increase in level of the cleaved caspase 3 at 10 and 30 µM (Fig. 9A). Western analysis also showed induced cleavage of PARP with 1.3-DNP (Fig 9B). In contrast, 1.8-DNP did change neither caspase 3 nor PARP. See figure 9A and B.

Inhibition of cell death; zVAD-FMK

zVAD-FMK is a cell-permeable caspase inhibitor that irreversibly binds to the catalytic site of caspase-1 and caspase-3 related proteases, and can inhibit induction of apoptosis (Chen et al. 2011). Light microscopy and fluorescence microscopy analysis after Hoechst 33342 and PI staining showed partly inhibition of 1.3-DNP induced cell death (Fig. 9C) . Cell death was reduced from 31 % in the positive control to 19 % in samples incubated with Z-VAD-FMK

Cell cycle alterations

To detect any alterations of cell cycle by nitro-PAHs Hepa1c1c7 cells were analyzed by flow cytomety, to determine the relative amount of cells in G1, S and G/M phase after 24 hrs of exposure. All three compounds induced a decrease of cells in G1 and an increase of cells in S phase. 1.3-DNP was most potent of the three, as it gave a significant G2 increase even at lower concentrations, from 11% in control to 32% and 33% in 3 µM and 10 µM respectively. Together with a decrease in G1 phase down from 59% in control to 25% in 10 µM, and S phase increase from 27% in control to 42% in 10 µM. 1.8-DNP also show more potent than 1-NP, it gave a significant decrease of cells in G1 phase, down from 57% in control to 42% at 10 µM and up from 28% in control to 42% at 10 µM in S phase. 1-NP gave a G1 reduction from 57% in control to 34% at 10 µM (Fig 10).

DNA damage

DNA damage indicating DNA strand breaks and oxidative damage was investigated using the comet assay method. In its traditional form it measures DNA strand-breaks (SSBs) and alkali-labile sites (ALS), but by including the bacterial formamidopyrimidine DNA N-glycosylase (Fpg) one can measure oxidized bases such as 7.8-dihydro-8-oxoguanine (8-oxoG) and various ring-opened purines (Collins, Dusinska, et al. 1997; Hansen et al. 2010). Samples were both untreated and treated with the lesion-specific endonuclease FPG, which recognizes specific lesions and converts them to strand breaks. Hepa1c1c7 cells were treated with 1, 3, 10 or 30 µM 1-NP, 1.3-DNP or 1.8-DNP, 10 µM B[*a*]P or DMSO as a control for 24 hrs. Treatment without FPG gave no increase in DNA damage, except for B[*a*]P that was used as a positive control (Fig. 11A). After FPG treatment a nice dose response was seen for all the compounds. 1-NP > 1.3-DNP > 1.8-DNP was the order of damage potential. 1-NP gave an

increase from 4 % in the control to 18 % in the highest concentration, 1.3-DNP from 3 % to 17 %, and 1.8-DNP increased from 3 % to 12 % (Fig. 11B).

DNA damage response

To investigate the different DNA damage signal pathways that can be triggered by 1.3-DNP and 1.8-DNP, we looked at the phosporylation of Chk1, H2AX and p53. Phosphorylation was investigated with western analysis, and p53 phosphorylation was also investigated with immunocytochemistery using specific antibody coupled with secondary antibody (Flour Alexa 488-A, horseradish peroxidase-conjugated goat-anti-rabbit).

Checkpoint Chk 1 is an effector kinase activated by ATR in response to single strand breaks. It is important in many response to DNA replication stress and some forms of DNA damage (Meuth 2010). Analysis of DNA response with western blotting showed an increase of phosporylation of Chk1 after 1.8-DNP exposure. 1.3-DNP gave no response to phosphorylation of Chk1 (Fig. 12B).

H2AX will become phosphorylated at serine 139 when double-strand (DSB) is induced in mammalian cells. This modified form is termed γ-H2AX, and can be easily identified with antibodies as a sensitive indicator of DNA DSB formation (Dickey et al. 2009). In our study, H2AX was detected after both exposure to 1.3-DNP and 1.8-DNP. Western blotting analysis showed an increase in the amount of phosphorylated protein with a molecular weight and antibody binding properties corresponding to γH2AX (i.e. the phosphorylated H2AX), and suggest an induction of γH2AX. The amount was increasing with increasing concentration of the compounds (Fig. 12A).

p53 is a tumor suppressor protein that is a transcription factor, particularly for genes that control progression through cell cycle or that initiate apoptosis. As a regulator of apoptosis it can act as an induction component to both the extrinsic and intrinsic pathway (Oda, Arakawa, et al. 2000; Tampio et al. 2008; Brady and Attardi 2010; Boehme and Blattner 2009). After exposure for 24 hrs, western blotting revealed an increase in phosphorylated p53 with exposure to 1.8-DNP. This increase is already seen at 1 µM, whereas increase after 1.3-DNP

exposure was only at 10 µM (Fig. 12). Immunocytochemi illustrated further that there was a translocation of p53 to the nucleus after 1.8-DNP exposure (Fig 14).

NOXA is a primarily a p53-response gene, but can also be activated in the absence of p53. It contributes to apoptosis initiated by p53 or DNA damage (Ploner, Kofler, and Villunger 2008). NOXA encodes a Bcl-2 homology 3 (BH3). When expressed it localizated to mitochondria and interacted with anti-apoptotic Bcl-2 family members, resulting in activation of caspase 3 (Oda, Ohki, et al. 2000). When exposed to 1.3-DNP for 24 hrs the apparent levels of NOXA decreased with increasing concentration, when analyzed by western blotting. In contrast, 1.8-DNP exposure did not show any change in protein level (Fig. 12).

Inhibitor of cell death by pifithrin-α and µ

Pifithrin-α (PFT- α) is a chemical compound known for its ability to suppress p53-mediated transactivation. It can protect cells from p53-mediated apoptosis induced by various stimuli, by inhibiting both pathways from p53 (Komarova et al. 2003; Strom et al. 2006). Light microscopy and fluorescence microscopy analysis after Hoechst 33342 and PT staining showed that PFT- α almost completely inhibited 1.3-DNP-induced cell death (Fig. 13A), suggesting a p53-mediated apoptosis, since it suppress the transactivation of p53.

Pifithrin-µ (PFT- µ) inhibits p53 binding to mitochondria by reducing its affinity to anti-apoptotic proteins Bcl-xl and Bcl-2, but has no effect on p53-dependent transactivation (Komarova et al. 2003). Light microscopy and fluorescence microscopy analysis after Hoechst 33342 and PI staining suggested rather and increase in of 1.3-DNP induced cell death (Fig. 13B). Thus, mitochondrial translocation of p53 seems to be of minor importance.

DISCUSSION

1-NP, 1.3-DNP and 1.8-DNP are known to induce tumors in rats, and of the three nitro-PAHs 1.8-DNP is generally thought to be the one that is the most genotoxic and carcinogenic (Imaida et al. 1991; Ohgaki et al. 1984; Takayama et al. 1983). Although BEAS-2B cells are lung cells, their response to the toxic effects of nitro-PAHs were not so relevant. While Hepa1c1c7 cells did show a metabolism close to what is seen *in vivo* even as it is a hepatoma cell line. In accordance to previous studies we find that 1.8-DNP, which is the most carcinogenic compound, caused little cell death. In contrast to the previous study, p53 was found to translocate to the nucleus, and we also found an adequate DNA damage response suggesting that other parallel cell death pathways are lacking. These possible cell death signaling pathways seemed to be activated by 1.3-DNP, in which a smaller p53 response seemed to be enough to trigger apoptosis.

The compounds tested induced cell death to various extents in the two different cell lines. In BEAS-2B cells only 1-NP resulted in a slight increase in cell death, while in Hepa1c1c7 cells also the other two nitro-PAHs; 1.3-DNP and 1.8-DNP, induced cell death. The far most cytotoxic of the three compounds was 1.3-DNP. An increased toxicity was observed at concentration as low as 3 µM, and as early as between 12 and 20 hrs the cell death started to occur. The response after 1.8-DNP exposure was notably smaller than the other two, so after further investigation over 72 hrs time course we saw that it did in fact induce cell death.

Cytokines regulates inflammation in two ways, both pro-inflammatory and anti-inflammatory; they can also affect tumor development. Acute inflammation after a bacterial or viral infection may have beneficial outcome to stop the infection. However, chronic inflammation is often harmful and can lead to several diseases including cancer. Also, inflammation after exposure to particles does not benefit the cells as the immune system cannot get rid of them. Instead this will lead to a chronic state which can result in tissue damage, due to formation of ROS and other active metabolites In this study we saw only a cytokine response after 1-NP exposure of BEAS-2B cells. Earlier studies have shown a great increase in CXCL8 levels after 1-NP (Ovrevik et al. 2010). CXCL8, the cytokine measured, can contribute to human cancer progression.

It is the caspases that gives apoptotic cells their characteristic appearance of condensed nuclei (Bratton and Cohen 2001). Caspase 3, one of the effector caspases, cleaves regulatory proteins, including PARP, which is mainly involved in DNA repair and apoptosis (Diefenbach and Burkle 2005). In this study we observed that exposure to 1.3-DNP resulted in cleaving of both Caspase 3 and PARP. This correlates well with the cell death observed with fluorescence microscopy. The necrosis observed with apoptosis after exposure to 1-NP, 1.3-DNP and 1.8-NP can be due to an incomplete apoptotic process and a switch from apoptosis to necrosis, most probably due to inhibition of mitochondrial respiration or inactivation of caspases (Nicotera, Leist, and Ferrando-May 1999; Solhaug et al. 2004). When mitochondrial respiration is inhibited or disturbed in some way the cells will be ATP depleted, as apoptosis is an energy requiring process the cell cannot fully execute it as have to undergo another process, necrosis (Leist et al. 1997). An interesting note is that DNA strand breaks events results in the activation of PARP. And PARP participates in the repair process of DNA but uses nicotinamide adenine dinuclotide (NAD) as a substrate in ADP ribosylation of damaged nuclear proteins and DNA, and ADP ribosylation of itself. To maintain the NAD stores the cells use ATP, then activation of PARP can contribute to excessive consumption of ATP, leading to necrotic cell death (Nanavaty et al. 2002). Our study showed that zVAD-FMK did partially inhibit 1.3-DNP induced cell death; this supports the need for caspase activation in the induced cell death. But as the inhibition was not complete there must be an alternative caspase-independent pathway as well. Oxidative damage to mitochondria seems to be important for the fate of the cells as mitochondria is not only “the energy production unit” of a cell but it is also a key participant in many signal pathways, like the extrinsic apoptotic pathway (Fariss et al. 2005). Moderate production of ROS can then lead to the production of inflammatory mediators, whereas oxidative stress to high levels of ROS have been shown to induce cytotoxicity (Boland et al. 2000).

