Toksiske effekter av nitro-PAH og amino-PAH i humane bronkiale epitelceller (BEAS-2B):

Betydningen av DNA-skaderesponsen

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SAMMENDRAG

I denne studien har vi evaluert og sammenlignet effektene av ulike nitro-polisykliske aromatiske hydrokarboner (nitro-PAH), som er mutagene og karsinogene forbindelser, og finnes på forurensningspartikler fra blant annet dieseleksos. Humane bronkiale epitelceller (BEAS-2B) ble eksponert for 1-nitropyren (1-NP), 3-nitrofluoranten (3-NF) og 3-nitrobenzantron (3-NBA), og deres korresponderende amino-former, 1-AP, 3-AF og 3-ABA, i tillegg til 2-nitrobenzantron (2-NBA) og benzo[*a*]pyren (B[*a*]P). Etter eksaminering med fluorescens mikroskopi viste det seg at 3-NBA var mest cytotoksisk, og at nitro-forbindelsene var mer potente enn sine korresponderende amino-former. Bedømt ut fra nukleær kondensering og kløyving av caspase 3 og PARP, induserte 3-NBA hovedsaklig apoptotisk celledød. Alle forbindelsene, med unntak av 2-NBA og B[*a*]P, førte til en signifikant økning i frigjøringen av cytokinet CXCL8 (IL-8). Generelt induserte nitro-forbindelsene CXCL8 i større grad enn amino-forbindelsene. Amino-formene derimot gav økt frigjøring av CCL5 (RANTES). B[*a*]P førte til økt ekspresjon av AhR/ARNT-regulerte gener som CYP1A1/1B1, mens 3-NBA, 3-NF og 1-NP alle reduserte disse genuttrykkene, noe som indikerer AhR/ARNT-antagonisme. 3-NBA forårsaket mest DNA-skade, bedømt ut fra mengden DNA-addukter, DNA enkelttrådbrudd og akkumulering av celler i S-fase. Videre analyse av DNA-skaderesponsen (DDR) viste at 3-NBA aktiverte ATM, Chk2 og p53. Aktiveringen av p53 ble vist ved økt fosforylering og translokasjon til kjernen. 2-NBA, 1-NP og B[a]P førte ikke til fosforylering av disse proteinene. p53-hemmeren, pifithrin-α, reduserte mengden 3-NBA-indusert celledød, hvilket tyder på at DNA-skade/DDR-signalisering og aktivering av p53 er sentrale komponenter i slik celledød. Studien illustrerer at de ulike PAH har svært forskjellige toksiske potensialer (målt i celledød og cytokindannelse), og små endringer i molekylstruktur, så som plassering av nitro-grupper eller substitusjon av nitro- med en aminogruppe, har dramatisk innvirkning på toksisiteten.

FORKORTELSER

AD, aerodynamisk diameter; AhR, arylhydrokarbonreseptor; AhRR, arylhydrokarbonreseptor-repressor; APAF-1, *apoptotic peptidase activating factor 1*; ARNT, arylhydrokarbon nukleærtranslokator; ATM; *ataxia-telangiectasia-mutated*; ATR, *ATM and Rad3-related*; ATP, *Adenosine-5'-triphosphate*; B[a]P, benzo[a]pyren; humane bronkiale epitelceller, BEAS-2B; CYP, cytokrom P450; CXCL8, IL-8 (interleukin 8); CCL5, RANTES; Chk, *checkpoints*; CO, karbonmonoksid; DEP, dieseleksospartikkel; DDR, DNA skaderespons; DSB, DNA dobbeltrådbrudd, ELISA, *Enzyme-Linked ImmunoSorbent Assay*; 1-AP, 1-aminopyren; 3-AF, 3-aminofluoranten; 3-ABA, 3-aminobenzantron; 1-NP, 1-nitropyren; 3-NF, 3-nitrofluoranten; 3-NBA, 3-nitrobenzantron; 2-NBA, 2-nitrobenzantron; nitro-PAH, nitrosubstituerte-polysykliske aromatiske hydrokarboner; NAT1 og NAT2, *N,*O-acetyltransferaser; NR, nitroreduktaser; NO_x, nitrogenoksider; NF-κB, nukleær faktor-kappa B; FACS, *fluorescence-activated cell sorting*; IκB, hemmer av kappa B; ICAD, *Inhibitor of Caspase activated deoxyribonuclease*; γH2AX, phospho-*histone H2A.X*; NOS, *nitric oxide synthase*; *NQO1, NAD(P)H:quinone oxidoreductase*; O_3, ozon; PAH, polysykliske aromatiske hydrokarboner; PARP, poly(ADP-ribose) polymerase; PCR, polymerase kjedereaksjon; PI, propidium iodid; PFT, pifithrin; PM, partikulært materiale; ROS, reaktive oksygenforbindelser; SSB, DNA enkelttrådbrudd; SO_2, svoveldioksid; SULT1A1 og SULT1A2, sulfotransferaser; TNF, tumornekrose faktor; XRE, xenobiotisk responselement, *XO, xanthine oxidase*;

1. BAKGRUNN

1.1 Introduksjon

Epidemiologiske studier har assosiert eksponering for luftforurensning og dieseleksos med økt risiko for utvikling av lungekreft (Arlt 2005). Dieseleksos inneholder ulike polysykliske aromatiske hydrokarboner (PAH) og nitrosubstituerte-PAH (nitro-PAH), og enkelte av disse er potente mutagener og karsinogener. Deteksjon av disse forbindelsene i lungene til ikke-røykere med lungekreft, har medført en betydelig interesse for å vurdere deres potensiale til å forårsake negative helseeffekter hos mennesker (Arlt et al. 2005, IARC 1989, Tokiwa et al. 1993).

1.2 Luftforurensing og helseeffekter

Det er velkjent at luftforurensning kan resultere i negative helseeffekter. Allerede i 1775 oppdaget Sir Percival Pott en sammenheng mellom kreft på pungen og soteksponering hos Londons feiere (BROWN & THORNTON 1957), og von Volkman rapporterte i 1875 om økt insidens av hudkreft blant arbeidere i kulltjære-industrien (Guengerich 2005). Meuse-dalen i Belgia ble rammet av økt dødelighet fra 1. til 5. desember 1930, da en tykk toksisk tåke dekket store deler av dalen. Denne episoden var den første pekepinnen på at atmosfærisk forurensning kan forårsake sykdommer og dødsfall (Nemery, Hoet, & Nemmar 2001). Senere inntraff en forurensningshendelse i London, *den store Londontåken*, som medførte en katastrofal økning i morbiditet og mortalitet blant byens innbyggere, fra 5. til 9. desember 1952. Kombinasjonen av økt vedfyring på grunn av sterk kulde, og mye biltrafikk, gav en midlertidig, kraftig økning i luftforurensning. Luftkvaliteten var så elendig at det ble estimert ca 4 000 dødsfall i løpet av disse få dagene og de påfølgende to ukene. Ytterligere 8 000 mennesker døde de neste ti ukene, som en konsekvens av tåken (Black 2003, Casarett 2001, Schwartz 1994, WILKINS 1954). En følge av dette ble etablering av betegnelsen *smog*. Ordet er satt sammen av de engelske ordene *smoke* og *fog*, og betegner en kjemisk kompleks type luftforurensning. Denne blandingen av røyk og tåke finner man fremdeles i mange storbyer over hele verden, som for eksempel Los Angeles, Beijing, Mexico City og Tokyo (Black 2003, SFT 2007, WHO 1987).

I et forsøk på å kontrollere det økende luftforurensingsproblemet ble *The Clean Air Act* innført, og den gav senere opphav til USAs miljøverndepartement (USEPA). USEPA er ansvarlig for å beskytte befolkningen mot skadelig utendørs luftforurensing (Black 2003,

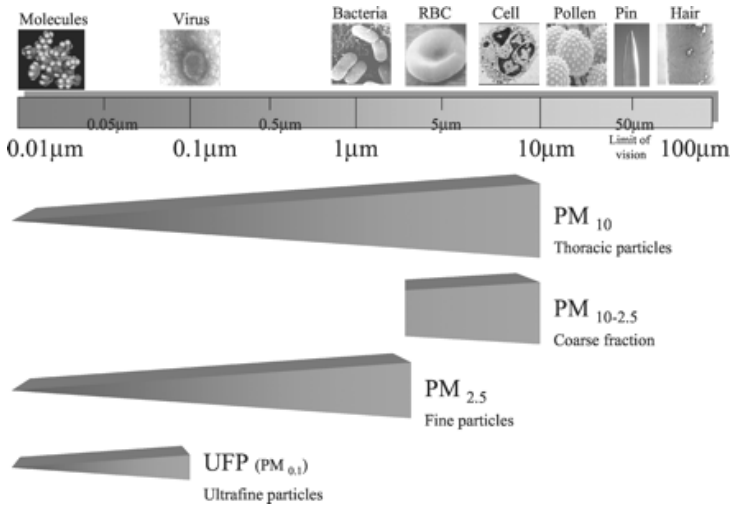
Casarett 2001, WILKINS 1954). Selv i dag indikerer flere befolkningsundersøkelser at luftforurensing er en stor risikofaktor i forhold til kreftutvikling. Pope og medarbeidere har dokumentert, ved bruk av epidemiologiske undersøkelser, at det finnes en sammenheng mellom økt finpartikulær luftforurensning og lungekreft (Pope, III et al. 2002). Lungekreft er den vanligste kreftformen, og er ansvarlig for flest kreftrelaterte dødsfall verden rundt (Hoek et al. 2002, Vineis et al. 2004). Sigarettrøyk anses å være den viktigste enkeltfaktoren, men andre faktorer som luftforurensning i inne- og uteluft, er sannsynligvis også med å bidra til den høye forekomsten (Toriba et al. 2007).

Luftforurensning er en heterogen, kompleks blanding av ulike komponenter som varierer lokalt og regionalt. De viktigste komponentene er svoveldioksid (SO_2), nitrogenoksider (NO_x), karbonmonoksid (CO), ozon (O_3) og svevestøv (Brook et al. 2004). Svevestøv eller partikulært materiale (PM), er betegnelsen på små partikler som holder seg svevende i lufta over en lengre periode. En stor andel av svevestøvet er forbrenningspartikler, som er bygd opp av ulike komponenter bundet til en karbonkjerne (Fig. 1). Det foreligger flere hundre befolkningsstudier hvor svevestøv vurderes som en viktig komponent i luftforurensning, med stor betydning for menneskers helse (Casarett 2001, SFT 2007, Vineis and others 2004).

Figur 1: Forenklet bilde av en forbrenningspartikkel (Nasjonalt folkehelseinstitutt 2004).

Svevestøvpartikler stammer ofte fra ufullstendig forbrenning av organisk materiale, som f eks vedfyring eller bensin i bilmotorer, som kan resultere i dannelsen av polysykliske aromatiske hydrokarboner (PAH). PAH er da lokalisert på overflaten av partiklene. PM beskrives ofte med tall som tar utgangspunkt i aerodynamisk diameter (AD), som varierer med størrelse og fasong (Fig. 2). Den aerodynamiske diameteren er ulik fra den geometriske

diameteren, og defineres som den teoretiske diameteren til en sfærisk partikkel med egenvekt 1, som vil falle like fort i luft som den egentlige partikkelen. Ulik egenvekt gir ulik aerodynamisk diameter på partikler som geometrisk sett er identiske. De viktigste størrelsesfraksjonene som benyttes er PM_{10} (AD≤10 μm), $PM_{2,5}$ (AD≤2,5 μm), PM_1 (AD≤1 μm) og $PM_{0,1}$ (AD≤0,1 μm). Svevestøv fordeles normalt i tre størrelseskategorier; grovfraksjon (2,5-10 μm), finfraksjon (≤2,5 μm) og ultrafine partikler (≤0,1 μm) (Brook and others 2004, Casarett 2001, Ormstad & Lovik 2002, Vineis and others 2004). De biologiske responsene overfor inhalerte partikler bestemmes av partiklenes størrelse, hvilke kjemiske komponenter partiklene består av, og partiklenes overflateegenskaper. Partiklenes størrelse lar oss til en viss grad anslå hvor i luftveiene de deponeres. Generelt avsettes store partikler i de øvre luftveiene, mens finfraksjon og ultrafine partikler deponeres helt nederst i lungeblærene, der hvor gassutvekslingen foregår. Figur 2 illustrerer ulike partikkelfraksjoner.



Figur 2: Illustrasjon av partikkelfraksjonens størrelse i forhold til utvalgte biologiske materialer. PM_{10} tilsvarer størrelsen på en celle, $PM_{2,5}$ en bakterie, og $PM_{0,1}$ tilsvarer i størrelse virus og molekyler. Figuren er hentet fra Brook og medarbeidere (Brook et al. 2004).

En artikkel i Lancet (2002) kategoriserte urban luftforurensning som et av verdens største helseproblemer. Man antar at mer enn 800 000 mennesker årlig dør som følge av svevestøveksponering (Ezzati et al. 2002, SFT 2007). Sammenhengen er skjønt ikke like tydelig som det den var for 30 år siden, hvilket er en konsekvens av at luftforurensningen generelt har minket og at kildene i den vestlige verden har endret seg. Partiklene kommer nå hovedsakelig fra biltrafikk, og ikke først og fremst fra industrielle kilder, slik som tidligere. I

tillegg har det forekommet en reduksjon i antallet store partikler. Blant de mest betydningsfulle kildene til svevestøv finner vi dieseleksos og partikler fra veistøv, i tillegg til vedfyring (Ormstad & Lovik 2002).

1.3 Dieseleksos

Siden 1970 har det vært bekymring for at inhalering av dieseleksos kan forårsake forverring av lungesykdommer og lungekreft hos mennesker. I den senere tid har epidemiologiske studier bekreftet en økt lungekreftsrisiko ved eksponering for dieseleksos, f eks hos yrkessjåfører i Sverige (Garshick et al. 2008, Jakobsson, Gustavsson, & Lundberg 1997). En lignende undersøkelse ble utført på ansatte i jernbanene i USA, etter innføringen av dieseldrevne lokomotiv, og viste en signifikant økning i antall lungekrefttilfeller hos arbeidere som jobbet i nærheten av operative linjer (Garshick et al. 2004, Garshick and others 2008, IARC 1989, Vineis and others 2004). Dieseleksos er klassifisert som sannsynligvis kreftfremkallende i mennesker (IARC Gruppe 2A) av International Agency for Research on Cancer (IARC 1989, Toriba and others 2007).

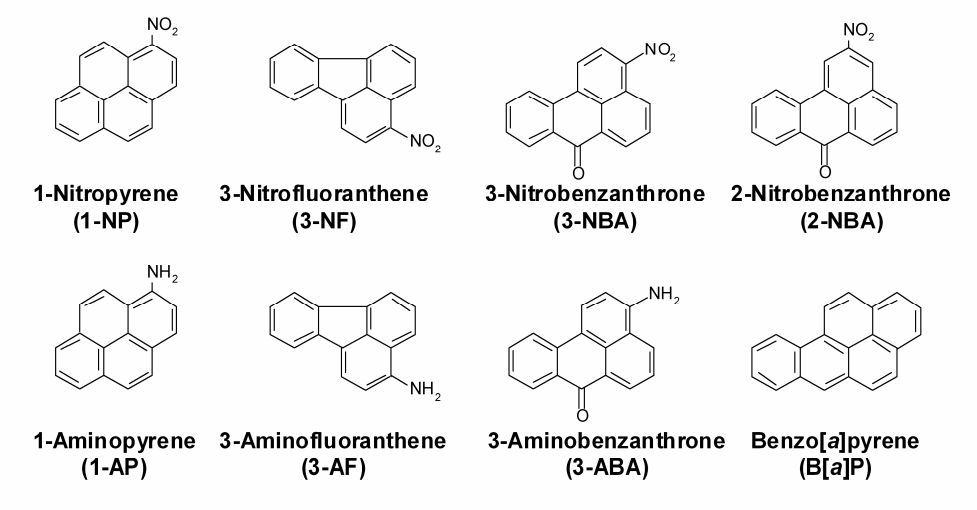
I urbane områder er dieseleksospartikler hovedkomponenten i finfraksjonen av svevestøvet ($PM_{2,5}$) (Kawasaki et al. 2001). Dieseleksospartikler (DEP) inneholder mutagene og karsinogene forbindelser festet til en karbonkjerne (Garshick and others 2004, Pandya et al. 2002, Schenker 1980). Av disse er ulike PAH og nitrosubstituerte-PAH (nitro-PAH) interessante. 1-Nitropyren (1-NP) er et av de vanligste nitro-PAH i dieseleksospartikler (DEP), og har blitt rapportert å være blant de som bidrar mest til mutagenisiteten (Salmeen et al. 1982, Scheepers et al. 1995). 1-NP er samtidig det mest studerte nitro-PAH og klassifiseres av IARC i gruppe 2B karsinogener, dvs mulig kreftfremkallende overfor mennesker (IARC 1989, Purohit & Basu 2000, Rosenkranz et al. 1980). Relatert til det høye nivået av denne mutagene komponenten, har 1-NP blitt foreslått som en kjemisk markør for dieseleksos (Scheepers and others 1995, Toriba and others 2007). Det er imidlertid viktig å påpeke at bensin- og dieselmotorer er under kontinuerlig utvikling slik at sammensetningen av f eks dieseleksos varierer mye.

1.3.1 Polysykliske aromatiske hydrokarboner (PAH)

PAH er en stor gruppe strukturelt beslektede organiske forbindelser, med to eller flere aromatiske ringer (benzenringer). De forekommer i forurenset luft, både i dampfase og bundet til partikler. Det er registrert flere hundre ulike PAH, såkalte *tjærestoffer* (Dybing et al. 2005, Yaffe et al. 2001). Disse benzenringene kan være substituerte med ulike kjemiske grupper. Ulike substitusjoner, som tilstedeværelsen av en nitro- eller aminogruppe i visse posisjoner, medfører en økt eller senket biologisk potens av forbindelsen (Bostrom et al. 2002, Holme et al. 2007), og gir dermed opphav til vidt forskjellige helseeffekter. PAH er et uønsket biprodukt av ufullstendig forbrenning av organisk materiale. Viktige kilder til PAH er vedfyring, bil- og flytrafikk, grillet mat og sigarettrøyk (Bostrom and others 2002, Dybing and others 2005, Fu 1990). Benzo[*a*]pyren (B[*a*]P) er den PAH som er best kartlagt, og denne er inkludert i gruppe 2A karsinogener av IARC (IARC 1989).

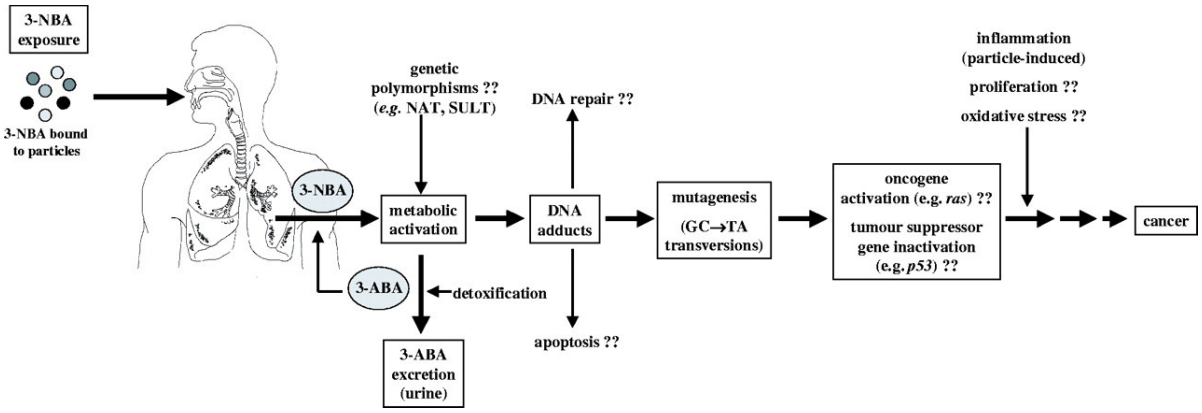
1.3.2 Nitro-polysykliske aromatiske hydrokarboner (nitro-PAH)

Et nitro-PAH består av én eller flere nitrogrupper bundet til et polysyklisk aromatisk hydrokarbon. Mange av forbindelsene er mutagene og karsinogene. Nitro-PAH omtales også som *nitroarener*, og inkluderer bl a 1-nitropyren (1-NP), 3-nitrofluoraten (3-NF), 2-nitrobenzantron (2-NBA) og 3-nitrobenzantron (3-NBA) (Fu 1990, Purohit & Basu 2000). Nitro-PAH dannes også ved ufullstendig forbrenning av organisk materiale, eller ved atmosfæriske reaksjoner hvor PAH reagerer med nitrogenoksider (Fu 1990, Yaffe et al. 2001). En metabolsk aktivering er nødvendig for at nitro-PAH skal ha mutagene eller karsinogene effekter på mennesker. Denne aktiveringen er avhengig av forbindelsenes struktur, metabolisme, evne til å danne DNA-addukter og hvor lett denne skaden repareres (Fu et al. 1994, Purohit & Basu 2000). De ulike PAH brukt i denne studien illustreres i figur 3.



Figur 3: Strukturformel og navn på PAH brukt i denne studien.

Nitroketon 3-nitrobenzantron (3-nitro-7*H*-benz[*de*]anthracen-7-one eller *3-NBA*) er et av de mest potente mutagenene, og er et potensielt humant karsinogen. 3-NBA ble oppdaget i Tokyo for noen få år siden, i dieseleksos bundet til overflaten på partikulært materie, derav kallenavnet *the devil in the diesel* (Arlt 2005). Denne toksiske forbindelsen har DNA-skadende egenskaper i både animale og humane modellsystemer. I urban uteluft finner man nivåer av 3-NBA i et omfang rundt 0.6-6.6 ppm, og det estimerte daglige inntaket hos mennesker er ca 90 pg (Nagy et al. 2007). Hovedmetabolitten til 3-NBA er 3-aminobenzantron (3-ABA), som skilles ut i human urin (Fig. 4). 3-ABA kan i seg selv ha et gentoksisk potensiale, og er dermed ikke nødvendigvis et rent detoksifiseringsprodukt som tidligere antatt. Både 3-NBA og 3-ABA har evnen til å produsere oksidativt stress i humane lungeepitelceller (Arlt et al. 2008, Hansen, Seidel, & Borlak 2007a).



Figur 4: Postulert mekanisme for karsinogeniteten av 3-NBA i gnagere og mennesker (Arlt 2005).

3-NBA kan i atmosfæren omdannes til isomeren 2-nitrobenzantron (2-NBA), som eksisterer i en konsentrasjon ca sytti ganger høyere enn 3-NBA. Gentoksisiteten til 2-NBA er imidlertid bare en tredjedel av 3-NBA. Dette indikerer at posisjonen til nitrogruppen spiller en avgjørende rolle for gentoksisiteten. 2-NBA er tilsynelatende mindre vannløselig, mer lipofil og mer biologisk stabil, sammenlignet med 3-NBA, noe som er avgjørende for biotilgjengeligheten. Den lipofile egenskapen bidrar til at 2-NBA kan bioakkumulere. Siden 2-NBA finnes i høye konsentrasjoner i luft, og på grunn av dens økte lipofile karakter, kan den muligens utgjøre en minst like stor trussel som 3-NBA (Nagy and others 2007).

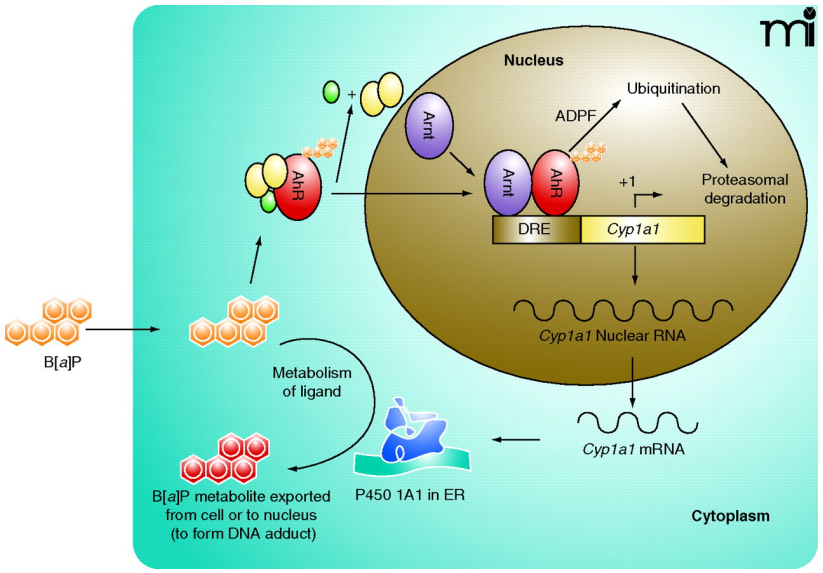
1.3.3 Metabolisme

PAH blir absorbert gjennom lunger, mage-/tarmsystemet og huden. I kroppen omdannes lipofile PAH av ulike enzymer til vannløselige og lett utskillbare forbindelser. Imidlertid kan metabolisme av PAH via cytokrom P450 (CYP) enzymer også resultere i dannelsen av reaktive elektrofile intermediater, som kovalent binder seg til makromolekyler (DNA, RNA og proteiner). B[*a*]P biotransformeres via CYP1A1 til det ultimate karsinogen benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoksid. Diolepoksider kan binde seg kovalent til nukleofile seter i DNA og på denne måten danne PAH-DNA addukter, hvilket er en av årsakene til at det oppstår mutasjoner (Bostrom and others 2002, Dybing and others 2005). Nitro-PAH kan biotranformeres via CYP-enzymer, nitroreduktaser og fase-II-biotransformasjonsenzymer, og kan gi opphav til reaktive metabolitter, på samme måte som andre PAH. Imidlertid kan disse enzymene også resultere i detoksikering av PAH, hvor de da hydrofile PAH skilles ut via galle eller urin (Arlt 2005, Arlt and others 2005, Bostrom and others 2002, Chae et al. 1999, Guengerich 2000, Hatanaka et al. 2001).

1.3.4 CYP1

Ulike CYP-enzymer kan være involvert i den metabolske aktiveringen av PAH og nitro-PAH, hvorav CYP1A1 og CYP1B1 er av stor betydning (Aklillu et al. 2005, Gautier et al. 1996, Guengerich 2000). CYP1 er ansvarlig for både metabolsk aktivering og detoksikering av et antall PAH (Nebert et al. 2004). Gjennom en oksidasjonsreaksjon kan CYP-enzymer omdanne PAH til epoksider og/eller dioler (Aklillu et al. 2005), men de kan også resultere i en reduksjon av nitrogruppen som kan gi opphav til den korresponderende aminoformen (Chae and others 1999, Landvik et al. 2007).

Arylhydrokarbonreseptor (AhR) er en ligand-aktivert transkripsjonsfaktor, som regulerer CYP1 og en rekke andre proteiner. AhR aktiveres ved at kjemiske induktorer som B[*a*]P bindes til, slik at et kjernelokaliseringssignal blottlegges (Safe 2001), deretter translokeres AhR til kjernen hvor den danner et kompleks med arylhydrokarbon-nukleærtranslokator (ARNT). Det ligandaktiverte AhR/ARNT-komplekset bindes så til xenobiotisk responselement (XRE) i promotorregionen til de aktuelle genene. Konsekvensen er økt transkripsjon av gener involvert i metabolismen av PAH. Dermed spiller AhR en sentral rolle i toksisiteten til PAH, og på denne måten fremmer PAH sin egen metabolisme. Figur 5 viser hvordan CYP-enzymer induseres via AhR. AhR regulerer også uttrykket av arylhydrokarbonreseptor-repressor (AhRR), som man antar at undertrykker AhR-funksjonen gjennom en negativ feedback loop (Haarmann-Stemmann & Abel 2006). Flere nyere studier foreslår også at AhR, da ikke kompleksbundet til ARNT, spiller en viktig rolle i reguleringen av flere cytokingener (Haarmann-Stemmann, Bothe, & Abel 2009a).



Figur 5: CYP1 induksjon via AhR (Ma 2007).

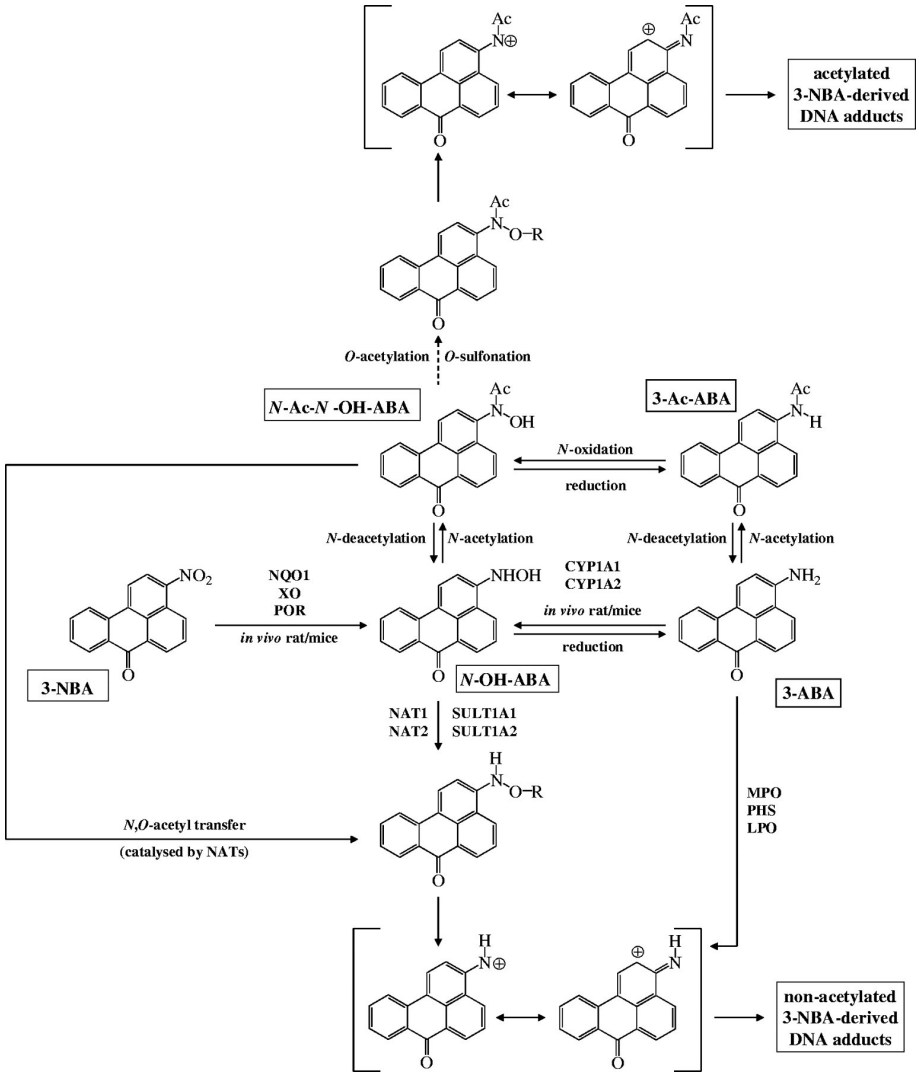
1.3.5 Nitroreduktaser

Reduksjon av nitrosubstituerte aromatiske forbindelser kan skje via nitroreduktaser (NR), hvorav type I NR ikke krever oksygen og type II NR krever oksygen (Ask et al. 2003, Ask et al. 2004, Purohit & Basu 2000). Reduksjon av nitroforbindelsen ($R\text{-}NO_2$) til den korresponderende aminoformen ($R\text{-}NH_2$) (amino-PAH) skjer gjennom en seks-elektron reduksjonsreaksjon (Arlt and others 2005, Moller 1994). Denne reaksjonen kan føre til

dannelse av elektrofile reaktive mellomprodukter (nitroso og *N*-hydroksylamin) eller reaktive oksygenforbindelser (ROS). Metabolismen av nitrosubstituerte aromatiske forbindelser medfører dannelsen av nitroso (R-NO), som reduseres av type I NR med overføring av to elektroner. Nitroso-derivatet kan deretter reduseres med to nye elektroner, til *N*-hydroksylamin (R-NHOH). Derivater av både nitroso og *N*-hydroksylamin er reaktive forbindelser som kan bindes kovalent til vitale makromolekyler. *N*-hydroksylamin reduseres videre med to elektroner, til det korresponderende aminet. Ved tilstedeværelse av oksygen (type II NR) kan molekylært oksygen oksidere nitro-anion ($R\text{-}NO_2^{\bullet -}$) radikalet. Utfallet blir en redoks-syklus som gir regenererte nitroforbindelser og superoksidanion (Ask and others 2003, Ask and others 2004).