Earlier work have showed 1.8-DNP to be a more potent mutagen and carcinogen than 1.3-DNP, however, the damage 1.8-DNP causes is great enough to halter further cell replication (Imaida et al. 1991; Takayama et al. 1983). Since 1.8-DNP halts cell cycle it is possible that it induces DNA damage that in most cases should result in cell death. One hypotesis is that the cells survive the DNA damage, but with an increased probability of having mutations and chromosomal aberrations. Abilities like this may contribute to an explanation to the increased mutagenic and carcinogenic properties we find in 1.8-DNP (Landvik et al. 2007). In this study 1.3-DNP and 1.8-DNP were the most potent compounds with regard to cell cycle alterations.

1-NP and 1.3-DNP gave a significant accumulation of cells in G_2-phase, whereas 1.8-DNP gave an increase of cells in S-phase. Large amount of DNA adducts and SSBs, which in turn halter DNA replication, can be the reason why the cell cycle is stalled. Previous studies have shown a detection of a major DNA adduct after exposure to 1.8-DNP (Landvik et al. 2007). Differences in nitroreductases can be one basis for the differences in responses of nitropyrenes to DNA adduct formation. 1-NP and 1.3-DNP are reduced by a nitroreductase that transfers a single electron, 1.8-DNP, however, is reduced by an enzyme that transfers two electrons (Eddy et al. 1986).

To further investigate the damage that could lead to alterations in cell cycle we used the comet assay. Previous studies have shown that 1-nitrosopyrene, a metabolite of 1-NP, are reduced by NADH and can then produce significant amounts of ROS through cyclic redox reactions, resulting in oxidative damage (Cherng et al. 2006). This is verified in our study as we detected oxidative damage from exposure with 1-NP, but also from the other two compounds, 1.3-DNP and 1.8-DNP. In the present study we showed an increase in SSBs when cells were exposed to 1.8-DNP at 30 µM, and a minor increase when exposed to 1.3-DNP. Oxidative DNA damage, detected as 8-oxoG, was high in all the compounds cells were exposed to. This correlates with the finding of accumulation of cells in S-phase that is described earlier. DNA damage may activate cell cycle checkpoints and stall replication.

PAHs may induce apoptosis resulting from DNA damage, and/or cytotoxic damage (Landvik et al. 2007). We wanted to investigate the mechanisms and pathways that exposure to 1.3-DNP and 1.8-DNP would give, but also to see if there was any difference between the two. 1-NP has already been more thoroughly investigated and are known to activate a set of different signaling pathways. DNA damage can initiate different cell signaling pathways as a response to the damage. These include activation of checkpoints to the cell cycle and delay of the cycle progression, which will lead to an increase in S-phase, enabling time to repair the damaged DNA or induction of cell death. Characterization of signaling routes involved in response to DNA damage detection and execution, commonly called the DNA damage response.

In this study, we observed an increase in phosphorylation of H2AX, phosphorylation of serine 15 at p53, activation of Chk1, NOXA, however gave no marked decrease in 1.8-DNP exposed cells. 1.3-DNP gave response in H2AX and a notably activation of p53, however, none in

Chk1 and NOXA. There is an indication that points to formation of γH2AX as an early chromatin modification following initiation of DNA fragmentation during apoptosis (Rogakou et al. 2000). This finding is interesting as 1.8-DNP only gave cell death after longer exposure. p53 may trigger apoptosis, and expression of several different apoptotic genes involved in the mitochondrial apoptotic pathway are stimulated by p53 (Meek 2004). BCL-2 family proteins regulate the release of cytochrome c from mitochondria and the following activation of caspases.

p53 inhibition with pifithin-α (PFT- α) did show a full inhibition of cell death induced by 1.3-DNP. PFT- α does inhibit nuclear accumulation of p53. This correlates with p53 being involved in the 1.3-DNP-induced cell death, and that the nuclear translocation and the following transciption is playing a central role in the apoptotic pathway. Pifithrin-μ (PFT-μ) gave no decrease in inhibition of cell death, suggesting that translocation of p53 to the nucleus is not needed to execute the apoptotic prcess (Komarova et al. 2003). This supports our findings from the immunocytochemistery that the phosphorylated p53 accumulates in the nucleus, and hence implies a transactivation of p53.

Immunocytochemistery showed no translocation of p-p53 after 1.3-DNP exposure but after 1.8-DNP exposure a translocation was seen. This implies that there have to be some kind of inhibition of the death signal further down the signal pathway, as the cells do not die.

Treatment to 1-NP did show a formation of vacuoles as described earlier by (Asare et al. 2009). In the present study, a difference in the cellular effects the PAHs caused was seen. Different number of nitro amines and also their configuration seem to have an important significance in how they affect the cells. The reason for this could be that they interact with different enzymes, or that the affinity for the same enzyme is higher or lower. It is therefore interesting to see that there is such a dramatic difference regarding the cytotoxicisity between 1.3-DNP and 1.8-DNP.

As conclusion, the study showed that the different nitro-PAHs had very different toxic potential (cell death), and that a small change in molecular structure, such as a substitution from 3rd position to 8th position can have such a massive impact to the cellular response. Most interesting were that 1.8-DNP induced low cytotoxcicity, while it still had almost the same level of DNA damage as the rest of the compounds tested. The DDR is activated, represented

by Chk1 and p53, suggesting that other parallel pathways may be lacking. In contrast to a previous study, p53 was found to translocate to the nucleus. 1.3-DNP, however, seemed to activate the cell death signaling pathway, but the amount of p53 response required to trigger apoptosis seemed smaller.

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REFERENCES

Arlt, V. M. 2005. 3-Nitrobenzanthrone, a potential human cancer hazard in diesel exhaust and urban air pollution: a review of the evidence. *Mutagenesis* 20 (6):399-410.

Asare, N., X. Tekpli, M. Rissel, A. Solhaug, N. Landvik, V. Lecureur, N. Podechard, G. Brunborg, M. Lag, D. Lagadic-Gossmann, and J. A. Holme. 2009. Signalling pathways involved in 1-nitropyrene (1-NP)-induced and 3-nitrofluoranthene (3-NF)-induced cell death in Hepa1c1c7 cells. *Mutagenesis* 24 (6):481-93.

Ask, K., N. Decologne, N. Asare, J. A. Holme, Y. Artur, H. Pelczar, and P. Camus. 2004. Distribution of nitroreductive activity toward nilutamide in rat. *Toxicol Appl Pharmacol* 201 (1):1-9.

Ask, K., S. Dijols, C. Giroud, L. Casse, Y. M. Frapart, M. A. Sari, K. S. Kim, D. J. Stuehr, D. Mansuy, P. Camus, and J. L. Boucher. 2003. Reduction of nilutamide by NO synthases: implications for the adverse effects of this nitroaromatic antiandrogen drug. *Chem Res Toxicol* 16 (12):1547-54.

Boehme, K. A., and C. Blattner. 2009. Regulation of p53--insights into a complex process. *Crit Rev Biochem Mol Biol* 44 (6):367-92.

Boland, S., V. Bonvallot, T. Fournier, A. Baeza-Squiban, M. Aubier, and F. Marano. 2000. Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 278 (1):L25-32.

Brady, C. A., and L. D. Attardi. 2010. p53 at a glance. *J Cell Sci* 123 (Pt 15):2527-32.

Bratton, S. B., and G. M. Cohen. 2001. Apoptotic death sensor: an organelle's alter ego? *Trends Pharmacol Sci* 22 (6):306-15.

Chen, S. Y., L. Y. Chiu, M. C. Maa, J. S. Wang, C. L. Chien, and W. W. Lin. 2011. zVAD-induced autophagic cell death requires c-Src-dependent ERK and JNK activation and reactive oxygen species generation. *Autophagy* 7 (2):217-28.

Cherng, S. H., S. L. Hsu, J. L. Yang, C. T. Yu, and H. Lee. 2006. Suppressive effect of 1-nitropyrene on benzo[a]pyrene-induced CYP1A1 protein expression in HepG2 cells. *Toxicol Lett* 161 (3):236-43.

Clarke, P. R., and L. A. Allan. 2009. Cell-cycle control in the face of damage--a matter of life or death. *Trends Cell Biol* 19 (3):89-98.

Collins, A., M. Dusinska, M. Franklin, M. Somorovska, H. Petrovska, S. Duthie, L. Fillion, M. Panayiotidis, K. Raslova, and N. Vaughan. 1997. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ Mol Mutagen* 30 (2):139-46.

Collins, A. R., V. L. Dobson, M. Dusinska, G. Kennedy, and R. Stetina. 1997. The comet assay: what can it really tell us? *Mutat Res* 375 (2):183-93.

Coster, G., and M. Goldberg. 2010. The cellular response to DNA damage: A focus on MDC1 and its interacting proteins. *Nucleus* 1 (2):166-178.

Darzynkiewicz, Z., and X. Huang. 2004. Analysis of cellular DNA content by flow cytometry. *Curr Protoc Immunol* Chapter 5:Unit 5 7.

Dickey, J. S., C. E. Redon, A. J. Nakamura, B. J. Baird, O. A. Sedelnikova, and W. M. Bonner. 2009. H2AX: functional roles and potential applications. *Chromosoma* 118 (6):683-92.

Diefenbach, J., and A. Burkle. 2005. Introduction to poly(ADP-ribose) metabolism. *Cell Mol Life Sci* 62 (7-8):721-30.

Eddy, E. P., E. C. McCoy, H. S. Rosenkranz, and R. Mermelstein. 1986. Dichotomy in the mutagenicity and genotoxicity of nitropyrenes: apparent effect of the number of electrons involved in nitroreduction. *Mutat Res* 161 (2):109-11.

Fariss, M. W., C. B. Chan, M. Patel, B. Van Houten, and S. Orrenius. 2005. Role of mitochondria in toxic oxidative stress. *Mol Interv* 5 (2):94-111.

Garshick, E., F. Laden, J. E. Hart, B. Rosner, T. J. Smith, D. W. Dockery, and F. E. Speizer. 2004. Lung cancer in railroad workers exposed to diesel exhaust. *Environ Health Perspect* 112 (15):1539-43.

Ghosh, S., H. Narang, A. Sarma, H. Kaur, and M. Krishna. 2011. Activation of DNA damage response signaling in lung adenocarcinoma A549 cells following oxygen beam irradiation. *Mutat Res*.

Hansen, S. H., A. K. Olsen, E. J. Soderlund, and G. Brunborg. 2010. In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes. *Mutat Res* 696 (1):55-61.

Hurley, P. J., and F. Bunz. 2007. ATM and ATR: components of an integrated circuit. *Cell Cycle* 6 (4):414-7.

Hussain, S. P., P. Amstad, K. Raja, M. Sawyer, L. Hofseth, P. G. Shields, A. Hewer, D. H. Phillips, D. Ryberg, A. Haugen, and C. C. Harris. 2001. Mutability of p53 hotspot

codons to benzo(a)pyrene diol epoxide (BPDE) and the frequency of p53 mutations in nontumorous human lung. *Cancer Res* 61 (17):6350-5.

Imaida, K., M. S. Lee, C. Y. Wang, and C. M. King. 1991. Carcinogenicity of dinitropyrenes in the weanling female CD rat. *Carcinogenesis* 12 (7):1187-91.