1.3.6 Metabolisme av 3-NBA

Bioaktivering av 3-NBA er overveiende katalysert av nitroreduktaser som *nitric oxide synthase* (NOS), *NAD(P)H:quinone oxidoreductase* (NQO1), *xanthine oxidase* og *aldehyde oxidase*, og kan resultere i dannelsen av reaktive oksygen typer (ROS). 3-NBA omdannes til sin reaktive metabolitt *N*-hydroksyl-3-aminobenzantron (*N*-OH-3-NBA) via nitroreduktasene (Arlt and others 2005). Konjugeringsenzymer som *N*,*O*-acetyltransferaser (NAT1 og NAT2) eller sulfotransferaser (SULT1A1 og SULT1A2) er involvert i de påfølgende aktiveringstrinnene, som kan være med på å danne reaktive nitreniumioner, som igjen kan kovalent bindes til DNA og gi skader (Arlt et al. 2002a, Arlt et al. 2003, Arlt and others 2005). Metabolsk aktivering av 3-NBA vises i figur 6.



Figur 6: Foreslått metabolsk aktivering av 3-NBA (Arlt 2005).

1.4 DNA-skade

DNA-skade kan forekomme på grunn av enten eksogen eller endogen gentoksisk påvirkning, henholdsvis påvirkning fra omgivelsene (UV-stråling og kjemikalier), og biprodukter fra normal cellemetabolisme (reaktive oksygen spesies; ROS). Slik påvirkning kan forstyrre opprettholdelsen av den genomiske integriteten. Om enkel- eller dobbeltråd-brudd i DNA, DNA-addukt-dannelse eller oksidative skader forblir uoppdaget eller ureparert, kan mutasjoner og tumorgenese bli vanskelig å forhindre. Aktivering av onkogener eller tap av tumorsupressorgener resulterer i DNA-replikasjonsstress og DNA-skade, som videre signaliserer til den cellulære DDR (DNA-skaderesponsen) (Bartek, Bartkova, & Lukas 2007, Kriek et al. 1998, Maslov & Vijg 2009).

1.4.1 DNA-skaderesponsen (DDR)

DDR starter ofte med en aktivering av signalveiene ATR-Chk1 og ATM-Chk2, og har som hovedoppgave å forsinke cellesyklus-progresjonen for å gi mer tid til DNA-reparasjon. DDR induserer cellulær senescence eller celledød, dersom skaden er for stor (Bartek, Bartkova, & Lukas 2007, Harper & Elledge 2007).

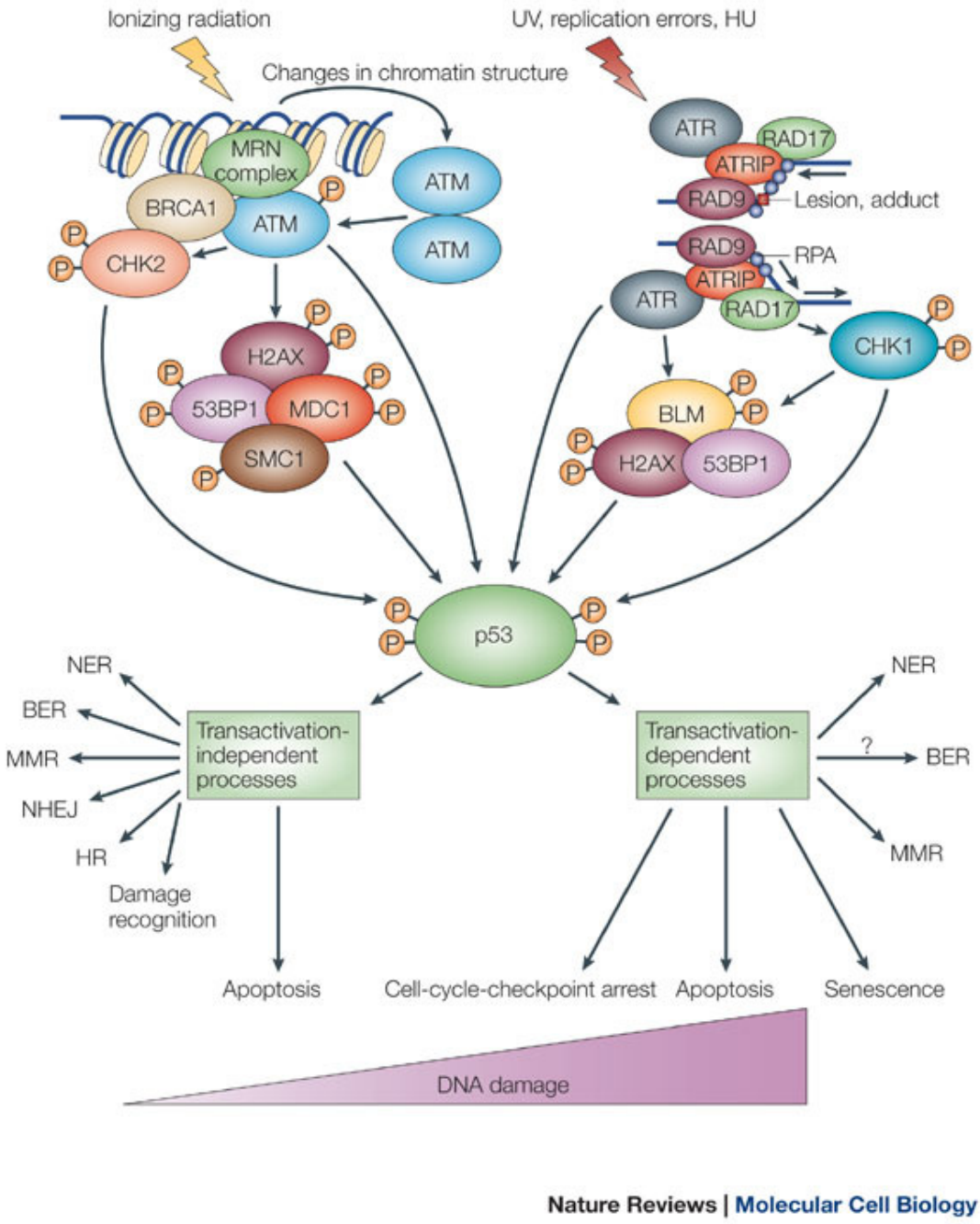
ATM (*ataxia-telangiectasia-mutated*) og ATR (*ATM and Rad3-related*) proteinkinaser er sensorer som primært aktiveres som respons på kromatinforandringer i DNA, henholdsvis dobbeltrådbrudd (DSB) og enkelttrådbrudd (SSB), det sistnevnte forårsakes hovedsakelig av UV-stråling, replikasjons- eller transkripsjonsstress (Cann & Hicks 2007, Ljungman 2005, Tanaka et al. 2007, Yang, Yu, & Duerksen-Hughes 2003). Både ATM og ATR har evnen til å direkte fosforylere og aktivere p53. ATM og ATR regulerer DNA-skade-transduksjonskaskaden ved å kontrollere cellesyklusoverganger, DNA-replikasjon, DNA-reparasjon og apoptose (Bartek & Lukas 2003b, Cimprich & Cortez 2008).

DNA i eukaryoter er pakket som nukleosomer, som utgjør byggesteinene i kromatin. Hvert nukleosom består av DNA surret rundt en kjerne av åtte histon-proteiner. H2AX er et medlem av H2A familien (Takahashi & Ohnishi 2005). DNA-skade induserer fosforylering av H2AX, mediert av ATM og ATR. Aktivert H2AX (betegnet γ-H2AX) spiller en kritisk rolle i opprettholdelsen av den genomiske stabiliteten og γH2AX akkumulerer på steder i DNA med DSB, hvor den antas å rekonstruere kromatin og assistere i rekrutteringen av DNA-reparasjons- og –signaliseringsfaktorer. ATR aktiverer H2AX ved DSB som oppstår i forbindelse med replikasjon, mens ATM er den viktigste kinasen med tanke på å fosforylere H2AX etter DSB som oppstår på grunn av eksogen påvirkning (Mattsson et al. 2009).

Checkpoint kinase 1/2 (Chk1/2) er to serin/threonin kinaser som aktiveres som respons på DNA-skade og replikasjonsstress, og induserer cellesyklus-arrest. De er antatt å være tumor-supressorer. Chk1 og Chk2 blir aktivert henholdsvis av ATR og ATM, og formidler signalet videre (*transducers)* (Bartek & Lukas 2003a, Zhang et al. 2004). ATR-Chk1 og ATM-Chk2 signalveiene er ikke parallelle veier i DDR, men det er en høy grad av *cross-talk* mellom de to signalveiene. Chk1/2 overfører skadesignalet videre til effektor-proteiner, som tumor supressor proteinet p53 (Bartek & Lukas 2003a, Sengupta & Harris 2005).

p53 er mutert i mer enn 50 % av alle humane kreftformer (Romer et al. 2006). p53 fungerer som en *guardian of the genome,* hvilket innebærer å oppdage og reagere på DNA-skade gjennom ATM/ATR og Chk1/Chk2 kinaser, i tillegg til å være *policeman of the oncogenes,* hvilket består i å respondere på onkogene signaler (Efeyan & Serrano 2007). p53 er en transkripsjonsfaktor med en sentral rolle i regulering av cellesyklus, DNA-reparasjon, DNA-rekombinasjon, senescence og apoptose. (Sengupta & Harris 2005). p53 regulerer transkripsjonen av proteiner involvert i cellereparasjon og celledød ved å binde seg til promotorregioner til de enkelte sentrale genene. I respons på DNA-skade kan ATM, ATR, Chk1 og Chk2 direkte fosforylere p53 på *Ser*15, som påskynder akkumulering og funksjonell aktivering av p53 (Fig. 7). Ved å øke transkripsjonen av Bax, som induserer frigjøringen av cytokrom c fra mitokondriene, trigger p53 den apoptotiske prosessen. I tillegg kan p53 direkte interagere med proteiner og regulere deres aktivering på en transkripsjons-uavhengig måte. p53 regulerer også celledød via dødsreseptorer og dødsligander (Roos & Kaina 2006). Defekt p53 resulterer i manglende celledød (Bratton & Cohen 2001, Romer and others 2006).

p21 blir betraktet som et av de viktigste og mest potente effektormolekylene til p53, og p53 regulerer p21ved å binde seg til dens promotorregion. p21 kan arrestere celler som er på vei inn i G1-fasen i cellesyklus (Abukhdeir & Park 2008). Forstyrrelse i balansen mellom celleoverlevelse og celledød har stor innvirkning på mutasjonsfrekvens og kreftutvikling. Flere PAH er i stand til å skade DNA, men DNA-skade forårsaket av PAH kan noen ganger resultere i færre celler arrestert i G1 enn forventet. p53-aktivering etter slik skade medfører også mindre aktivering av cellesyklus-inhibitoren p21 enn normalt. Konsekvensene av mislykket p21-indusering og G1-arrest, kan være at celler replikerer sitt DNA med addukter festet til, at celler akkumulerer i S-fasen, og til slutt økt sannsynlighet for genomiske mutasjoner.



Figur 7: p53 sin funksjon i DNA-skaderesponsen (Sengupta & Harris 2005).

1.5 Cytokiner

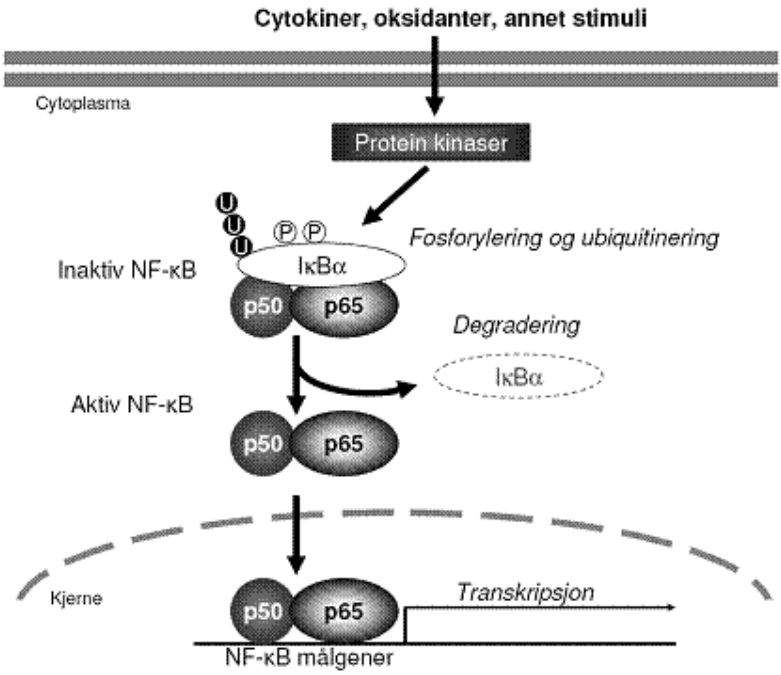
Cytokiner er for det meste lavmolekylære proteiner eller glykoproteiner, som virker som kjemiske signalmolekyler mellom celler. En rekke cytokiner produseres under immun- og inflammasjonsprosesser i respons på cellulært stress, forårsaket av enten eksogene eller endogene agens. De kan føre til systemiske og lokale effekter, hvis hensikt er å kontrollere

eller minimere cellulær skade. En ukontrollert og vedvarende cytokindannelse kan lede til forandringer i cellevekst, differensiering og apoptose (Hussain & Harris 2007). Aktive immunceller kan øke det oksidative stressnivået og dermed kan DNA-skade induseres, og mutasjoner oppstå (Emmendoerffer et al. 2000). Det er bred enighet om at kronisk inflammasjon kan medvirke til utvikling av astma, kronisk obstruktiv lungesykdom (KOLS) og lungekreft (Coussens & Werb 2002, Hussain & Harris 2007). Påvirkning fra partikler i dieseleksos og luftforurensning har blitt vist å høyne inflammasjonsnivået i humane lunger (Casillas et al. 1999). Dieseleksos innehar karsinogene egenskaper som kan potenseres av inflammatoriske stimuli (IARC 1989, Park & Park 2009a) Det finnes også forskning som tyder på at PAH i seg selv kan ha inflammatorisk potensiale (Pei et al. 2002).

Interleukin-8 (IL-8) (CXCL8) har en sentral rolle i flere patologiske tilstander, inkludert kronisk inflammasjon, astma, fibrose og kreft. RANTES (CCL5) derimot, er et cytokin med *anti-tumor* aktivitet (Henriquet et al. 2007, Moran et al. 2002, Mukaida 2003, Xie 2001).

Tumor nekrose faktor-α (TNF-α) er et proinflammatorisk cytokin, som spiller en sentral rolle i flere cellulære mekanismer (Coussens & Werb 2002). En av de viktige proinflammatoriske oppgavene til TNF-α er å aktivere av NF-κB. I noen celletyper kan apoptose og nekrose initieres av TNF-α (Marx 2004).

En av de viktigste transkripsjonsfaktorene for cytokiner er nukleær faktor-kappa B (NF-κB), inklusivt for CXCL8, CCL5 og TNF-α. NF-κB er en gruppe transkripsjonsfaktorer som aktiveres av ulike typer stressignaler, og deltar i reguleringen av stressresponser, inflammasjon, proliferasjon, DNA-reparasjon, apoptose og cellesyklus. Den kan imidlertid også aktiveres av DNA-skaderesponsen (DDR) (de, Schmid, & Hofer-Warbinek 1999). I cytoplasma er inaktivt NF-κB festet til inhibitoren av κB (IκB). Degradering av IκB tillater kjernetranslokering av NF-κB, hvor den kan feste seg til promotorregioner og øke transkripsjonen av ulike gener (Janssens & Tschopp 2006) (Fig. 8). NF-κB-aktivitet vil ofte beskytte celler mot apoptose. Imidlertid har det også blitt rapportert at NF-κB kan øke utrykket av p53, og at en slik aktivering kan resultere i økt apoptose (Kim et al. 2002).

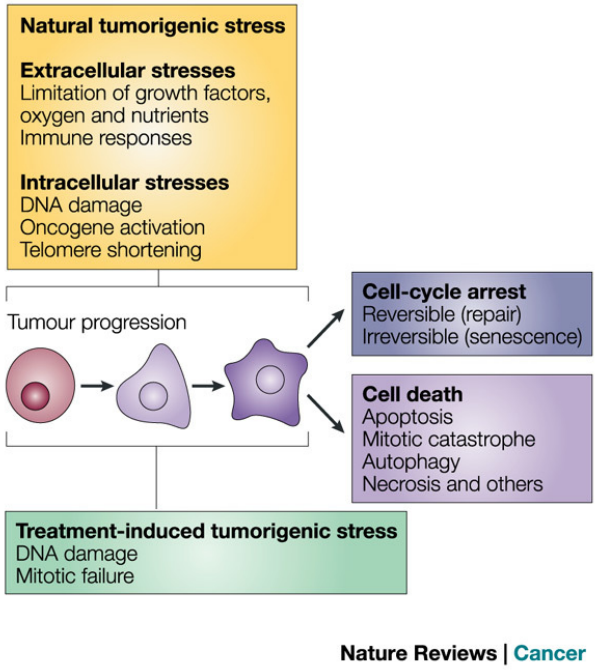


Figur 8: NF-κB-aktivering involverer fosforylering og ubiquitinering, etterfulgt av proteolytisk degradering av inhibitor-proteinet IκB. Fritt NF-κB translokeres til kjernen hvor den binder til sine målgener og aktiverer transkripsjonen av disse (Johan Øvrevik, Nasjonalt folkehelseinstitutt).

Flere studier indikerer at AhR kan interagere med ulike typer NF-κB, som RelB. Det har blitt påvist at AhR/RelB heterodimeren regulerer cytokin-produksjon, blant annet av CXCL8 (Baglole et al. 2008, Vogel & Matsumura 2009, Xia et al. 1997).

1.6 Celledød

Celledød ble tidligere inndelt i kategoriene apoptose (Type I), autofagisk celledød (Type II) og nekrose (Type III) (Lockshin & Zakeri 2004). Denne inndelingen har imidlertid vist seg for enkel, og det har kommet til et stort antall nye klassifiseringssystemer for celledød (Kroemer et al. 2009, Okada & Mak 2004, Tang et al. 2008). Figur 9 illustrerer hvordan en celle responderer på ulike former for stress, og deretter følger en kort presentasjon av fem distinkte typer celledød, med størst vekt på apoptose.



Figur 9: Cellenes respons på stress (Okada & Mak 2004).

1.6.1 Permanent cellesyklusarrest (senescence)

En celles respons på en DNA-skade er avhengig av skadens omfang og hvorvidt den kan repareres. Hvis skaden er for stor dør som regel cellen, men dersom skaden kan repareres induseres en midlertidig cellesyklusarrest. Hvis reparasjonen mislykkes og cellen ikke går inn i senescence, dør ofte cellen slik at den unngår å få mutasjoner. En vedvarende cellesyklusarrest kalles iblant *levende celledød*, ettersom cellene tilsynelatende lever, men ikke kan dele seg videre (Kroemer and others 2009). p53 er et tumor supressor protein som er involvert i permanent cellesyklusarrest, og som blir aktivert ved enkel- eller dobbeltrådbrudd i DNA. Aktivert p53 kan virke som transkripsjonsfaktor, eller direkte regulere proteiner involvert i celledeling og reparasjon. Uttrykk av gener som inhiberer cellesyklus blir ofte kontrollert av p53. $p21^{WAF1/CIP1}$ reguleres av p53 og er sentral i cellesyklusarrest hvor den hemmer aktiviteten til cyklinavhengige kinaser (cdk-er), som er pådrivere for celledeling. I tillegg er det blitt foreslått at p21 er involvert i reguleringen av apoptose (Campisi 2005, Gartel 2005). Risikoen for utvikling av kreft reduseres ved stopp i celledeling, som gir tid til DNA-reparasjon (Okada & Mak 2004).

1.6.2 Autofagi

Ordet autofag er av gresk opprinnelse og betyr selvspisende (Gozuacik & Kimchi 2004). Autofagi er en mekanisme for degradering av cellulære komponenter i cytoplasma, og fungerer som en celleoverlevelsesmekanisme i utsultede/døende celler. Nye studier indikerer at autofagi også er involvert i celledød, men dets eksakte rolle i den katabolske prosessen i døende celler er ennå ikke avklart (Baehrecke 2005). De morfologiske forandringene er delvis kromatinkondensering og akkumulering av autofage vesikler (vakuolisering), men det forekommer ingen DNA-fragmentering (Lockshin & Zakeri 2004, Okada & Mak 2004, Tang et al. 2008). I følge Kroemer og medarbeidere fungerer autofagi som en pro-overlevelse signalvei. *Autofagisk celledød* kan derfor være en misvisende betegnelse, selv om det definerer celledød som skjer med pågående autofagi. De to kan ganske enkelt være parallelle hendelser (Kroemer and others 2009).

1.6.3 Mitotisk katastrofe

En mitotisk katastrofe er en type celledød som skjer under, eller etter, en mislykket mitose. Resultatet av en mislykket mitose er mikro- eller multinukleære celler som inneholder ukondenserte kromosomer, eller induksjon av apoptose eller nekrose. En anbefalt, mer korrekt og informativ betegnelse av denne type celledød er *celledød som oppstår under metafasen* (Kroemer and others 2009, Okada & Mak 2004).

1.6.4 Nekrose

Apoptotisk og autofagisk celledød blir ofte betegnet som aktive prosesser på grunn av at de krever proteinsyntese, i motsetning til nekrotisk celledød som ofte er en passiv prosess (Asare et al. 2008, Kroemer and others 2009, Tang and others 2008). Nekrose er en patologisk type celledød karakterisert av irreversibel svelling av cytoplasma og degradering av organellene, inkludert mitokondriene. Etter hvert forekommer tap av membranintegriteten, hvilket resulterer i at cellen går i stykker, og i at det frigjøres skadelige cellulære komponenter. Dette påvirker cellene omkring og inflammasjonsutvikling inntrer i omliggende vev (Fadeel & Orrenius 2005). Nekrose gir ødeleggelse av plasmamembranen, degradering av DNA og organeller, samt svelling av mitokondrier, hvilket medfører forstyrrelser i ionetransport, energiproduksjon og pH-balanse. Infeksjoner og andre irritative forhold kan forårsake en slik type celledød (Okada & Mak 2004). Senere forskning har vist

at det også forekommer en rekke typer programmert nekrose, som *necroptosis* og *paraptosis* (Asare and others 2008, Asare et al. 2009a, Kroemer and others 2009).

1.6.5 Apoptose

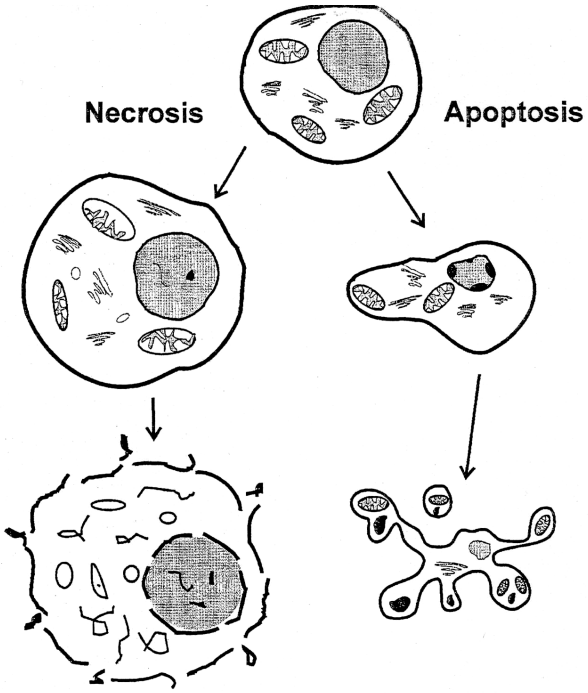
Programmert celledød (PCD) er en veldefinert prosess hvor cellene begår selvmord, gjennom apoptose eller alternative signalveier. Celledød som oppstår på et bestemt tidspunkt i utviklingsprosessen er definert som programmert. Apoptose er ikke synonymt med PCD. Apoptose er ofte, men ikke nødvendigvis, en type PCD (Chipuk & Green 2005, Tang and others 2008).

Apoptose beskrives ofte som en form for celledød som fjerner overflødige, skadede eller infiserte celler, gjennom aktive prosesser. Apoptose er essensielt i normal celleutvikling, differensiering og homeostase hos multicellulære organismer (Leist & Jaattela 2001, Tang and others 2008). Vekst og utvikling hos multicellulære organismer involverer en viktig balanse mellom cellevekst, celledeling og celledød. Denne balansen er avhengig av at celledød skjer på riktig sted, til riktig tid og i riktig mengde. Akkumulering av uønskede celler på grunn av mangelfull apoptose kan gi overdreven og unormal cellevekst, i ytterste konsekvens kreft, mens celletap relatert til overdreven apoptotisk respons kan gi neurodegenerering og hjertefeil (Baehrecke 2005, Bratton & Cohen 2001).

Apoptose kjennetegnes også ved ulike karakteristiske morfologiske og biokjemiske forandringer, så som skrumping av cellen, kromatinkondensering og desintegrasjon av cellen til små fragmenter (Jaattela 2004, Roos & Kaina 2006) (Fig.10). Siden prosessen er ATP-avhengig holdes organellene intakte. Aktivering av caspaser er involvert i apoptose, caspasene er cystein-proteaser som kutter proteiner i spesifikke fragmenter. De fører ofte til en aktivering av *Inhibitor of Caspase activated deoxyribonuclease* (ICAD), som kutter opp DNA i spesifikke lengder. Under forvandlingen fra levende celler til apoptotiske legemer bevares cellemembranen intakt, slik at cellulære komponenter ikke lekker ut. De apoptotiske cellene blir raskt gjenkjent og fagocytert av andre celler (naboceller eller immunceller). På denne måten forhindres inflammasjon av omkringliggende vev (Fadeel & Orrenius 2005). Denne typen celledød induseres blant annet når en DNA-skade er for alvorlig til å kunne repareres.

Apoptose kan aktiveres via en utvendig (dødsreseptor-indusert) eller en innvendig (mitokondrie-indusert) signalvei (Tang and others 2008). Utvendig signalvei induseres av endogene ligander som fester seg til dødsreseptorer på celleoverflaten. Den innvendige signalveien blir trigget av ekstra- eller intracellulært stress, så som fravær av vekstfaktorer, hypoxi, DNA-skade eller induksjon av onkogener. Signaler som overføres som respons på disse formene for stress konvergeres hovedsakelig via mitokondriene (Okada & Mak 2004, Tang and others 2008).

Apoptose karakteriseres ofte av frigjøringen av cytokrom c fra mitokondriene som aktiverer spesifikt initiator-caspase 9. I de fleste celledød-signalveier er rollen til caspaser heller å fremme ødeleggelsen av celler som allerede er forpliktet til å dø, enn å avgjøre om de skal dø eller ikke (Jaattela 2004). Celleoverlevelse/-død reguleres delvis av bcl-proteiner, som kan være både pro- og anti-apoptotiske. Noen bcl-proteiner er pro-apoptotiske, som for eksempel Bax. Når Bax translokeres og bindes til mitokondriemembranen resulterer det i frigjøring av cytokrom c til cytosol og aktivering av apoptosen. Cytokrom c i kompleks med *apoptotic peptidase activating factor 1* (APAF-1) og initiator-caspase 9 (apoptosom) gir apoptose, siden apoptosomet kløyver blant annet effektor-caspase 3. Bax er igjen under kontroll av p53 (Bratton & Cohen 2001, Robertson & Orrenius 2000). Poly (ADP-ribose) polymerase (PARP), ser ut til å være involvert i DNA-reparasjon, og er et av de viktigste proteinene som kløyves av caspase 3. PARP er foreslått som markør for apoptotiske celler (Oliver et al. 1998) (Bratton & Cohen 2001, Roos & Kaina 2006).



Figur 10: Skjematisk representasjon av apoptose og nekrose. Nekrose er karakterisert av oppsvulming av cellen og lysering, i motsetning til apoptose som kjennetegnes av at cellen skrumper inn og fragmenteres, og deretter blir de apoptotiske legemene fagocytert av immunceller (Robertson & Orrenius 2000).

2. MÅLSETNINGER

Målsetningen med denne studien var å kartlegge toksisiteten til ulike nitro- og amino-PAH i den humane bronkiale epitelcellelinjen BEAS-2B. Ut fra første *screening* ble det satt et spesielt fokus på å belyse betydningen av DNA-skaderesponsen for PAH-indusert celledød (apoptose) og inflammasjon (cytokinfrigjøring).

Delmål:

- Å undersøke toksisiteten til de ulike nitro- og amino-PAH-forbindelsene, og å studere i hvilken grad de induserer celledød (apoptose og nekrose) og cytokinfrigjøring.

- Å undersøke om noen av stoffene aktiverer NF-κB, og om dette er forbundet med deres evne til å indusere frigjøring av cytokiner, inkludert CXCL8, CCL5 og TNF-α.

- Å studere nitro- og amino-PAH sin evne til å indusere AhR, ARNT og AhR/ARNT-regulerte gener (CYP1A1, CYP1B1 og AhRR), og dets betydning for DNA-skade, celledød og cytokinproduksjon.

- Å undersøke alle forbindelsenes effekter på cellesyklus og DNA-skade.

- Å karakterisere DNA-skaderesponsens betydning for cellesyklus, apoptose og inflammasjon.

Dette mastergradsarbeidet er en del av et større forskningsprosjekt, *Importance of components and sources in particle-induced lung inflammation: Role of PAHs and metals*, som involverer flere samarbeidspartnere. Bronkiale epitelceller BEAS-2B eksponert for ulike PAH-forbindelser ble sendt til Institute of Cancer Research i Surrey, UK for DNA-skadeanalyser. Dette arbeidet ble utført av Dr. Ezter Nagy (*comet assay*) (Artikkel-figur 17) og Dr. Volker Arlt (^{32}*P-postlabelling*) (Artikkel-figur 16) ved Section of Molecular Carcinogenesis. Real-time RT-PCR målinger av AhR, ARNT, AhRR, CYP1A1 og CYP1B1 (Artikkel-figur 11) ble analysert av Dr. Steen Mollerup ved Statens arbeidsmiljøinstitutt, Avdeling for kjemisk og biologisk arbeidsmiljø, Toksikologisk gruppe, som også veiledet mastergradsstudenten med tanke på tidskurven (Artikkel-figur 12). Dr. Johan Øvrevik ved

Nasjonalt folkehelseinstitutt, Divisjon for miljømedisin, Avdeling for luftforurensning og støy (MILS) utførte CXCL8- og CCL5-målingene (*ELISA*) (Artikkel-figur 9), og veiledet studenten med tanke på TNF-α-analysen (data ikke vist).