Kang, Y., H. M. Cheong, J. H. Lee, P. I. Song, K. H. Lee, S. Y. Kim, J. Y. Jun, and H. J. You. 2011. Protein phosphatase 5 is necessary for ATR-mediated DNA repair. *Biochem Biophys Res Commun* 404 (1):476-81.

Komarova, E. A., N. Neznanov, P. G. Komarov, M. V. Chernov, K. Wang, and A. V. Gudkov. 2003. p53 inhibitor pifithrin alpha can suppress heat shock and glucocorticoid signaling pathways. *J Biol Chem* 278 (18):15465-8.

Landvik, N. E., M. Gorria, V. M. Arlt, N. Asare, A. Solhaug, D. Lagadic-Gossmann, and J. A. Holme. 2007. Effects of nitrated-polycyclic aromatic hydrocarbons and diesel exhaust particle extracts on cell signalling related to apoptosis: possible implications for their mutagenic and carcinogenic effects. *Toxicology* 231 (2-3):159-74.

Leist, M., B. Single, A. F. Castoldi, S. Kuhnle, and P. Nicotera. 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J Exp Med* 185 (8):1481-6.

Leng, S. X., J. E. McElhaney, J. D. Walston, D. Xie, N. S. Fedarko, and G. A. Kuchel. 2008. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. *J Gerontol A Biol Sci Med Sci* 63 (8):879-84.

Ma, J. Y., and J. K. Ma. 2002. The dual effect of the particulate and organic components of diesel exhaust particles on the alteration of pulmonary immune/inflammatory responses and metabolic enzymes. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 20 (2):117-47.

Malhi, H., M. E. Guicciardi, and G. J. Gores. 2010. Hepatocyte death: a clear and present danger. *Physiol Rev* 90 (3):1165-94.

Meek, D. W. 2004. The p53 response to DNA damage. *DNA Repair (Amst)* 3 (8-9):1049-56.

Meuth, M. 2010. Chk1 suppressed cell death. *Cell Div* 5:21.

Nanavaty, U. B., R. Pawliczak, J. Doniger, M. T. Gladwin, M. J. Cowan, C. Logun, and J. H. Shelhamer. 2002. Oxidant-induced cell death in respiratory epithelial cells is due to DNA damage and loss of ATP. *Exp Lung Res* 28 (8):591-607.

Nicotera, P., M. Leist, and E. Ferrando-May. 1999. Apoptosis and necrosis: different execution of the same death. *Biochem Soc Symp* 66:69-73.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 288 (5468):1053-8.

Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura, and Y. Taya. 2000. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 102 (6):849-62.

Ohgaki, H., C. Negishi, K. Wakabayashi, K. Kusama, S. Sato, and T. Sugimura. 1984. Induction of sarcomas in rats by subcutaneous injection of dinitropyrenes. *Carcinogenesis* 5 (5):583-5.

Otsuki, Y., Z. Li, and M. A. Shibata. 2003. Apoptotic detection methods--from morphology to gene. *Prog Histochem Cytochem* 38 (3):275-339.

Ovrevik, J., V. M. Arlt, E. Oya, E. Nagy, S. Mollerup, D. H. Phillips, M. Lag, and J. A. Holme. 2010. Differential effects of nitro-PAHs and amino-PAHs on cytokine and chemokine responses in human bronchial epithelial BEAS-2B cells. *Toxicol Appl Pharmacol* 242 (3):270-80.

Ploner, C., R. Kofler, and A. Villunger. 2008. Noxa: at the tip of the balance between life and death. *Oncogene* 27 Suppl 1:S84-92.

Purohit, V., and A. K. Basu. 2000. Mutagenicity of nitroaromatic compounds. *Chem Res Toxicol* 13 (8):673-92.

Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, J. B. Park, J. S. Rhim, and C. C. Harris. 1988. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. *Cancer Res* 48 (7):1904-9.

Rogakou, E. P., W. Nieves-Neira, C. Boon, Y. Pommier, and W. M. Bonner. 2000. Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J Biol Chem* 275 (13):9390-5.

Seviour, E. G., and S. Y. Lin. 2010. The DNA damage response: Balancing the scale between cancer and ageing. *Aging (Albany NY)* 2 (12):900-7.

Shimada, T., and Y. Fujii-Kuriyama. 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci* 95 (1):1-6.

Smith, J., L. M. Tho, N. Xu, and D. A. Gillespie. 2010. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res* 108:73-112.

Smits, V. A., P. M. Reaper, and S. P. Jackson. 2006. Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Curr Biol* 16 (2):150-9.

Solhaug, A., S. Ovrebo, S. Mollerup, M. Lag, P. E. Schwarze, S. Nesnow, and J. A. Holme. 2005. Role of cell signaling in B[a]P-induced apoptosis: characterization of unspecific effects of cell signaling inhibitors and apoptotic effects of B[a]P metabolites. *Chem Biol Interact* 151 (2):101-19.

Solhaug, A., M. Refsnes, and J. A. Holme. 2004. Role of cell signalling involved in induction of apoptosis by benzo[a]pyrene and cyclopenta[c,d]pyrene in Hepa1c1c7 cells. *J Cell Biochem* 93 (6):1143-54.

Solhaug, A., M. Refsnes, M. Lag, P. E. Schwarze, T. Husoy, and J. A. Holme. 2004. Polycyclic aromatic hydrocarbons induce both apoptotic and anti-apoptotic signals in Hepa1c1c7 cells. *Carcinogenesis* 25 (5):809-19.

Strom, E., S. Sathe, P. G. Komarov, O. B. Chernova, I. Pavlovska, I. Shyshynova, D. A. Bosykh, L. G. Burdelya, R. M. Macklis, R. Skaliter, E. A. Komarova, and A. V. Gudkov. 2006. Small-molecule inhibitor of p53 binding to mitochondria protects mice from gamma radiation. *Nat Chem Biol* 2 (9):474-9.

Takayama, S., M. Tanaka, Y. Katoh, M. Terada, and T. Sugimura. 1983. Mutagenicity of nitropyrenes in Chinese hamster V79 cells. *Gann* 74 (3):338-41.

Tampio, M., J. Loikkanen, P. Myllynen, A. Mertanen, and K. H. Vahakangas. 2008. Benzo(a)pyrene increases phosphorylation of p53 at serine 392 in relation to p53 induction and cell death in MCF-7 cells. *Toxicol Lett* 178 (3):152-9.

Zhang, X. P., F. Liu, and W. Wang. 2011. Two-phase dynamics of p53 in the DNA damage response. *Proc Natl Acad Sci U S A*.

FIGURE LEGENDS

Figure 1. Chemical structures of the test compounds.

Figure 2. Morphology changes determined by light microscopy. BEAS-2B cells were exposed to 1, 5, 10, 15 or 30 µM 1-NP, 1.3-DNP or 1.8-DNP, for 24hrs. Control cells were treated with DMSO only. Original magnification; 100X, examined by reverted microscope.

Figure 3. Morphology changes determined by light microscopy. Hepa1c1c7 cells were exposed to 1, 3, 10 or 30 µM 1.3-DNP or 1.8-DNP, for 24hrs. Control cells were treated with DMSO only. Original magnification; 100X, examined by reverted microscope.

Figure 4. Morphology changes determined by fluorescence microscopy. Both BEAS-2B and Hepa1c1c7 cells were treated with 1-NP, 1.3-DNP, 1.8-DNP or DMSO (control), for 24 hrs. Concentration was 30 µM. The cells are stained with Hoechst 33342 and propidium iodide (PI). Hoechst stains nucleus and DNA appear fluorescent blue. Hoechst can penetrate the cell membrane. PI is a red fluorescent dye. It can only stain cells with a disrupted membrane. V: viable cells. Nec: necrotic cells. Ap: apoptotic cells. Ap/Nec: apoptotic-necrotic cells. Original magnification; 400X?.

Figure 5. Cell death determined by fluorescence microscopy. BEAS-2B cells were exposed to 1-NP, 1.3-DNP, 1.8-DNP or DMSO (control), for 24 hrs. The concentration was 1, 5, 10, 15 or 30 µM. Cells were exposed and trypsinated, stained with Hoechst 33342 and propidium iodide (PI), and subsequently analyzed for apoptosis, necrosis and apoptosis/necrosis using fluorescence microscopy. The data presents mean ±SEM of independent experiments (n=5). No significant difference from DMSO-treated controls.

Figure 6. Cell death determined by fluorescence microscopy. Hepa1c1c7 cells were exposed to 1-NP, 1.3-DNP, 1.8-DNP or DMSO (control), for 24 hrs. The concentration was 1, 3, 10 or 30 µM. Cells were exposed and trypsinated, stained with Hoechst 33342 and propidium iodide (PI), and subsequently analyzed for apoptosis, necrosis and apoptosis/necrosis using fluorescence microscopy. The data presents mean ±SEM of independent experiments (n=3). * Significantly different from DMSO-treated controls.

Figure 7. Celle death determined by fluorescence microscopy. A) Hepa1c1c7 cells were exposed to 1.3-DNP or DMSO (control), for 24 hrs. The concentration was 1, 3, 10 or 30 µM. Cells were harvested after 0, 8, 12, 20 or 24 hrs. B) Cells were exposed to 1.8-DNP or DMSO (control) and harvested after 72 hrs. Cells were exposed and trypsinated, stained with Hoechst 33342 and propidium iodide (PI), and subsequently analyzed for apoptosis, necrosis and apoptosis/necrosis using fluorescence microscopy. The data presents mean ±SEM of independent experiments (n=3). * Significantly different from DMSO-treated controls.

Figure 8. IL-8 determined by ELISA. A) BEAS-2B cells were exposed to 1, 5, 10, 15, or 30 µM of 1-NP, 1.3-DNP or 1.8-DNP or DMSO for 24 hrs, and the media were subsequently analyzed. The data presents mean ±SEM of independent experiments (n=5). B)

Figure 9. Effects of PAHs on caspase 3 and PARP, determined by Western blotting, and inhibition of apoptosis by zVAD-FMK. In Hepa1c1c7 cells (A) Levels of PARP and caspase 3 were analyzed by Western blotting, after 24 hrs of exposure of various levels of 1.3-DNP, 1.8-DNP or DMSO. Picture is one out of three independent experiments. (B) Microscopic examination, following PI/Hoechst 33342 staining, of the effect of the caspase inhibitor zVAD-FMK on 1.3-DNP induced cell death (apoptosis and necrosis). The Hepa1c1c7 cells were pre-treated for 1 h with zVAD-FMK (µM) followed by co-exposure of 1.3-DNP (30 µM) or DMSO, for 24 hrs. Percentage of cell death was estimated by fluorescence microscopy counts. The data presents mean ±SEM of independent experiments (n=3). * Significantly different from DMSO-treated controls. # Significantly different from treatment without zVAD-FMK.

Figure 10. Celle cycle distributions measured by flow cytometri. Hepa1c1c7 cells were exposed to 1-NP, 1.3-DNP, 1.8-DNP or DMSO (control), for 24 hrs. The concentration was 0.1, 0.3, 1 or 3 µM. Cells were trypsinated and stained with Hoechst 33258. Data is presented as the relative proportions of cells (%) in the different cell cycle phases. Each bar represents mean ± SEM of independent experiments. (n=3). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 11. DNA damage measured by comet assay. Illustration of cells with different amount of tail intensity (A). Hepa1c1c7 cells were exposed to 1, 3, 10 or 30 µM of 1-NP, 1.3-DNP, 1.8-DNP, B[*a*]P or DMSO, for 24 hrs. DNA strand breaks were measured without enzyme treatment (B), and oxidative damage to DNA was measured after treatment with FPG enzyme (C). Values (% tail DNA) represent mean ± SEM of independent experiments (n=3).

Figure 12. Effects of PAHs on histone H2AX, Chk1, phosphorylated p53 and NOXA, determined by Western blotting. In Hepa1c1c7 cells: (A) Levels of phosphorylated H2AX (γH2AX) were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 1.3-DNP, 1.8-DNP or DMSO. The picture is representative for one out of three separate experimental set-ups. (B) Levels of p-Chk1 after exposure to the PAHs for 24 hrs (C) Levels of phosphorylated p53 after exposure to the PAHs for 24 hrs. (D) Levels of NOXA after exposure to 1.3-DNP and 1.8-DNP for 24 hrs.

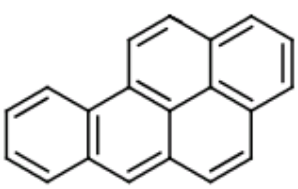
Figure 13. 1.3-DNP induced p53 activation and involvement if cell death, determined by fluorescence microscopy. In Hepa1c1c7 cells: (A) Cells were exposed to 10 µM 1.3-DNP for 24 hrs with or without pre-incubation for 1 h with pifithrin- α (PFT-α, 20 µM). Control cells were treated with DMSO only. (B) Cells were exposed to 10 µM 1.3-DNP for 24 hrs with or without pre-incubation for 1 h with pifithrin- µ (PFT-µ, 20 µM). Control cells were treated with DMSO only. * Significantly different from DMSO-treated controls. # Significantly different from treatment without inhibitor.

Figure 14. Effects on phosphorylated p53 determined by immunocytochemistery. In Hepa1c1c7 cells subcellular location of phosphorylated p53 was analyzed by

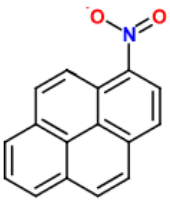
immunocytochemistery after 24 hrs of exposure to 30 µM of 1.3-DNP and 1.8-DNP. The picture is only acquired from one experimental set up. Original magnification; 1000X.

FIGURES

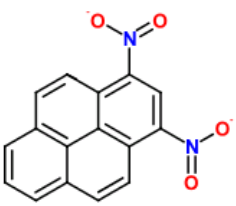
Figure 1



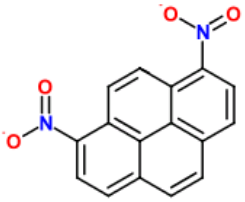
Benzo[*a*]pyrene (B[*a*]P)



1-nitropyrene (1-NP)



1.3-dinitropyrene (1.3-DNP)



1.8-dinitropyrene (1.8-DNP)

Figure 2

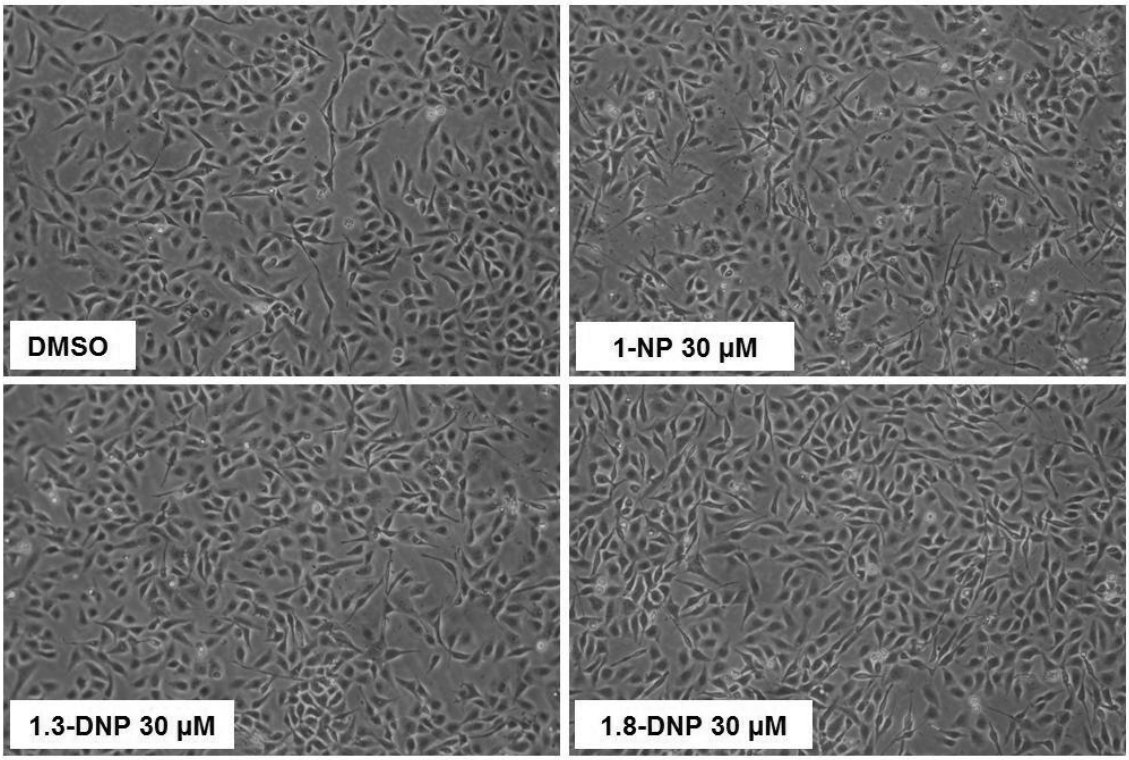


Figure 3

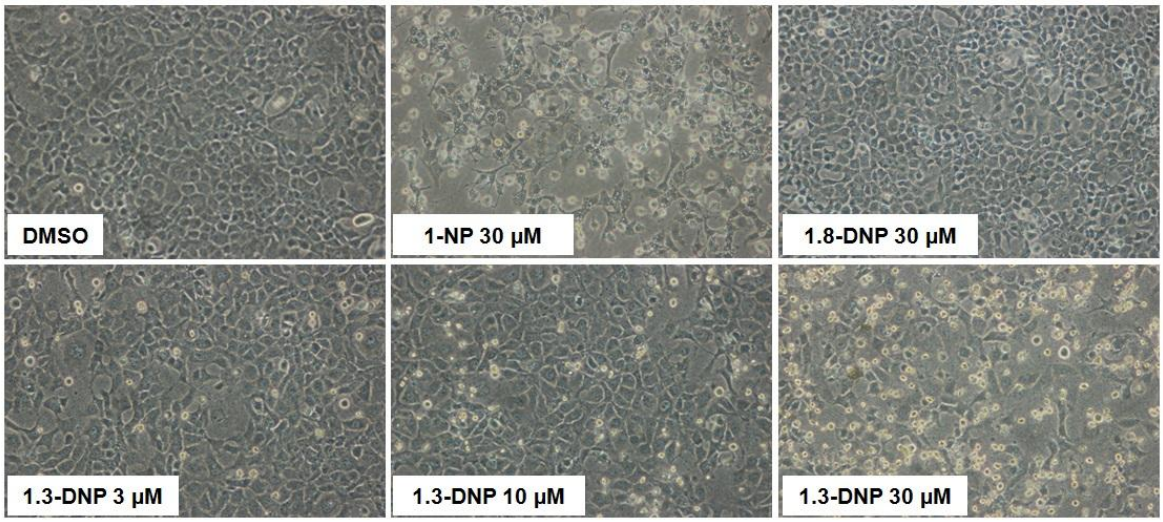


Figure 4

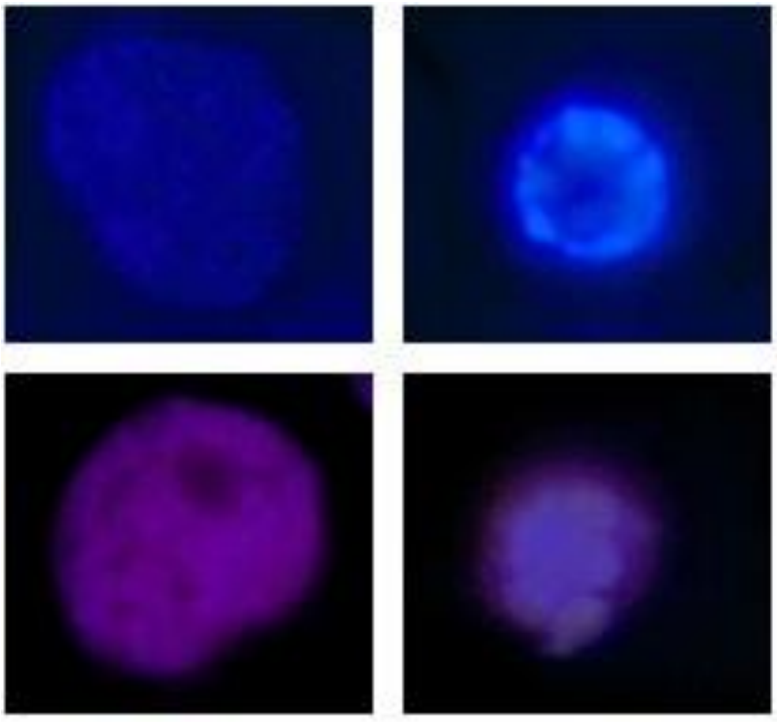
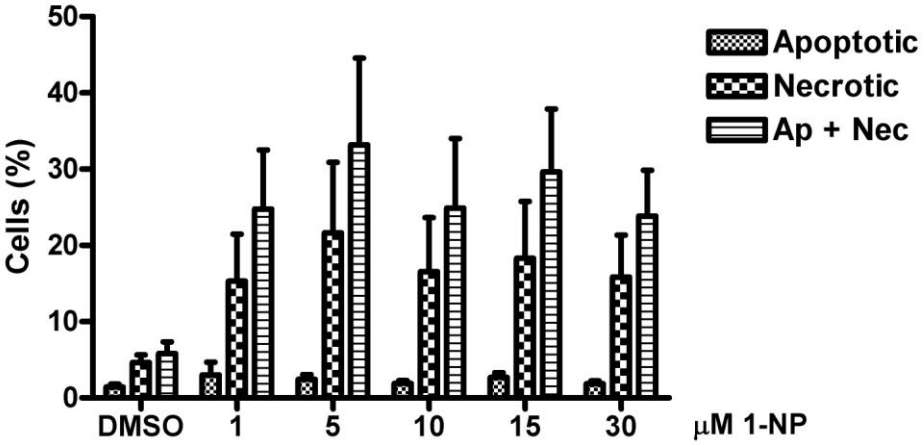
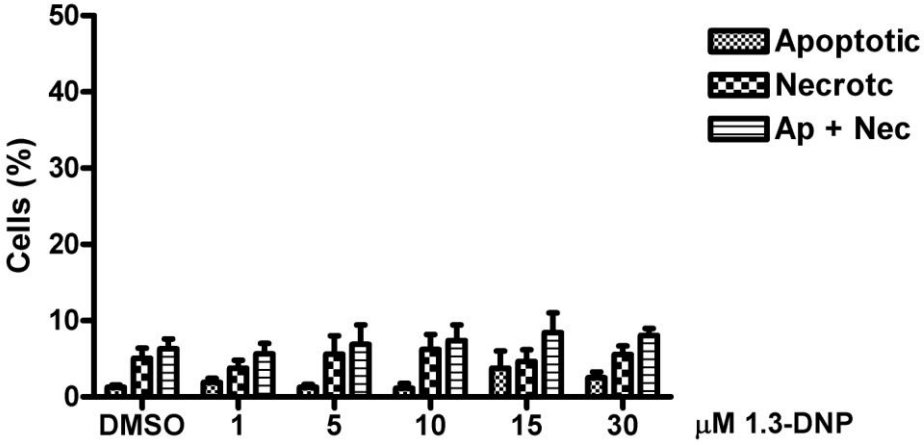