3. EKSPERIMENTELLE BETRAKTNINGER

Det eksperimentelle systemet, BEAS-2B celler

En bronkial epitelcellelinje (BEAS-2B), derivert fra normale humane lunger, er et spesielt relevant modellsystem for å studere toksisiteten av luftforurensninger relatert til mennesker. Dette på grunn av at mesteparten av inhalerte partikler deponeres i forgreiningene i bronkiene, slik at bronkiale epitelceller derfor er naturlige målceller for inhalert forurenset luft. Flere studier har vist at B[*a*]P og andre PAH kan indusere apoptose i BEAS-2B-celler. Denne cellelinjen har et basalt utrykk av CYP1A1, som er induserbart, og er et viktig enzym i metabolismen av PAH. BEAS-2B cellelinjen egner seg derfor godt til mekanistiske studier av PAH-indusert apoptose. BEAS-2B cellene har blitt transformert ved hjelp av et adenovirus (Ad12SV40). Tilstedeværelse av det virale onkoproteinet SV40 T antigen, i kompleks med p53 kan være nødvendig for immortialisering av cellelinjen. p53 er gjennom dette mutert i kodon 47, men det forandrer ikke dets funksjonelle egenskaper (Hussain et al. 2001, Lehman et al. 1993, Liao et al. 2007, Reddel et al. 1995).

Celledød

Mange teknikker er tilgjengelige for karakterisering av ulike former for celledød. I denne studien har *fluorescens mikroskopi* etter Hoechst 33342/PI farging vist seg å være en av de mest pålitelige teknikkene. Det er ved hjelp av fluorescens mikroskopi mulig å avgjøre om en celle er apoptotisk, apoptotisk-nekrotisk, nekrotisk eller levende, basert på opptak av fargestoff og den nukleære morfologien. Imidlertid er denne metoden tidkrevende, og det kan dessuten være krevende å oppdage og klassifisere apoptose med liten kjernekondensering og fragmentering, for en uerfaren person. *Flow cytometri* er en annen metode, som kan gi relevante resultater for denne studien, basert på det faktum at apoptotiske celler generelt har mindre cellestørrelse og mindre DNA-innhold, enn celler i G_1-fasen i cellesyklus, mens nekrotiske celler ofte har økt cellestørrelse. Kjernefargning med Hoechst 33258, tillater at cellene separeres i forhold til deres størrelse og DNA-innhold. Det er imidlertid viktig å skille ut (*gate out*) støy fra flow cytometri-dataene. Sammenlignet med fluorescens mikroskopi er denne metoden langt raskere, men den skiller ikke like enkelt ut nekrotiske celler fra normale celler og skiller heller ikke ut tidlige apoptotiske stadier. Dermed er det viktig å kombinere flere ulike metoder ved karakterisering av celledød.

Det er videre vanlig å identifisere aktiveringen av ulike caspaser som et ledd i karakteriseringen av den apoptotiske prosessen. Aktivering av apoptotiske markører som caspaser kan detekteres ved deres kløyving. Kløyving av PARP, et substrat for caspase-3, brukes også som indikator på caspase-aktivitet. *Western blotting* tilfører pålitelig informasjon om kløyving av caspase-3 og PARP. Western blotting (immunoblot) er en immunologisk teknikk som baserer seg på identifisering av et eller flere proteiner i et cellelysat. Gelelektroforese brukes for å separere denaturerte proteiner etter størrelse. Deretter overføres proteinene til en membran hvor de merkes med antistoff, som gjør det mulig å detektere de spesifikke proteinene. Western blotting gir informasjon om proteinenes uttrykk, noe som indikerer hvorvidt det er aktivert eller nedregulert (sammenlignet med en ubehandlet prøve; kontroll). Identifisering er mulig ved å sammenligne proteinenes størrelse med en størrelse-markør (stige i kDa). *FACS (fluorescence-activated cell sorting*) er en mer kompleks teknikk, som ikke bare kvantifiserer det fluorescerende signalet, men også separerer celler fra en blandet populasjon, ved hjelp av gitte karakteristika (f eks fluorescens-intensitet, størrelse og viabilitet). *Immunocytokjemi* muliggjør detektering, avgjør tilstedeværelse og visualiserer cellelokaliseringen av individuelle proteiner i cellene merket med antistoffer med fluorokrom-påheng. Aktivering av proteiner indikeres ved øket fluorescens i cellene eller forandret intracellulær lokalisasjon av fluorescensen. Kombinasjon av flere metoder, som Western blotting, flow cytometri og immunocytokjemi gir et mer pålitelig resultat enn de ulike metodene brukt hver for seg.

Cellulære organeller som mitokondrier, kan være involvert i ulike signalveier, og kan spille viktige roller i ulike typer celledød. Analyse med fluorescens mikroskopi etter farging med Mito Tracker® kan avsløre om PAH-eksponering har påvirket mitokondrie-integriteten, siden Mito Tracker® akkumuleres i funksjonelle mitokondrier.

DNA-kondensering og -fragmentering er sene hendelser i det apoptotiske forløpet. Ved å undersøke oppstrømsproteiner involvert i DNA-skaderesponsen, kan man tidligere avklare om en DNA-skade har oppstått, og hvorvidt denne har vært viktig for å igangsette apoptosen. ATR/ATM er sensorer øverst i kaskadereaksjonen, og aktiverer Chk1/2, som igjen resulterer i aktivering av p53. Deretter kan p53 aktivere en ny rekke proteiner som er involvert i cellesyklus, DNA-reparasjon eller apoptose. Disse nedstrømsproteinene, foruten p21 (regulator av cellesyklus), er ikke undersøkt i denne oppgaven.

Enzymatisk hemming

Før man tar i bruk kjemiske inhibitorer for å karakterisere cellesignaliseringsveier, er det viktig å kjenne til deres spesifisitet i det aktuelle testsystemet. Dermed er det viktig å bruke den anbefalte konsentrasjon og eksponeringstid for de ulike inhibitorene. Pifithrin-α (PFT-α) er en inhibitor av p53, som ble funnet å hemme PAH-indusert celledød. Tidligere studier (Solhaug et al. 2005) har vist at PFT-α hemmer det andre aktiveringstrinnet i metabolismen av B[*a*]P. Siden CYP1A1/1B1 ikke ser ut til å være involvert i aktiveringen av 3-NBA skulle ikke dette influere resultatene her.

Cytokinfrigjøring

Enzyme-Linked ImmunoSorbent Assay (ELISA) er en immunologisk metode som effektivt detekterer og kvantifiserer cytokinnivået i supernatanten til PAH-eksponerte celler. *Sandwich-ELISA,* også kalt *two-site analysis*, er et sensitivt system med to antistoffer, som forutsetter at antigenet har to epitoper. Antistoffene er gjerne konjugert med isotoper, enzymer eller fluorescerende forbindelser, eller med forbindelser som sender ut lys i form av luminescens. Denne dobbel-spesifisiteten kjennetegner *sandwich-ELISA*, som har høyere spesifisitet enn de to andre immunologiske teknikkene, Western blotting og flow cytometri. CXCL8, CCL5 og TNF-α var cytokinene som ble undersøkt i denne studien.

Genekspresjon

Real-time RT-PCR er en metode som måler genutrykket av utvalgte gener, ved å isolere den totale RNA-mengden av et bestemt gen, som deretter blir revers transkribert til cDNA, og til slutt kjøres det PCR (*polymerase chain reaction*) for å amplifisere opp mengden av det utvalgte genet. I dette arbeidet målte vi genuttryket til AhR, ARNT, AhRR, CYP1A1 og CYP1B1.

Cellesyklus

Cellesyklus-sjekkpunkter regulerer progresjonen mellom trinnene i cellesyklus (G1→S→G2→M) og garanterer stor grad av nøyaktighet i kritiske hendelser, som for eksempel segregering av kromosomer (M-fasen), DNA-replikasjon (G2 fasen) eller DNA-skade-indusert cellesyklusarrest og apoptose (G1/S fasen). Forstyrrelser i aktiviteten til sjekkpunktene kan resultere i akkumulering av DNA-mutasjoner, og kan videre føre til

genomisk ustabilitet, og tumorutvikling. Den prosentvise fordelingen av apoptotiske celler og celler i ulike faser av cellesyklusen kan bestemmes ved hjelp av flow cytometri, etter at de eksponerte cellene har blitt farget med Hoechst 33258. De ulike cellefasene kan skilles på grunnlag av deres DNA-innhold (Hoechst 33258 fluorescens), cellestørrelse (*Forward scatter*) og cellegranularitet (*Side scatter*). Akkumulering av celler i de ulike fasene kan indikere ulike reaksjoner i kjølvannet av en DNA-skade, og kan si noe om DNA-skaden/DNA-skaderesponsen som har oppstått.

DNA-skader

^{32}*P-postlabeling* analyse er en ultrasensitiv metode, som i denne studien ble brukt for å detektere og kvantifisere PAH–DNA addukter. Addukter kan gi opphav til DNA-replikasjonsstress, for eksempel ved å hindre DNA-replikasjonsgaffelen, eller ved å skape DNA-enkeltrådbrudd. *Comet-metoden* er en enkel teknikk til å måle DNA-trådbrudd og oksidative skader, i eukaryote celler. Oksidative skader ble vurdert ved å måle de trådbrudd-spesifikke reparasjonsenzymene DNA-glycoylase (FPG) og endonuklease III (Endo III).

Cellesignalisering

DNA-skaderesponsen (DDR) består av en signalkaskade av proteiner som blir aktivert etter at ulike former for DNA-skader har oppstått. De signaliserer videre for å indusere cellesyklus-arrest, senescence, DNA-reparasjon eller celledød, avhengig av hvor omfattende skaden er. ATM/ATR er sensorer av henholdsvis DNA-dobbeltrådbrudd og DNA-enkelttrådbrudd, og signaliserer videre til henholdsvis Chk2/1 for å indusere cellesyklus-arrest. Resultatet av denne signaliseringsveien kan være aktivering av p53. Ulike metoder kan brukes for å analysere aktiveringen av proteinene involvert i DDR, inkludert NF-κB, som er involvert i cytokinproduksjon. NF-κB er først og fremst et overlevelsessignal. *Western blotting, FACS* og *immunocytokjemi*, er de metodene som ble brukt for analysering av DDR.

Statistisk analyse

ANOVA er en generalisering av t-testen som gjør det mulig å sammenligne mer enn to gjennomsnitt samtidig. Ved bruk av ANOVA må følgende forutsetninger oppfylles:

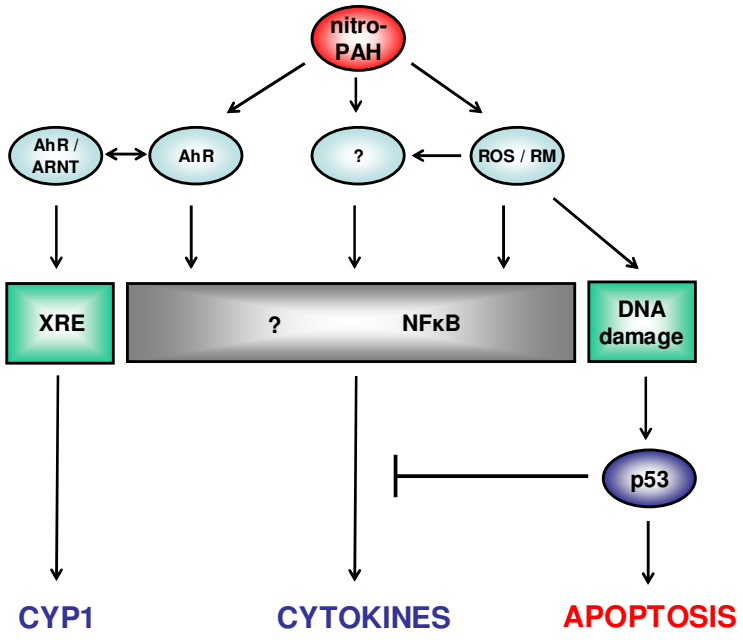
gjensidig uavhengige observasjoner, normalfordeling av dataene og lik varians for og i hver prøve.

4. KONKLUSJON

Det eksisterer flere regulatoriske systemer som bidrar til å opprettholde den genomiske integriteten. Slike systemer er nødvendige, siden mennesker daglig kommer i kontakt med en mengde ulike DNA-skadende agens, i tillegg til at spontane DNA-skader kontinuerlig oppstår i cellene våre. I denne studien har vi vist at forskjellige PAH induserer ulike responser i BEAS-2B celler, som er avgjørende for det endelige resultatet av en DNA-skade. Studien illustrerer at de ulike PAH har svært forskjellige toksiske potensialer (målt i celledød og cytokindannelse), og små endringer i molekylstruktur, så som plassering av nitro-grupper, eller substitusjon av en nitro- med en aminogruppe, har dramatisk innvirkning på toksisiteten.

- Nitro-PAH er mer cytotoksiske enn de korresponderende amino-formene. Nitro-PAH gav mest apoptose, mens forskjellene i nekrose var mindre mellom amino- og nitroforbindelsene. 3-NBA var den mest potente forbindelsen, og induserte massiv apoptose. Nitro-PAH induserte hovedsakelig frigjøring av CXCL8, og 1-NP og 3-NF var i så måte de mest potente. De korresponderende amino-formene 1-AP og 3-AF derimot, induserte mest CCL5.

- 3-NBA indikerte en mye sterkere NF-κB-aktivering i forhold til 1-NP, men på den annen side gav ikke 3-NBA spesielt mye cytokinproduksjon (CXCL8, CCL5 eller TNF-α) sammenlignet med 1-NP og 3-NF. Årsaken til dette kan være relatert til at DNA-skade kan aktivere NF-κB via p53, da disse preliminære resultatene medfører spekulasjoner om at p53 modulerer NF-κB sin normale funksjon, slik at den induserer et annet sett med gener, som ikke er involvert i cytokinfrigjøring (Fig. 11).

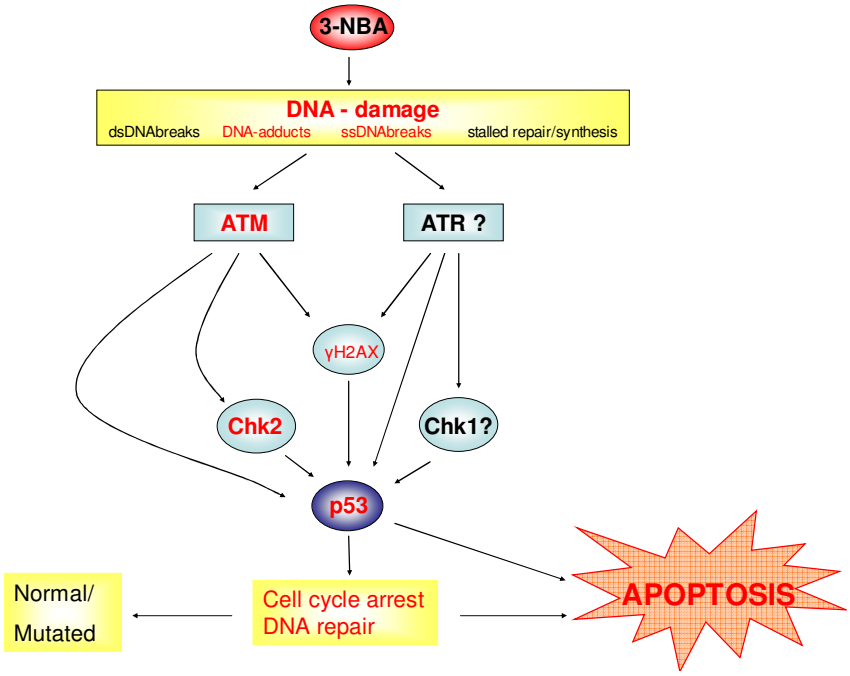


Figur 11: Postulert mekanisme for p53 sin negative påvirkning på NF-κB-mediert cytokin-frigjøring.

- B[*a*]P var den eneste forbindelsen som fremmet induksjon av genene AhRR, CYP1A1 og CYP1B1, mens det ser ut som at 1-NP, 1-AP, 3-NF og 3-NBA reduserte uttrykket av de samme genene. I denne studien fremstår det ingen direkte relasjon mellom AhR/ARNT signaliseringsveien og DNA-skade, celledød, forstyrrelser i cellesyklus eller cytokinfrigjøring.