Figure 5


Cells (%)
50
40
30
20
10
0
DMSO
1
5
10
15
30
μM 1-NP
Apoptotic
Necrotic
Ap + Nec


Cells (%)
50
40
30
20
10
0
DMSO
1
5
10
15
30
μM 1.3-DNP
Apoptotic
Necrotc
Ap + Nec

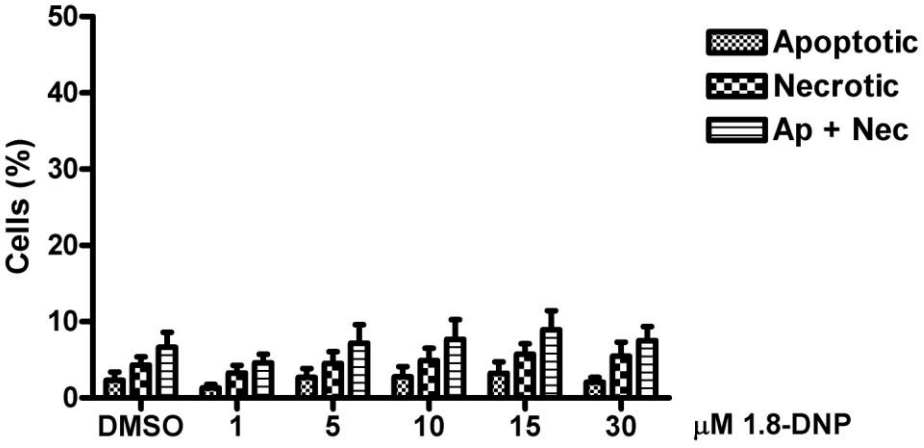
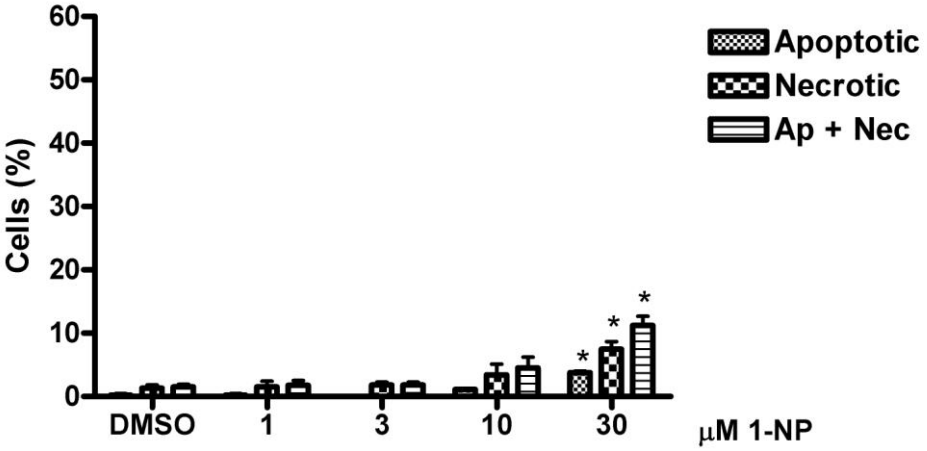
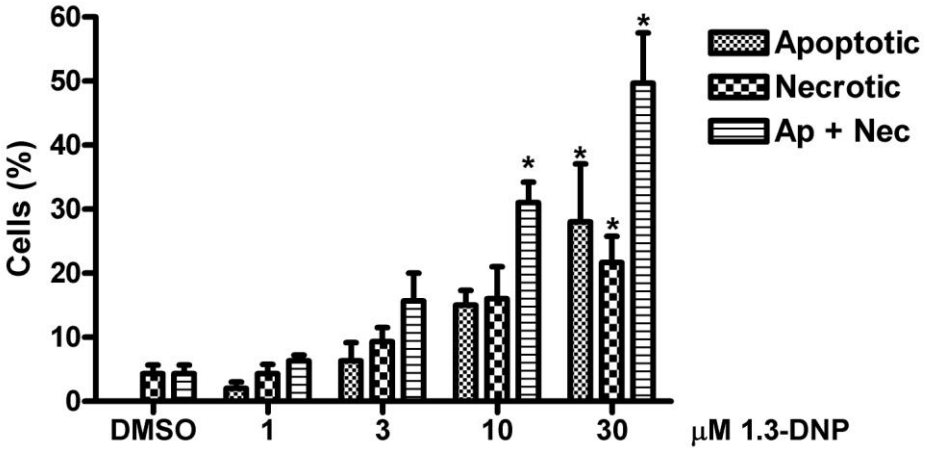

Cells (%)
50
40
30
20
10
0
DMSO
1
5
10
15
30
μM 1.8-DNP
Apoptotic
Necrotic
Ap + Nec

Figure 6


Cells (%)
60
50
40
30
20
10
0
DMSO
1
3
10
30
μM 1-NP
Apoptotic
Necrotic
Ap + Nec
*
*
*


Cells (%)
60
50
40
30
20
10
0
DMSO
1
3
10
30
μM 1.3-DNP
Apoptotic
Necrotic
Ap + Nec
*
*
*
*

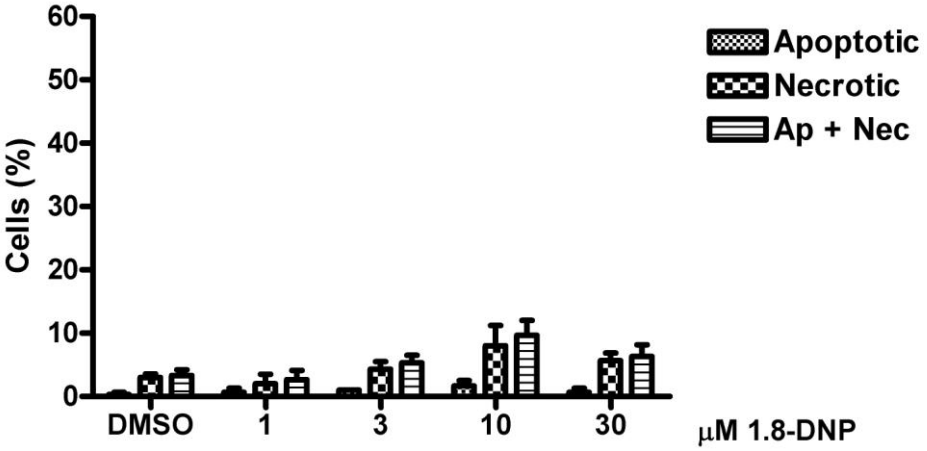
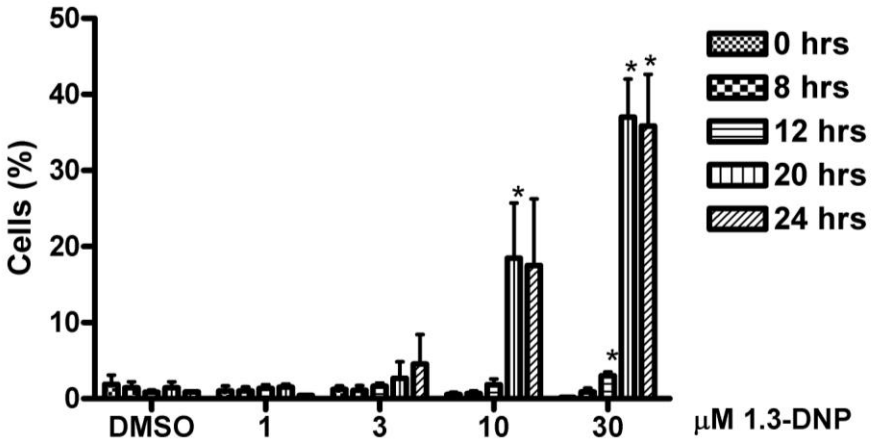

Cells (%)
60
50
40
30
20
10
0
DMSO
1
3
10
30
μM 1.8-DNP
Apoptotic
Necrotic
Ap + Nec

Figure 7

(A)


50
40
30
20
10
0
Cells (%)
DMSO
1
3
10
30
μM 1.3-DNP
0 hrs
8 hrs
12 hrs
20 hrs
24 hrs
*
*
*
*

(B)

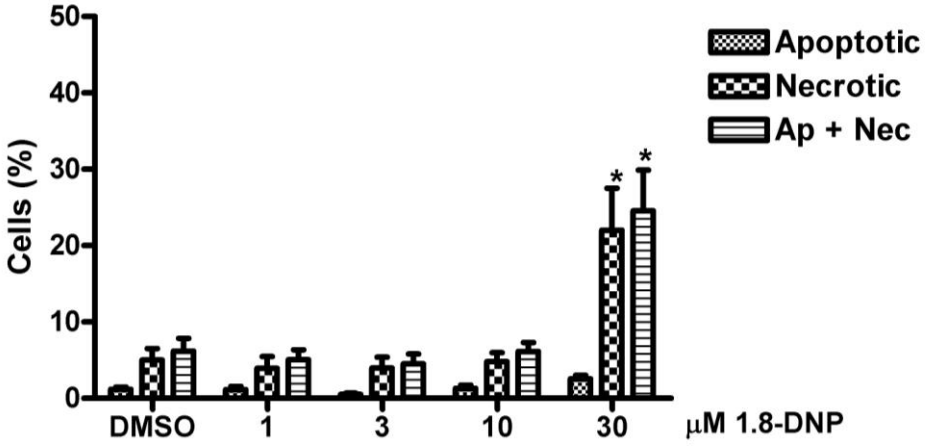

50
40
30
20
10
0
Cells (%)
DMSO
1
3
10
30
μM 1.8-DNP
Apoptotic
Necrotic
Ap + Nec
*
*