- 3-NBA og B[*a*]P gav akkumulering av celler i S-fase, hvilket indikerer at en DNA-skade har oppstått. 3-NBA gav flere DNA-addukter og flere DNA-enkelttrådbrudd enn noen av de andre testforbindelsene. 3-NBA induserte celledød og S-fase-akkumulering av cellene etter 24 timer, mens 2-NBA verken gav celledød eller S-fase-arrest på dette tidspunktet. Etter 72 timer, førte derimot 2-NBA til akkumulering av cellene i S-fase. Dette kan tyde på at 2-NBA langsomt metaboliseres til forbindelser som kan gi DNA-skader, selv om forbindelsen ikke førte til celledød. 3-NBA-indusert apoptose forårsakes av DNA-skade som aktiverer ATM/Chk2/p53 signaliseringsveien (Fig. 12). I tillegg var 3-NBA den mest potente forbindelsen med tanke på å fosforylere p53. p53 ser ut til å være et sentralt protein i den apoptotiske prosessen, noe som baseres på en kraftig økning av fosforylering og en hemming av 3-NBA-indusert celledød med p53-hemmeren, PFT-α.

PFT-α-resultatene er preliminære data, og det trengs flere repetisjoner av forsøket for å bekrefte disse. I tillegg hadde det vært ønskelig med siRNA studier mot p53.



Figur 12: Foreslått signaliseringsvei for 3-NBA-indusert apoptose. Skrift som er merket med rødt er funn fra denne studien.

VIDERE ARBEID

- Komparative analyser av disse PAH-forbindelsene i ulike cellesystemer, som f eks primære lungeceller, hepatocytter og andre cellelinjer. I*n vivo* studier ville være av spesiell interesse.

- Det at 2-NBA ser ut til å føre til DNA-skader etter lengre tid, mens den apoptotiske prosessen er hemmet, kan bidra til å forklare forbindelsens mutagene og kreftfremkallende egenskaper. Mangelen på celledød etter 2-NBA eksponering kan skyldes at DNA-skaden ikke så lett oppdages av DNA-skaderesponsen, eller eventuelt at 2-NBA skader p53-proteinet. Dette er en hypotese, og det kreves ytterligere studier for å få dette avklart.

- Årsaken til at 3-NBA så ut til å gi en mye sterkere NF-κB-aktivering enn 1-NP, men mindre cytokinproduksjon (CXCL8, CCL5 eller TNF-α) sammenlignet med 1-NP og 3-NF, kan være at DNA-skade aktiverer NF-κB via p53. Disse preliminære resultatene sammen med tidligere rapporterte funn, kan tyde på at p53 modulerer NF-κB sin normale funksjon, slik at den induserer et annet sett med gener, som ikke er involvert i cytokinfrigjøring. Denne hypotesen må imidlertid bekreftes f eks ved bruk av p53-hemmeren PFT-α og siRNA for p53.

- Cytokin-induserende og DNA-skade-induserende PAH kan forekomme sammen på dieseleksos partikler (DEP). Videre studier av de samvirkende effektene av disse er nødvendig for å undersøke om de DNA-skade-induserende PAH undertrykker effektene av de PAH som induserer cytokiner. Muligheten er tilstede for at DEP har et lavere inflammatorisk potensiale, enn det man kunne forvente ut fra de individuelle forbindelsene.

- Hva som forårsaket vakuoledannelsen observert etter 2-NBA-eksponering bør undersøkes videre. Evaluering av eventuell DNA-skade, og skader på intracellulære organeller kan være aktuelle arbeidsområder.

- Mangelen på p53-indusert p21-respons, etter eksponering for 3-NBA, hvilket ble observert som en manglende G1-arrest, er et aktuelt tema for videre 3-NBA-forskning.

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Toxic effects of nitro-PAHs and amino-PAHs in human bronchial epithelial BEAS-2B cells: role of DNA damage response.

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KEY WORDS: Nitro-PAHs, 3-nitrobenzanthrone, DNA damage, apoptosis.

ABBREVIATIONS

AhR, aryl hydrocarbon receptor; AhRR, AhR repressor; ARNT, AhR nuclear translocator; ATM, serine/threonine kinases ataxia-telangiectasia-mutated; ATR, ATM and Rad3-related; B[*a*]P, benzo[*a*]pyrene; Chk, checkpoint kinases; CYP, cytochrome P450; cyt c, cytochrome c; DDR, DNA damage response; DSBs, double-strand DNA breaks; DEP, diesel exhaust particles; DMSO, dimethyl sulphoxide; FCS, foetal calf serum; IκB, inhibitor of κB; NFκB, nuclear factor-kappa B; NQOI, NAD(P)H:quinone oxidoreductase; 1-AP, 1-aminopyrene; 1-NP, 1-nitropyrene; 3-AF, 3-aminofluoranthene; 3-NF, 3-nitrofluoranthene; 3-ABA, 3-aminobenzanthrone; 3-NBA, 3-nitrobenzanthrone; 2-NBA, 2-nitrobenzanthrone; nitro-PAH, nitrosubstituted-polycyclic aromatic hydrocarbons; NOS, nitric oxide synthase; NR, nitroreductases; PAH, polycyclic aromatic hydrocarbons; PARP, poly(ADP-ribose)polymerase; PFT, pifithrin; PI, propidium iodide; ROS, reactive oxygen species; SSBs, single-strand DNA breaks; XO, xanthine oxidase.

ABSTRACT

In the present study we have examined and compared the effects of some chemically related nitro-polycyclic aromatic hydrocarbons (nitro-PAHs), which are mutagenic and carcinogenic environmental pollutants, found in diesel exhaust and urban air pollution particles. Human bronchial epithelial cells (BEAS-2B) were exposed to 1-nitropyrene (1-NP), 3-nitrofluoranthene (3-NF) and 3-nitrobenzanthrone (3-NBA), and their corresponding amino forms, 1-AP, 3-AF and 3-ABA; in addition 2-nitrobenzanthrone (2-NBA) and benzo[*a*]pyrene (B[*a*]P) were included in this work. Examination by microscopy revealed that 3-NBA was the most cytotoxic, and that the nitro compounds were more potent than their corresponding amino compounds. Judged by nuclear condensation, and cleavage of caspase 3 and PARP, 3-NBA induced mainly apoptotic cell death. All the test compounds, except 2-NBA and B[*a*]P, significantly increased the release of the cytokine CXCL8 (IL-8), and the highest levels were induced by 1-NP and 3-NF. In addition, the corresponding amino forms, 1-AP and 3-AF, induced increased release of CCL5 (RANTES). B[a]P induced increased CYP1A1/1B1 gene expression, while 3-NBA, 3-NF and 1-NP all reduced CYP1A1/1B1 expression, suggesting AhR/ARNT antagonism. 3-NBA caused the most DNA damage, judged by the amount of DNA adducts and DNA single-strand breaks (SSBs), giving an explanation for the observed accumulation of cells in S-phase. Further analysis of the DNA damage response (DDR) showed that 3-NBA activated ATM and Chk2. p53 was found to be activated by phosphorylation and translocation to the nucleus. The p53 inhibitor, pifithrin-α, inhibited the 3-NBA-induced apoptosis, suggesting that the cell death was a result of triggering of DDR signalling pathways, ending up with an activation of p53. In conclusion, the different PAHs had very different toxic potentials (cell death and cytokines), and small changes in the molecular structures, such as a change of the nitro-group from position 3 to 2 or a substitution of a nitro- with an amino-group, had dramatic effects.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are generated by the incomplete combustion of organic material such as gasoline and diesel fuel, and are ubiquitous contaminants in ambient air. They are also found in grilled food, emissions from domestic heating, and cigarette smoke (IARC 1989, Landvik and others 2007, Pope, III and others 2002, Purohit & Basu 2000). Nitro-PAHs, which are an important subgroup of the PAHs, and are found in diesel exhaust, are often located on ambient air particles. These environmental pollutants have been shown to be highly mutagenic in bacterial systems (Rosenkranz and others 1980, Rosenkranz & Mermelstein 1983), and to induce tumors in animal models (el-Bayoumy 1992, Guengerich 2000). Different epidemiological studies suggest a correlation between exposure to diesel exhaust and urban air pollution, and the development of lung cancer in humans (Arlt 2005, Garshick and others 2004, IARC 1989).

Nitro-PAHs are most commonly metabolized by nitro reduction, although ring oxidation and conjugation reactions are frequent as well (Fu 1990, Purohit & Basu 2000, Rosenkranz & Howard 1986). The nitro group can be reduced to the corresponding amino form (amino-PAHs), mainly through six-electron reduction reactions. When oxygen is available, redox cycling can regenerate the nitro compound and produce reactive oxygen species (ROS), for instance superoxide anions ($O_2^{\bullet -}$). Reactions like this can be catalyzed by cytosolic nitroreductases (NR), like nitric oxide synthase (NOS), NAD(P)H:quinone oxidoreductase (NQO1), xanthine oxidase (XO) and aldehyde oxidase (Arlt and others 2003, Arlt and others 2005, Ask and others 2003, Silvers et al. 1997). Nitro reduction and ring oxidation may lead to formation of detoxified products, or dangerous electrophilic reactive metabolites. Ring hydroxylation may result in reactive epoxides which can react with essential macromolecules and be further catalyzed by microsomal cytochrome P450 enzymes like CYP1A1, 1A2 and 1B1 (Yamazaki et al. 2000). Furthermore, the aryl hydrocarbon receptor (AhR) is known to be activated by several aromatic compounds, and has the ability to induce these CYP-enzymes (Arlt et al. 2004, Chen et al. 2003, Nebert et al. 2000, Nebert and others 2004). AhR may also bind nitro-PAHs resulting in up-regulation of AhR-regulated genes (Iwanari et al. 2002, Landvik et al. 2009). Some CYP-enzymes have the ability to *N*-hydroxylate the nitro-PAHs. The result may be the formation of nitroso, *N*-hydroxylamine and the corresponding amine. A further acetylation or sulphatation of the *N*-hydroxylamine can give

rise to reactive nitrenium ions which have the potential to form DNA adducts (Arlt and others 2005, Hatanaka and others 2001) The outcome of both ring oxidation and nitro reduction can be severe DNA damage, related to the covalent binding between reactive metabolites and DNA, however, other macromolecules may also be affected.

Cells respond to DNA damage by activating DNA repair and DNA damage signalling pathways (Ljungman 2005). DNA damage response (DDR) involves mechanisms of regulation, and is important for both DNA repair and replication control. In this response, ATM (ataxia-telangiectasia-mutated) and ATR (ATM and Rad3-related) protein kinases are independent molecular complexes, that respond to different types of DNA damage (Harper & Elledge 2007). The ATM kinase is a major sensor of DNA double-strand breaks (DSBs) and larger chromatin alterations, and organizes cell cycle arrest and apoptosis via further activation of the checkpoint kinase, Chk2, and p53. ATR on the other hand, is the main sensor of stalled replication forks and is associated with DNA single-strand breaks (SSBs) and DNA adducts. Thus, it appears to be the main activator of the replication stress response. ATR plays a pivotal part in DNA repair, apoptosis and cell cycle arrest, via the Chk1 and p53 pathway (Bartek, Bartkova, & Lukas 2007, Bartek & Lukas 2007, Cann & Hicks 2007, Cimprich & Cortez 2008, Efeyan & Serrano 2007, Ljungman 2005, Zgheib et al. 2005).

PAHs, like B[*a*]P, are known to cause an accumulation of the tumor suppressor protein p53, which has an important role in the induction of temporary and permanent cell cycle arrest, DNA repair and apoptosis (Bjelogrlic et al. 1994, Campisi 2005). DNA damage, oncogene activation, telomere erosion and cellular stress, are factors that can activate p53. The main molecular function of p53 is its ability to act as a transcription factor, inducing transcription of proteins involved in cell cycle arrest and cell death (Efeyan & Serrano 2007, Romer and others 2006, Sengupta & Harris 2005). Furthermore, p53 is involved in various non-transcriptional pathways (Speidel, Helmbold, & Deppert 2006).

There is no simple correlation between DNA damage, mutations and cancer. The consequence of DNA damage could be reparation, mutations, or induction of cell death such as apoptosis, if the damage is too extensive, or if the safety valves fail (Campisi 2005, Norbury & Zhivotovsky 2004). However, cell death, i.e. protection of the organism against mutations and cancer development, is not a fail-safe. Proliferation of cells with fixed DNA damage, and selection of apoptosis resistant cells, which play an important part in tumor

development, may not always be prevented (Huc et al. 2004, Solhaug, Refsnes, & Holme 2004).

Apoptosis is distinguished from necrosis as the former involves the activation of specific pathways which result in characteristic morphological features, including rounding-up of the cell, cell shrinkage, nuclear fragmentation (karyorrhexis), chromatin condensation, cytoplasmic and nuclear pyknosis, and the formation of apoptotic bodies (Kroemer et al. 2009, Rogakou et al. 2000). A key feature of apoptosis is the activation of cysteine-aspartic acid proteases (caspases) that cleave other protein substrates within the cell. Caspase activation can be carried out either by the extrinsic (death receptor mediated) or the intrinsic (mitochondrion mediated) pathway (Tang and others 2008). Meanwhile, necrosis is characterized by a gain of cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents, and is considered an uncontrolled or pathological form of cell death (Kroemer and others 2009, Orrenius & Zhivotovsky 2006).

Cytokines are mainly low molecular proteins or glycoproteins, which act as chemical signaling molecules between cells. An important cellular response to stress is an increased production of cytokines. Cytokines are key regulators of inflammation with pro- and anti-inflammatory functions, and are considered to be an important part of tumor growth/progression, chronic obstructive pulmonary disease (COPD) and asthma (Hussain & Harris 2007). PAHs have been reported to induce cytokine release, including cytokines such as CXCL8 (interleukine-8; IL-8) and CCL5 (RANTES) (Oppenheim et al. 1997, Xie 2001). Nuclear factor-kappa B (NF-κB) is a central transcription factor involved in the regulation of these cytokines, and represents an important molecular link between inflammation and cancer (Henriquet and others 2007, Hussain & Harris 2007).

In several studies, Holme and co-workers (Asare and others 2008, Asare and others 2009a, Asare et al. 2009b, Holme and others 2007, Landvik and others 2007, Landvik and others 2009, Solhaug et al. 2004, Solhaug and others 2005, Solhaug, Refsnes, & Holme 2004) have found that PAHs and nitro-PAHs have different effects on cell death, DNA damage and cell signaling, in Hepa1c1c7 cells. In the present study we used immortalized human bronchial epithelial cells (BEAS-2B), a commonly used model for studying the cytotoxicity of air pollution components. We have examined the toxic effects of many of the same PAHs, with special focus on mechanisms involved in 3-nitrobenzanthrone (3-NBA)-induced apoptosis.