Figure 8

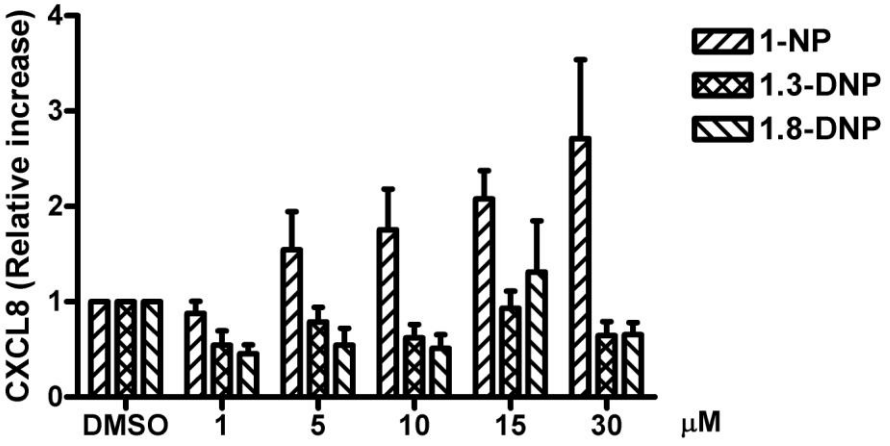
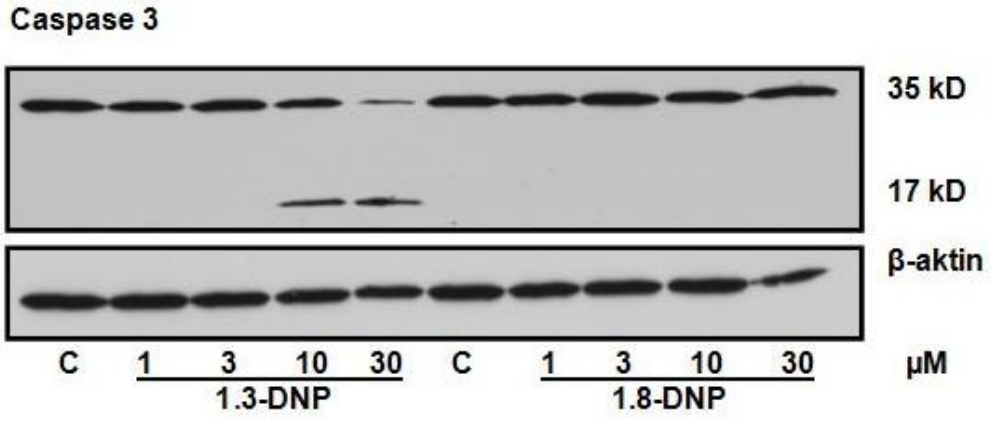
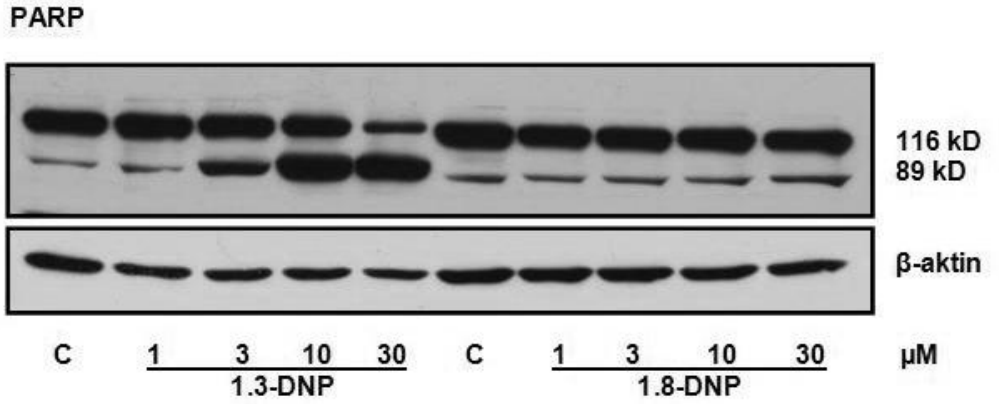

CXCL8 (Relative increase)
0
1
2
3
4
DMSO
1
5
10
15
30
μM
1-NP
1.3-DNP
1.8-DNP

Figure 9

(A)


Caspase 3
35 kD
17 kD
β-aktin
C 1 3 10 30 C 1 3 10 30 µM
1.3-DNP
1.8-DNP

(B)


PARP
116 kD
89 kD
β-aktin
C 1 3 10 30 C 1 3 10 30 µM
1.3-DNP
1.8-DNP

(C)

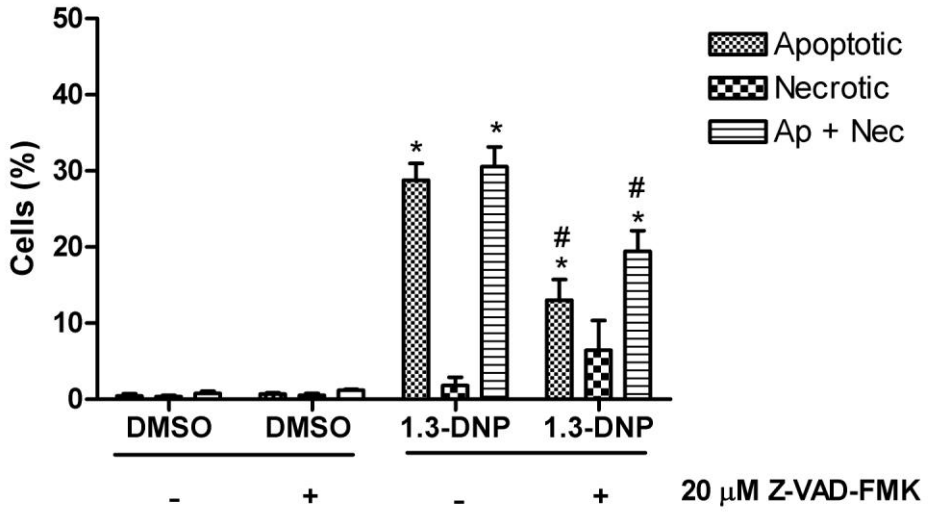

Cells (%)
50
40
30
20
10
0
Apoptotic
Necrotic
Ap + Nec
DMSO DMSO 1.3-DNP 1.3-DNP
- + - +
20 µM Z-VAD-FMK

Figure 10

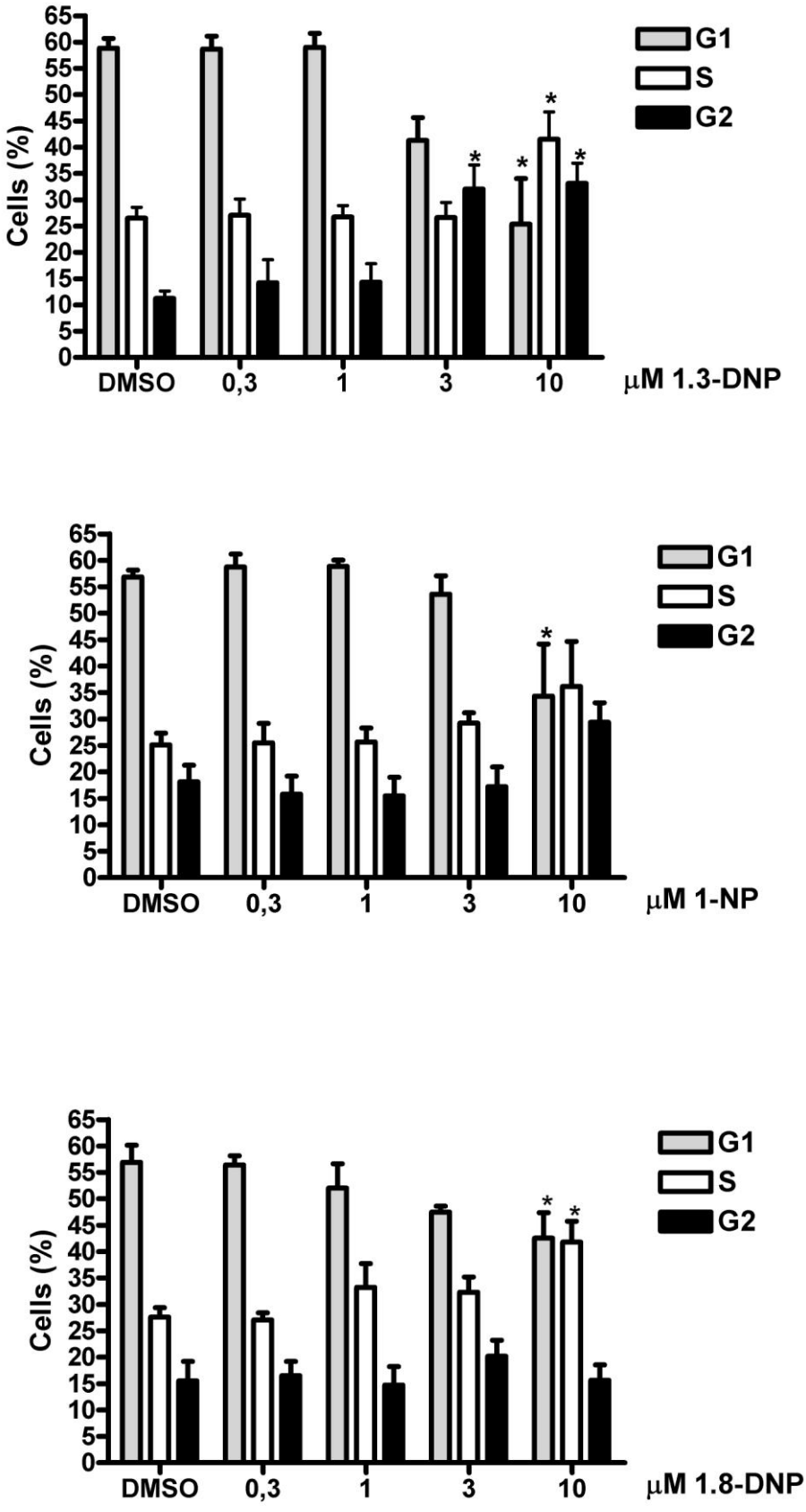
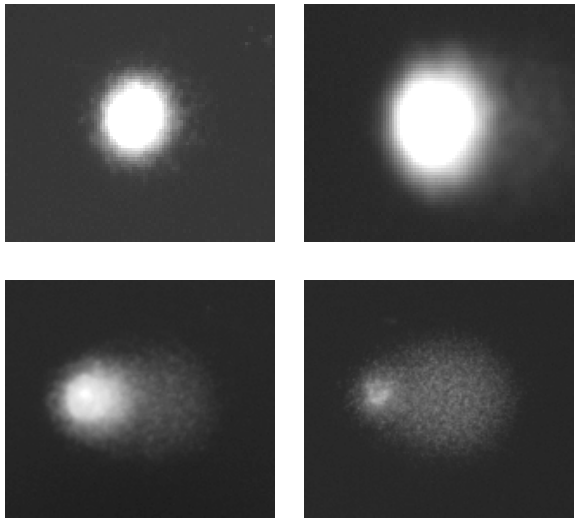
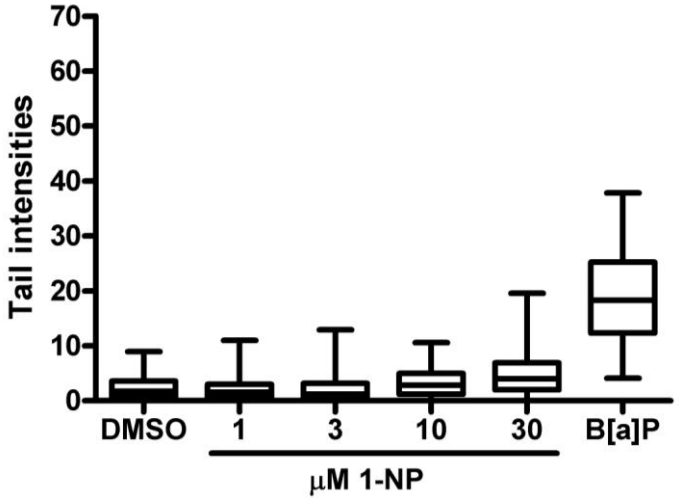

Cells (%)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
G1
S
G2
DMSO
0,3
1
3
10
μM 1.3-DNP
μM 1-NP
μM 1.8-DNP

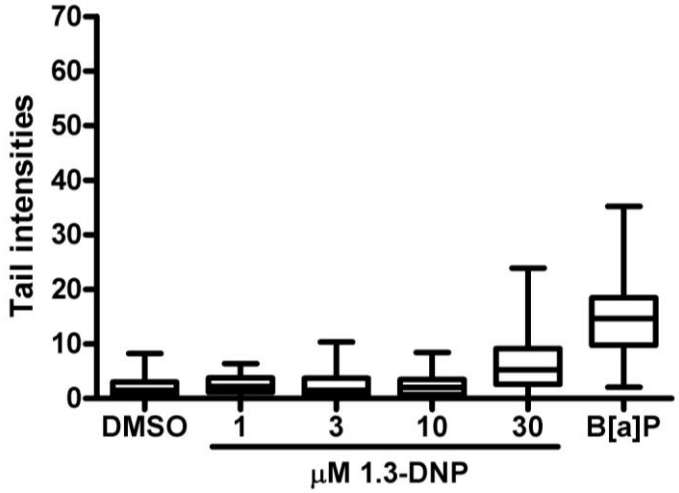
Figure 11

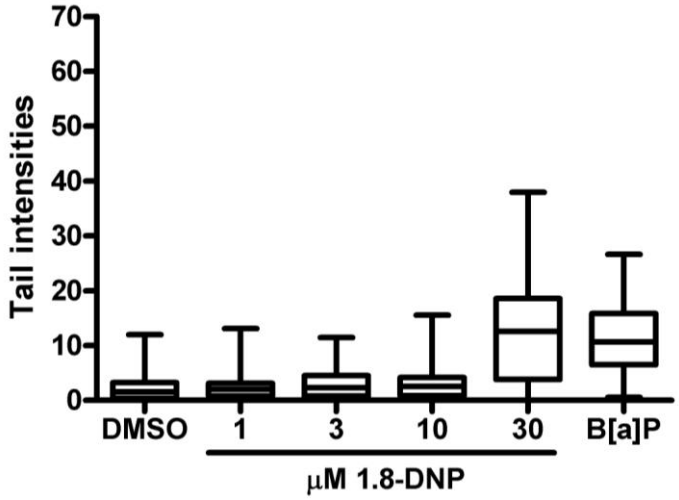
(A)



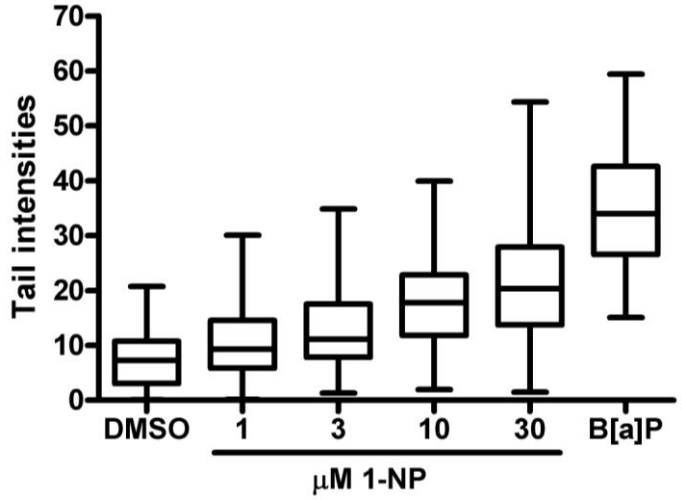
(B)

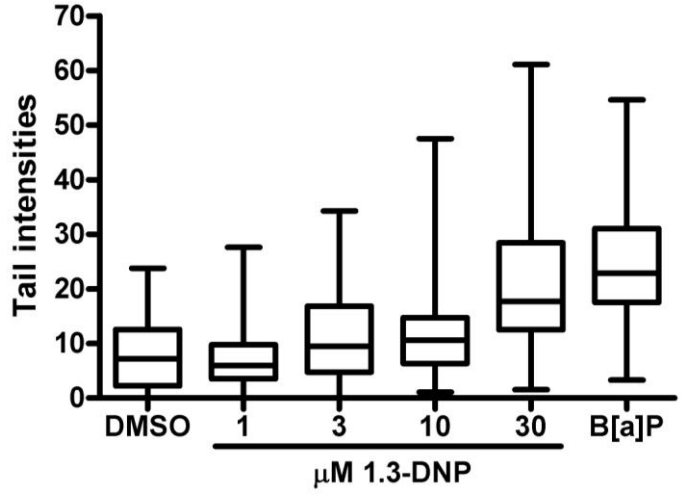

Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1-NP


Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1.3-DNP


Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1.8-DNP

(C)


Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1-NP


Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1.3-DNP

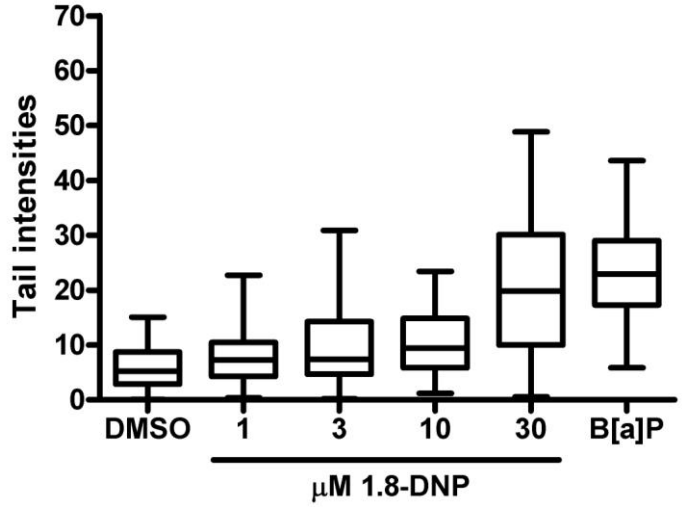
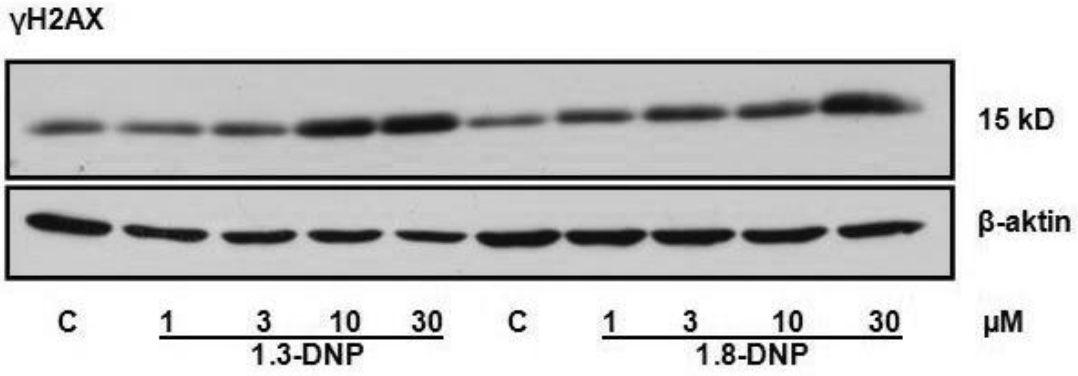
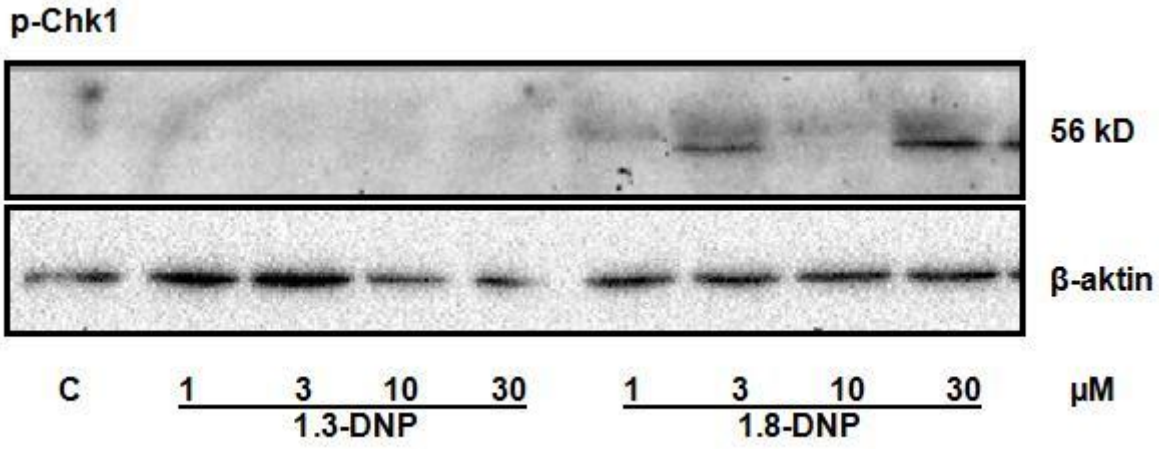