MATERIALS AND METHODS

Chemicals

LHC-9 cell culture medium was from Invitrogen (Carlsbad, CA, USA). Foetal calf serum (FCS) was from Gibco BRL (Paisley, Scotland, UK). Sterile HBS and purified collagen, PureCol™ were from Inamed Biomaterials (Freemont, CA 94538 USA). 3-nitrobenzanthrone (3-NBA) and 3-aminobenzanthrone (3-ABA) were prepared as described (Arlt and others 2003), and 2-nitrobenzanthrone (2-NBA) synthesized as reported (Arlt et al. 2007). 3-nitrofluoranthene (3-NF) and 3-aminofluoranthene (3-AF) were obtained from Chiron AS (Trondheim, Norway). Benzo[a]pyrene (B[a]P), 1-nitropyrene (1-NP), 1-aminopyrene (1-AP), bovine serum albumin (BSA), Ponceau S, dimethyl sulfoxide (DMSO), propidium iodide (PI), phenylmethylsulfonyl fluoride (PMSF), Hoechst 33258, Hoechst 33342, aprotinin, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and polyoxyethylene octyl phenyl ether (Triton X-100) were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Pifithrin-α (PFT-α) and Pepstatin A were from Calbiochem (Cambridge, MA, CA, USA). Mito Tracker® was obtained from Invitrogen (Carlsbad, CA). Leupeptin from Amersham Biosciences (Uppsala, Sweden). SYBR®Green I nucleic acid gel stain was obtained from Cambrex Bio Science (Rockland, USA). UltraPure™ Low Melting-Point Agarose was purchased from Invitrogen (Paisley, UK). Diagnostic slides with 3 wells, 14 mm diameter, with PTFE (Teflon) coating were obtained from Menzel-Gläser Glasbearbeitungswerk GmbH (Braunschweig, Germany). Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained from commercial sources as reported previously (Phillips & Arlt 2007). Cytokine ELISA assays for CCL5 (Human RANTES Cytoset), CXCL8 (Human IL-8 Cytoset) and TNFα (Human TNFα Cytoset) were purchased from Biosource International (Camarillo, CA, USA). FPG and Endo III enzymes were kindly donated by Prof. Andrew Collins (Department of Nutrition, University of Oslo, Norway). Antibodies against: ATR, p53, phospho-Chk1 (Ser345), phospho-Chk2 (Thr68), cleaved caspase-3, Cleaved PARP (Asp214), β-actin, phospho-p53 (Ser15), phospho-Histone H2A.X (Ser139), p21 Waf1/Cip1 (12D1), IκB were obtained from Cell Signaling (Berverly, MA, USA); antibody against CYP1A1 from Santa Cruz Biotechnology, Inc. (CA, USA); CYP1B1

from alpha diagnostics (San Antonio, USA); phospho-ATM (Ser1981) from R&D Systems, Inc. (Minneapolis, MN, USA). As secondary antibodies horseradish peroxidise-conjugated goat-anti-rabbit Sigma-Aldrich Chemical Company (St. Louis, MO, USA), horseradish peroxidise-conjugated rabbit anti-mouse IgG from Dako (Glostrup, Denmark) was applied. Flour Alexa 488-A and 647-A was obtained from Invitrogen (Paisley, UK). All other chemicals were purchased from commercial sources and were of analytical grade.

Cell culture

BEAS-2B cells, an immortalized SV40-adenovirus-hybrid (Ad12SV40) transformed human bronchial epithelial cell line kindly provided to the Dept. of Toxicology at the National Institute of Occupational Health by Dr C.C. Harris (Reddel et al. 1988), are commercially available from the American Tissue Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown at 37°C in a humidified incubator with a 5 % CO_2 atmosphere, where they were kept in logarithmic growth (1-9x10^6 cells/75 cm^2 flasks), and split twice a week. Cells were cultured in serum-free LHC-9 medium on collagen (PureCol^{TM})-coated culture dishes and flasks. When splitting the cells, they were washed with phosphate-buffered saline (without Ca^{2+} and Mg^{2+}; PBS) and added 0.0025 % trypsin, which was removed shortly afterwards. The cells were then incubated for 3 min at 37°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 Thursdays respectively) of the cells were transferred into new coated flasks for further growth. The day after the splitting procedure, fresh medium was added.

Exposure

BEAS-2B cells were plated in 35 mm 6-well culture dishes (8x10^4 or 10x10^4 cells/well), 50 mm culture dishes (10x10^4 cells/well) or 90 mm culture dishes (5x10^5 cells/dish), and grown to near confluence. Fresh medium was added the day after they were seeded and right before exposure. When inhibitors were assessed, cells were pre-incubated with the inhibitor for one hour before adding test substance. Cells were treated with 1-NP/1-AP, 3-NF/3-AF, 3-NBA/3-ABA, 2-NBA and B[*a*]P (0.3, 1, 3, 10, 15, or 30 μM) as described below. All the compounds used were dissolved in DMSO, whereas control cultures were treated with

vehicle (DMSO) only. The final concentration of DMSO in culture medium was ≤ 0.5 % (v/v). After exposure cells were analysed by light microscopy, fluorescence microscopy, flow cytometry, ELISA, real-time RT-PCR, ^{32}P-postlabelling, comet assay, immunocytochemistry or western blotting.

Light microscopy (cell morphology)

After exposure to selected polycyclic aromatic hydrocarbons (PAHs) for 24 and 72 hrs, the morphology of the cultures was recorded with a digital camera (Nikon D40) coupled to a light microscope, making it possible to examine the living cell cultures. The toxicity was observed and categorized as the relative amount of floating cells (dead cells) in the cultures versus attached cells (living cells). Cell shape and vacuoles were recognized and documented.

Fluorescence microscopy

Cell death

Cytotoxicity was further characterized by fluorescence microscopy, i.e. plasma membrane damage and changes in nuclear morphology associated with apoptosis or necrosis.. The cells were exposed to various concentrations (0.3-30 µM) of different PAHs for 24 and 72 hrs. Following exposure, the cells were trypsinated and stained with Hoechst 33342 (5 µg/mL) and propidium iodide (PI) (10 µg/mL). After incubation for 30 min, in the dark, at room temperature, the cells (~0.5 x 10^6 cells) were centrifuged, resuspended in foetal calf serum (FCS) and prepared onto microscope slides and air dried quickly. Both compounds can stain DNA, but only Hoechst 33342 will penetrate an intact plasma membrane. Cell morphology was evaluated using a Nikon Eclipse E 400 fluorescence microscope, with an UV-2A excitation filter 330-380 nm (magnification x 1000), and cells were classified as apoptotic, necrotic or viable. Cells with distinct condensed nuclei, segregated nuclei and apoptotic bodies were counted as apoptotic (PI-negative cells), and the fraction compared to the total number of cells was determined. Apoptotic-necrotic cells have condensed nuclei, equivalent

to apoptotic cells, but additionally have secondary damage to their plasma membranes. PI-positive apoptotic cells (apoptotic-necrotic) were categorized as apoptotic cells. Non-apoptotic cells, excluding PI, were categorized as viable cells. PI-stained cells with a round morphology and homogeneously stained nucleus due to the loss of plasma membrane integrity were termed necrotic (PI-positive) (Asare and others 2009a, Solhaug and others 2004). A minimum of 300 cells per slide were counted.

Mitochondrial membrane integrity

Visualization of mitochondrial morphology was studied after staining with Mito Tracker®. This method was used to evaluate PAHs impact on mitochondrial functions. After exposure to 2-NBA and 3-NBA (10 μM) for 24 hrs, cells were labelled with Mito Tracker® (25 μM), incubated at 37°C for 30 min, and subsequently washed with PBS and fixated with ice cold methanol. Finally, a drop of mounting medium (DAPI) was added, and a cover glass placed over, before microscopic examination with an Tx Red excitation filter 540-580 nm (magnification x 1000).

Flow cytometry

Flow cytometry is a technique for the analysis of multiple parameters of individual cells, by passing thousands of cells per second through a highly focused beam of UV light and capturing the light that emerges from each cell as it passes through. Flow cytometry measures both the deviation of light (light scatter) as a function of size and optical properties of the cells, as well as absorption/emission of the fluorescence from cell components stained with fluorescent dyes (Otsuki, Li, & Shibata 2003).

Cell staining for apoptosis and cell cycle analyzes by flow cytometry

After treatment the cells were trypsinated and prepared for flow cytometry as described previously (Wiger et al. 1997). Briefly, the DNA of the cells was 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. Hoechst 33258 stains DNA and Triton X-100 makes the cells

permeable. By using an Argus 100 Flow cytometer (Skatron, Lier, Norway), the blue fluorescence could be measured. Different cell phases as well as apoptotic cells/bodies were distinguished on the basis of their DNA content (Hoechst fluorescence) and cell size (forward light scatter). Percentages of cells in different phases of the cell cycle as well as apoptotic cells were estimated from DNA histograms using the Multicycle Program (Phoenix Flow system, San Diego, CA, USA). The apoptotic index was determined as the percentage of signals between the G1 peak and the channel positioned at 20% of the G1 peak (sub-G1 population).

Due to chromatin condensation and nuclear fragmentation during the process of apoptosis, the apoptotic cells and bodies arising from G_1 emit fluorescent signals that are lower than those of G_1 cells. The fluorescent signals, which are detected and recorded, can be much smaller than G_1 cells and thus represent only a fraction of the original amount of DNA in a whole nucleus. Therefore, if a particular treatment causes a great increase in the percentage of apoptotic cells/bodies, it must be remembered that one G_1 nucleus can fragment into many smaller condensed chromatin units. As apoptosis progresses, the size of the fluorescent signal becomes progressively smaller and at some point will be gated out together with the background fluorescence.

Cell staining for FACS analysis

Probing with specific antibodies coupled with secondary antibodies with Alexa 488-A provides the possibility to measure the phosphorylation-state of individual cells, observed as a shift in fluorescence.

Following trypsination, the cells were washed once in PBS, fixed in 1% paraformaldehyde (PFA) in PBS for 15 min on ice, and post-fixed in 90% ice cold methanol for at least two days, at – 20 °C. The cells were then washed two times in 5% BSA in PBS and incubated with primary antibody: phospho-Chk1 (Ser345) 1:50, phospho-Chk2 (Thr68) 1:25, cleaved caspase-3 1:200, phospho-ATM (Ser1981) 1:100, phospho-p53 (Ser15) 1:100, phospho-Histone H2A.X (Ser139) 1:50 or p21 Waf1/Cip1 (12D1) 1:200, in 5 % BSA, 0.2 % Triton X-100 in PBS overnight at 4 °C. Cells probed with primary antibody were then rinsed twice

with 5 % BSA in PBS and incubated with secondary antibody conjugated to Flour Alexa 488 1:500 for 2 hrs at room temperature in the dark. After washing (2x) with 5 % BSA in PBS, cells were then analysed with LSRII flow cytometer (BD Biosciences); $1x10^4$ cells were acquired.

Cytokine measurements

Enzyme-Linked ImmunoSorbent Assay (ELISA) is the most widely used and best validated biochemical technique for cytokine measurements. This approach enables accurate and sensitive detection of the antigen, the cytokine of interest (Leng et al. 2008).
In short, cells were grown in 35 mm, six-well culture dishes, exposed to selected compounds and incubated for 24 hrs at 37°C. After exposure the medium was harvested and centrifuged at 250 x *g* to remove floating cells. The final supernatants were stored at – 70°C. Cytokine protein levels were determined by 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).

Gene expression analysis by real-time RT-PCR

To investigate the response of nitro- and amino-PAHs-metabolising genes, BEAS-2B cells were exposed to different nitro- and amino-PAHs, and gene expression analysis was conducted with real-time RT-PCR. In short, total RNA was isolated, reverse transcribed to cDNA and real-time PCR was performed.

Cells were exposed to selected compounds and incubated for 24 hrs at 37°C. For the analysis of CYP1A1, CYP1B1, AhR, ARNT, and AHRR gene expression, total RNA was isolated by use of the TRIzol reagent. 1 µg total RNA was reverse transcribed to cDNA by using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) on a PCR system 2400 (PerkinElmer). The cDNA was diluted to a final volume of 100 µl in nuclease-free water. Real-time PCR was performed according to the manufacturer's guidelines and carried out on an ABI PRISM 7900 Sequence Detection System using Power SYBR Green PCR MasterMix (Applied Biosystems). Gene expression levels were normalized to the expression

of β-actin (ACTB) and to the expression in DMSO-treated cells. Primers for CYP1A1, CYP1B1, AHR, and β-actin were as published previously (Berge et al. 2004, Mollerup et al. 2002, Mollerup et al. 2006). Primers for ARNT and AHRR were as described in Uppstad et al. (manuscript submitted for publication).

To evaluate if time of exposure influenced the CYP1A1 or CYP1B1-induction, we made measurements after 8, 12 and 24 hrs of exposure to 1-nitropyrene (1-NP) and Benzo[*a*]pyrene (B[*a*]P). Data is representative of three independent experiments.

DNA adduct analysis

DNA adduct formation induced by the test compounds was measured with the ^{32}P-postlabelling method, using both the nuclease P1 digestion and butanol extraction enrichment versions of the assay (Phillips & Arlt 2007). In short, cells were exposed to various test compounds for 15 hrs. After trypsination, DNA was isolated from the cells as described previously (Arlt, Schmeiser, & Pfeifer 2001). For analysis, DNA samples were digested, enriched and labelled as reported elsewhere. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) plates (Macherey-Nagel, Düren, Germany) were described earlier (Arlt et al. 2002b). After chromatography TLC sheets were scanned using a Packard Instant Imager (Dowers Grove, IL, USA) and DNA adduct levels (RAL, relative adduct labelling) were calculated from adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. An external B[*a*]P diol-epoxide-DNA standard was used for identification of B[*a*]P-DNA adducts (Phillips & Castegnaro 1999). 1-NP-DNA adducts were identified as reported (Landvik and others 2007b). 3-NBA-derived DNA adducts were identified using authentic standards as described previously (Arlt et al. 2006). Results are expressed as DNA adducts/10^8 nucleotides.

Single cell gel electrophoresis (comet assay)

The comet assay is a simple and effective technique for measuring DNA strand breaks in single eukaryotic cells (Nagy et al. 2005, Singh et al. 1988). DNA strand breaks were

detected by the comet assay, and the lesion-specific repair enzymes formamidopyrimidine DNA-glycoylase (FPG) and endonuclease III (Endo III) were employed to further detect oxidative DNA damage.

The comet assay was performed as described previously (Nagy and others 2005). In short, cells were exposed to various test substances for 15 hrs. After trypsination, cells were centrifuged, re-suspended in medium with 10% DMSO, frozen and stored at –80°C until analysis. Cells were thawed quickly in a 37°C water bath, washed and resuspended in PBS. A fraction of the cell suspension was embedded in 0.75% agarose (37°C) and the mixture was spread over a three-windowed microscope slide. The slides were immersed in lysis buffer, followed by the addition of either enzyme buffer with FPG or Endo III to each slide window. Incubations were performed in a humidity chamber (37°C). The activity of FPG was tested using H_2O_2-treated cells as a positive control.

DNA unwinding was preformed in alkaline buffer (0.3 M NaOH), on ice, in the dark. After electrophoresis, slides were neutralised, dried, and fixated in methanol. Slides were treated with ethidium bromide (stained nuclei), and DNA damage was scored using a Comet IV capture system (version 4.11; Perceptive Instruments, UK) counting 50 nuclei per gel window. The tail intensity (% tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was measured for each nucleus scored.

Western blotting immunoassay

Western blotting (or immunoblotting) is a method used for separation and identification of proteins (total or phosphorylated forms) under study. In western blotting, equal amounts of cellular proteins are separated by SDS-PAGE (sodium dodecyl sulphate polyachrylamide gel electrophoresis), and transferred electrophoretically from the gel to a nitrocellulose (used in our study) support membrane. 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. Furthermore, a secondary antibody, conjugated with HRP (horse raddish peroxidase) is added and binds to the primary antibody-antigen complex. Luminescent substrates are then used to visualize the bound components.

BEAS-2B cells were grown in 90 mm culture dishes ($5x10^5$ cells/dish) and exposed to

various test substances for 12 hrs and 24 hrs, as described earlier. Cells were frozen at -80°C 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, 1 mM Na_3VO_4, 1 mM NaF, 10 μg/mL leupeptin, 1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL pepstatin A. After cell lysis, solutions were sonicated, centrifuged (290 x *g*), and supernatants containing cellular proteins were collected. Protein concentration was measured using the Bio-Rad DC protein assay kit. Samples were adjusted to an equal protein concentration with lysis buffer. 5x SDS-PAGE sample buffer (0.312 M Tris-HCl, pH 6.8, 10% SDS, 25% β-mercaptoethanol, 0.05% bromophenol blue, 10% glycerol) was added. Samples were boiled for 5 min.

A sample of 12.5 μg protein in each well was subjected to 10% SDS-PAGE. Proteins were transferred by electroblotting to nitrocellulose membranes. Equal protein loading was controlled by Ponceau S staining. The blots were blocked with 3% fat-free dry milk, and incubated with primary antibodies (overnight at 4°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. Afterwards blots were incubated with horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies 1:5000 for 2 hrs at room temperature. Between each step, the cells were washed three times in cold 1xTBS (*Tris*-buffered saline). Western blots were developed using the ECL chemiluminescence system according to the manufacturer's instructions (Amsterdam Pharmacia, Little Chalfont, UK). Results are from one representative experiment out of three, or just one if the results are in accordance with immunocytochemistry and flow cytometric data.