Tail intensities
70
60
50
40
30
20
10
0
DMSO
1
3
10
30
B[a]P
μM 1.8-DNP

Figure 12

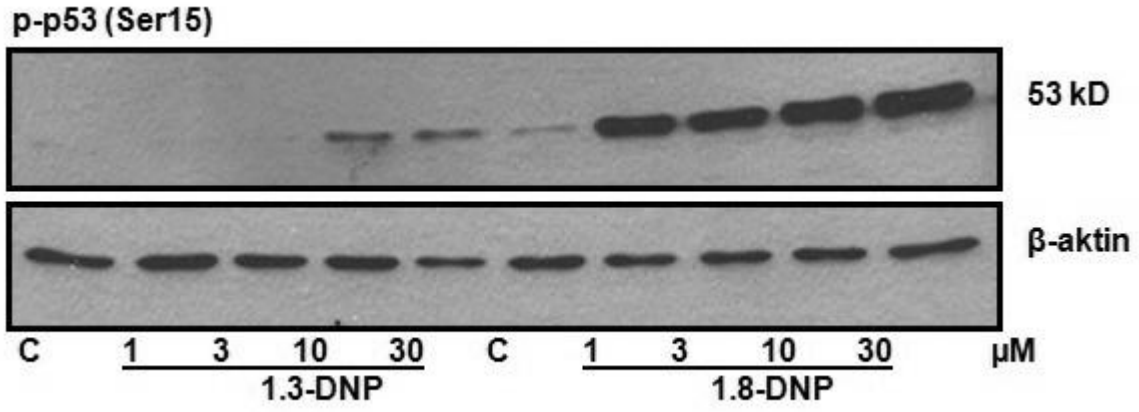
(A)


γH2AX
15 kD
β-aktin
C
1
3
10
30
C
1
3
10
30
μM
1.3-DNP
1.8-DNP

(B)


p-Chk1
56 kD
β-aktin
C
1
3
10
30
1
3
10
30
μM
1.3-DNP
1.8-DNP

(C)


p-p53 (Ser15)
53 kD
β-aktin
C
1
3
10
30
C
1
3
10
30
μM
1.3-DNP
1.8-DNP

(D)

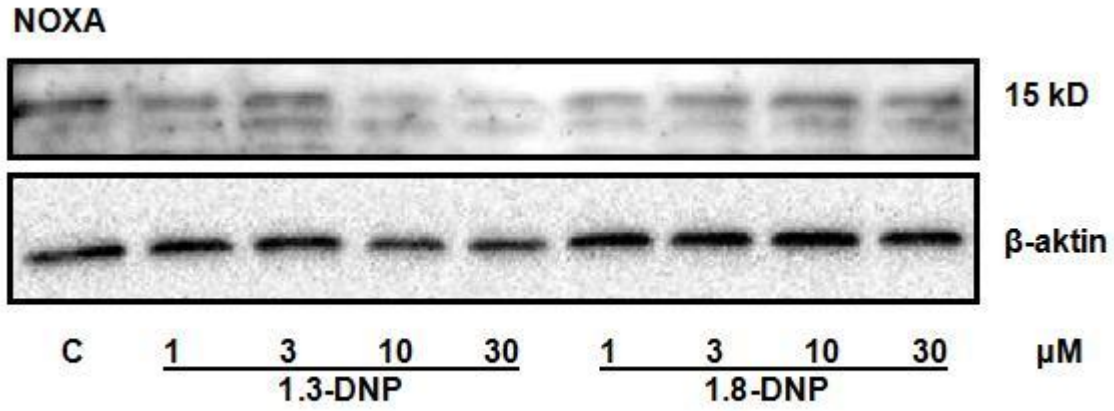
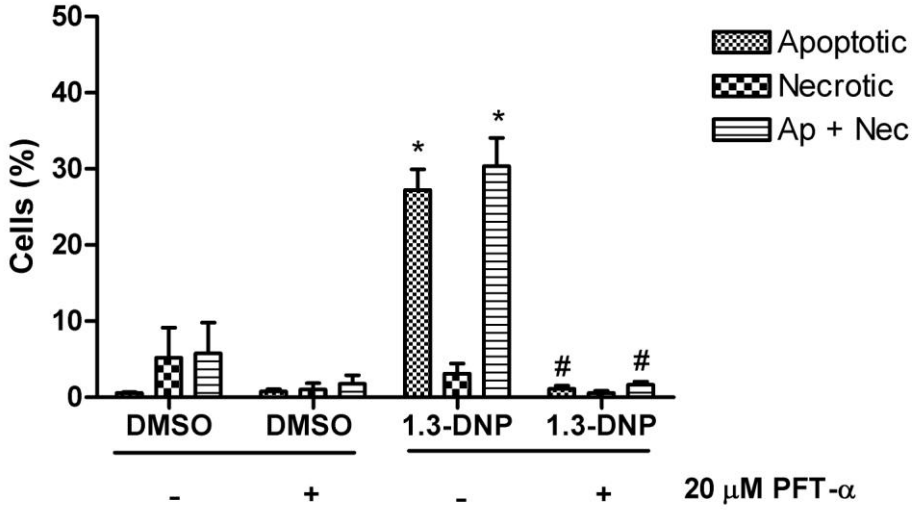

NOXA
15 kD
β-aktin
C
1
3
10
30
1
3
10
30
μM
1.3-DNP
1.8-DNP

Figure 13

(A)


Cells (%)
50
40
30
20
10
0
Apoptotic
Necrotic
Ap + Nec
*
*
#
#
DMSO
DMSO
1.3-DNP
1.3-DNP
-
+
-
+
20 μM PFT-α

(B)

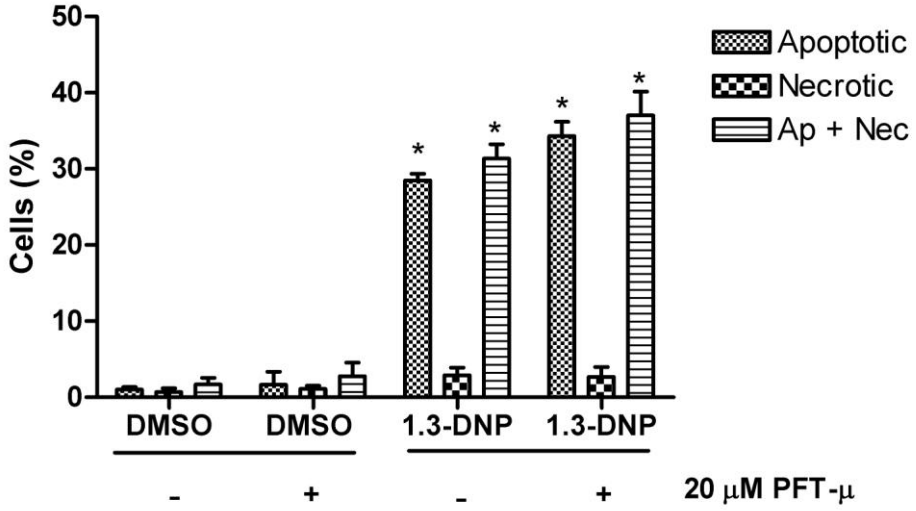
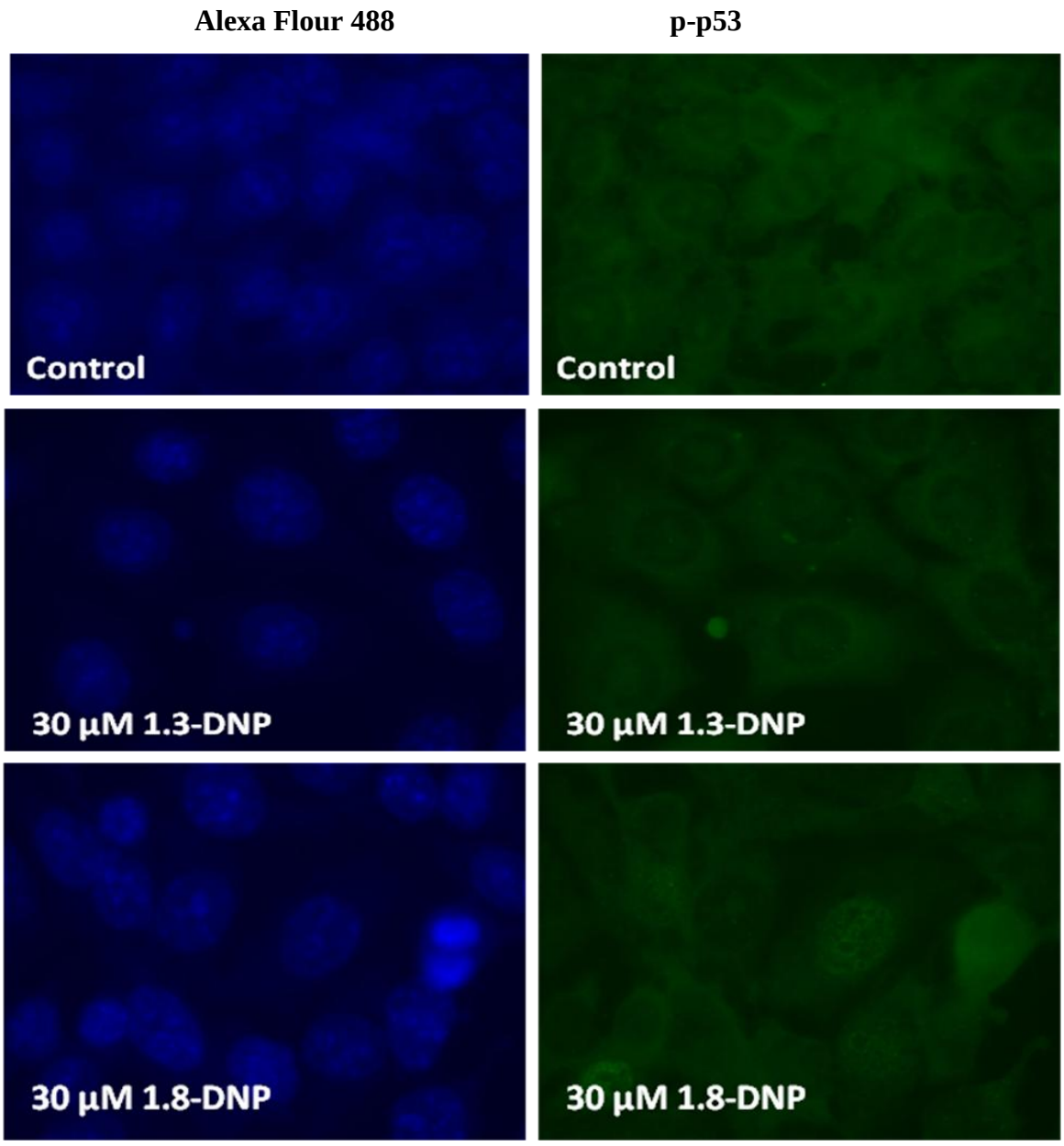

Cells (%)
50
40
30
20
10
0
Apoptotic
Necrotic
Ap + Nec
*
*
*
*
DMSO
DMSO
1.3-DNP
1.3-DNP
-
+
-
+
20 μM PFT-μ

Figure 14


Alexa Flour 488
p-p53
Control
Control
30 µM 1.3-DNP
30 µM 1.3-DNP
30 µM 1.8-DNP
30 µM 1.8-DNP