Immunocytochemistry

Immunocytochemistry is a technique which, like Western blotting, depends on specific antibody-antigen interactions for detection. This technique detects antigen in the treated cells, thus makes it possible to visualize the localization of the protein in question.

The cells were exposed to various test substances for 24 hrs as described earlier and seeded into 50 mm culture dishes ($10x10^4$ cells/well). After washing in cold phosphate-buffered saline (PBS), adherent cells were fixed with cold absolute methanol for 5 min in room

temperature. The cells in each dish were divided in 4 different areas that could be treated separately, by a PAP Pen, 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 at room temperature, in a humidified atmosphere, in the dark, over night, with primary antibodies diluted in PBS with 1% BSA. One square in the dish was the control area without primary antibody, for the purpose of detecting whether secondary antibodies have nonspecific binding, or if any of the compounds have autofluorescence. Finally, the cells were incubated with secondary antibody Flour Alexa 647 1:500 1 h under the same conditions as with primary antibodies. Between each step, the cells were washed three times in cold PBS. The last step in the procedure included adding a drop of DAPI, which stains the nuclei, as a mounting medium, and sealing with a cover glass to prevent drying and movement under the microscope. Data is representative of three independent experiments.

Statistical analysis

Statistical significance was evaluated using analysis of variance (ANOVA) with the Bonferroni post-test (two-way ANOVA) or the Dunnett post-test (one-way ANOVA). This made it possible to obtain p-values to determine statistically significant differences between controls and treated sets. $P<0.05$ was considered a statistically significant difference. Values are presented as means ± SEM. All calculations were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cell death

Morphology changes

BEAS-2B cells were treated with different nitro- and amino-PAHs for 24 hrs, or simply with DMSO as a negative control. The morphology of the cultures was examined using light microscopy. Cytotoxicity was evaluated by judging the relative amount of floating (dead) cells in the cultures, compared to attached (living) cells. As can be seen in figure 2, the nitro compounds were more cytotoxic than the amino compounds. 3-NBA induced the highest degree of cytotoxicity, followed by 3-NF and 1-NP, while B[a]P and 2-NBA, on the other hand, had no marked effects. Interestingly, 2-NBA induced the formation of large cytoplasmic vacuoles, which could indicate the swelling of cellular organelles like mitochondria. Cells were more square, flat and transparent after exposure to 3-ABA. No particular changes were observed after tests with the other compounds.

Apoptosis vs. necrosis

To further characterise the effects of various PAHs on cell death, we stained the cells with Hoechst 33342 and PI, and examined cell morphology by using fluorescence microscopy. This allowed us to differentiate between apoptotic and necrotic cell death.

Pictures in figure 3 show cell death, typical apoptotic, necrotic, as well as living cells, induced by some of the compounds tested. Some PI-positive cells showed partial chromatin condensation, indicative of apoptotic cells with secondary plasma membrane damage (apoptotic-necrotic cells). These cells were categorized and counted as apoptotic cells.

After 24 hrs of exposure, 1-NP, 3-NBA and 3-ABA significantly increased apoptosis in BEAS-2B cells (Fig. 4B), with 3-NBA being the most potent. For 3-NBA, a significant

increase could be seen already at 1 μM (Fig. 5A). While 1-NP and 3-ABA increased the number of apoptotic cells from 1% control to 6% and 4%, respectively, 3-NBA increased it to 43%, after exposure to concentrations of 30 μM (Fig. 4B). Significant increase in necrosis was induced already at 3 μM 3-NBA (Fig. 5A). 2-NBA did not induce neither apoptosis nor necrosis (Fig. 4B), while the rest of the compounds also induced necrosis. At a concentration of 30 μM, 3-NF increased the amount of necrotic cells from approximately 6% control to 25%, while 30 μM 3-NBA, 3-ABA and 1-NP increased it from 6 % control to 20%, 18% and 18%, respectively. 3-AF, B[a]P and 1-AP resulted in somewhat less necrosis, approximately 15%, 12% and 12%, respectively.

Furthermore, we also measured the fraction of apoptotic cells by flow cytometry. Due to chromatin condensation and nuclear fragmentation during the process of apoptosis, the apoptotic cells and bodies arising from G_1 emit fluorescent signals that are lower than those of G_1 cells. Different cell phases, as well as apoptotic cells/bodies, can in this manner be distinguished on the basis of their DNA content (Hoechst fluorescence) and cell size (forward light scatter). Apoptotic index was determined as the percentage of signals between the G1 peak and the channel positioned at 20% of the G1 peak (sub-G1 population). The data in figure 4A shows that 3-NBA was the most potent compound, followed by 3-ABA, 1-NP and 3-NF. Cytotoxicity related to apoptosis induced by 3-NBA (Fig. 5B) begins at 1 μM, which reflects the results acquired by fluorescence microscopy analysis. Negligible effects on apoptosis were seen with 2-NBA and B[a]P. In general, the results from flow cytometry correlate well with the findings from microscopy examinations.

The same set of experiments was carried out after 72 hrs of exposure. The results strengthen the notions obtained after 24 hrs exposure, as seen in figure 6.

Cleavage of Caspase 3 and PARP

Caspase 3 is an effector caspase and a key player in the apoptotic process. Cleaved caspase 3 is considered to be an apoptosis marker, and activates DNA endonucleases resulting in DNA fragmentation. When it is activated, caspase 3 participates in the proteolytic cleavage of several other proteins, such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP). Cleaved PARP is also considered to be a marker of apoptosis (Bratton & Cohen 2001)

Western analysis revealed that 24 hrs of exposure to 3-NBA increased amounts of proteins with a molecular weight and antibody binding properties corresponding to cleaved caspase 3, whereas none of the other compounds tested influenced this. Moreover, western analysis suggested that 3-NBA induced cleavage of PARP. Flow cytometry analysis revealed that 3-NBA exposure resulted in a major right shift in fluorescence of the whole peak, after staining for caspase 3, suggesting a large increase in cleaved caspase 3 for a subpopulation of the cells. Visualizing with immunocytochemistry following 24 hrs of exposure to 3-NBA, after staining with caspase 3 and PARP antibodies, further elucidated the results obtained with western blotting and flow cytometry analysis, showing that increased amounts of cleaved caspase 3 and cleaved PARP had accumulated in apoptotic cells. No changes were observed after exposure to 2-NBA, 1-NP or B[a]P. The highest concentration of 3-NBA was the most potent, as seen in figure 7.

Cytoplasmic vacuolization and mitochondrial damage

To further characterize the 2-NBA-induced cytoplasmic vacuolization and 3-NBA-induced cell death, the possible involvement of mitochondrial function was assessed by Mito Tracker® labelling, which accumulates in functional mitochondria. Exposure to 2-NBA or 3-NBA for 24 hrs did not affect mitochondrial integrity (Fig. 8). Similarly, no effect of 1-NP and B[*a*]P was observed by Mito Tracker® labelling (data not shown).

Cytokine release

CXCL8, CCL5 and TNF-α

The cytokine responses were examined with the ELISA technique. Based on previous screening studies of PAH-induced gene expression (Ovrevik et al. manuscript submitted for publication, results not shown), the release of CXCL8, CCL5 and TNF-α proteins were measured after 24 hrs of exposure. Cytokines may contribute to tumor growth, as in the case of CXCL8, while others like CCL5, may have tumor suppressive effects (Moran and others 2002, Xie 2001). Tumor necrosis factor alpha (TNF-α) has been shown to be one of the

major mediators of inflammation. It is induced by a wide range of pathological stimuli (Aggarwal et al. 2006), and is reported to be involved in the proliferation, survival, and apoptosis of cells (Gaur & Aggarwal 2003).

Notably, all of the test compounds, except 2-NBA and B[*a*]P, significantly increased the release of CXCL8 (Fig. 9A). The highest levels were induced by 1-NP and 3-NF, with 1-NP being the most potent. Only 1-AP and 3-AF induced increased release of CCL5 (Fig. 9B), and 2-NBA had no effects on CXCL8 or CCL5 release. Interestingly, 3-NBA also appeared to induce a somewhat higher level of CXCL8 release compared to the amino-PAHs. No effects were observed on release of TNF-α after 24 hrs of exposure (data not shown). However, further studies may be needed to verify whether TNF-α may be induced at earlier time-points.

NF-κB

Nuclear factor-kappa B (NF-κB), a family of multifunctional ubiquitous transcription factors, play an important role in the regulation of cellular stress responses, inflammatory reactions, the cell cycle and apoptosis (de, Schmid, & Hofer-Warbinek 1999). In the cytoplasm, NF-κB is kept in an inactive state by binding to IκB (inhibitor of κB). Degradation of IκB through the ubiquitin system allows transcriptional activity, where IκB releases and activates NF-κB, which then can translocate to the nucleus. Thus, degradation of IκB is an indicator of NF-κB-activation. Preliminary western analysis of cell lysates following 24 hrs of exposure to 3-NBA revealed a down-regulation in the amount of proteins corresponding to IκB (Fig. 10), thereby suggesting induced NF-κB-activity. In contrast, 2-NBA, 1-NP and B[*a*]P gave no marked effects on IκB. These experiments, however, need further verification.

Aryl hydrocarbon receptor and CYP-enzymes

AhR, and its DNA binding partner, the AhR nuclear translocator (ARNT), are central in mediating PAH induced gene expression and cytotoxicity. Therefore, we investigated whether the observed nitro- and amino-PAH-induced effects in the BEAS-2B cells could be related to this signalling pathway. We analysed the effects on AhR and ARNT gene

expression, as well as AhR/ARNT-regulated genes such as AhR repressor (AhRR), CYP1A1 and CYP1B1. None of the tested compounds induced increased expression of AhR or ARNT after 10 hrs of exposure (Fig. 11A), and only B[*a*]P induced elevated expression of AhRR, CYP1A1 and CYP1B1 (Fig. 11B). In fact, 1-NP, 1-AP, 3-NF and 3-NBA appeared to reduce the expression levels of AhRR, CYP1A1 and CYP1B1, but this reduction pattern bears no resemblance to the effects on cell death or cell cycle, or to the cytokine response patterns. Thus, neither cell death, nor cell cycle alterations or cytokine expression seem to be directly related to the AhR/ARNT pathway.

To further characterize the effects of nitro-PAHs the cells were exposed to 1-NP for 8 and 12 hrs, and analyzed by real-time RT-PCR. B[*a*]P was used as a positive control after 24 hrs. The results indicated a statistically significant down-regulation of CYP1A1, already after 8 hrs of exposure, and CYP1B1 after 12 hrs of exposure (Fig. 12).

Cell cycle alterations

PAHs may exert effects on the cell cycle directly via activation of receptors including AhR, and indirectly through the formation of reactive metabolites. In order to investigate whether nitro-PAH-induced cytotoxicity, DNA damage or cytokine responses correlated with effects on cell cycle, the proportions of BEAS-2B cells in G1, S and G2/M phase after 24 hrs of exposure were measured by flow cytometry. Most notably, both B[*a*]P and 3-NBA induced a marked decrease in cells in G1, and a larger increase in cells in S-phase, compared to the other compounds (Fig. 13). B[*a*]P only affected the cell cycle at its highest concentration (30 μM), but 3-NBA induced a significant S-phase accumulation even at low concentrations. At a concentration of 1 μM it appears to cause a significant S-phase arrest, which is the same concentration that induced apoptotic cell death, and 15 μM resulted in 45 % of cells accumulated in S-phase (Fig. 14). Higher concentrations of 3-NBA caused no further increase in cell cycle arrest. At a concentration of 30 μM, B[*a*]P arrested approximately 55% of the cells in S-phase, which indicated a huge cell cycle arrest. 3-ABA also reduced the amount of cells in G1, but with only a slight, non-significant effect on S-phase. Instead, 3-ABA significantly increased the number of cells in G2/M (35%). 3-AF appeared to induce a slight, but not significant, increase in S-phase. In comparison, 1-AP and 2-NBA had no apparent effect on BEAS-2B cell cycle distribution.

Furthermore, using flow cytometry analysis revealed some variations in cell cycle arrest, at unchanging concentrations, from 24 to 72 hrs of exposure (Fig. 15). After 72 hrs, no change had occurred for 3-NBA compared to after 24 hrs, but 2-NBA induced a marked S-phase accumulation after prolonged exposure. A delayed cell cycle alteration could indicate that 2-NBA is slowly metabolized, and could for that reason cause DNA damage after expanded exposure time.

DNA damage

DNA adduct formation

The accumulation in S-phase induced by 3-NBA and B[*a*]P suggests induction of DNA damage. To examine for any possible DNA damage, DNA adducts were detected by ^{32}P-postlabelling using both the butanol and nuclease P1 enrichment versions of the assay (Phillips & Arlt 2007). As seen from figure 16, adducts were formed by all test compounds, except for 3-AF and 2-NBA, or DMSO alone (control). The largest amount of DNA binding was observed with 3-NBA, at up to 876.4 ± 258.8 adducts per 10^8 nucleotides. In comparison, the remaining compounds formed only low levels of adducts, ranging between 10–40 adducts per 10^8 nucleotides. Adduct patterns observed after butanol extraction (Fig. 16, *upper panel*) was qualitatively similar to that found after nuclease P1 digestion (Fig. 16, *lower panel*), except for 3-NBA where the pattern was dependent on the enrichment procedure (Arlt and others 2002b, Arlt and others 2006).

DNA strand breaks and oxidative damage

DNA damage was further elucidated by the comet assay, which mainly detects single-stranded DNA breaks and alkali labelled sites. To assess the oxidative DNA base modifications we used the comet assay with lesion-specific endonucleases, FPG and Endo III, which recognise certain base damages and convert them to strand breaks (Dusinska & Collins 2008). BEAS-2B cells were treated with 15 μM of the test compounds for 15 hrs (Fig. 17). Without FPG or Endo III enzyme-treatment, a significant increase in DNA damage

relative to controls was observed for all chemicals, except 1-AP, 3-AF and 2-NBA. 3-NBA showed the highest capacity to induce comet formation (38.4 ± 1.7 *versus* 10.6 ± 4.6 % tail DNA). Following FPG treatment, only B[*a*]P induced a statistically significant increase in DNA damage after correction for effects in absence of enzyme treatment ($Tail_{[FPG]}$-$Tail_{[No\ enzyme]}$). No significant effects were observed following treatment with Endo III.

Notably, there was no clear correlation between DNA adduct formation, induced DNA strand breaks or formation of oxidative DNA damage, and the cytokine responses; the results rather support a negative role of the induced DNA damage on inflammatory responses. However, a strong link was observed between 3-NBA induced cytotoxicity, cell cycle arrest, DNA adduct formation, induced DNA strand breaks and formation of oxidative DNA damage. The mechanisms behind the 3-NBA-induced apoptosis and S-phase accumulation constitute the main subject of the continuing investigations in this thesis.

DNA damage response

To examine the involvement of DNA damage signaling pathways triggered by 3-NBA, we explored the possibly increased phosphorylation of ATR, ATM, chk1, chk2, H2AX and p53. 3-NBA effects were compared to the isomer 2-NBA, and to the well characterized 1-NP and B[*a*]P. Phosphorylation and total level of proteins were measured by flow cytometry, western blotting analysis and immunocytochemistry using specific antibody coupled with secondary antibody (Flour Alexa 488-A, horseradish peroxidise-conjugated goat-anti-rabbit/rabbit-anti-mouse IgG and Flour Alexa 647-A respectively).

ATM/ATR are phosphatidylinositol-3 kinases acting upstream of p53 as sensors/early effectors of DNA damage (Efeyan & Serrano 2007). Flow cytometry analysis showed that only 3-NBA exposure resulted in a slight right shift in fluorescence of the whole peak, after staining for p-ATM, suggesting some increased ATM phosphorylation in most cells. Immunocytochemistry following 24 hrs of exposure to 3-NBA, after staining for ATM, confirmed the results obtained with flow cytometry analysis, and showed that nuclei with DNA condensation resulted in an increase in fluorescence due to the accumulation of ATM. Some activation of ATR was indicated with immunocytochemistry analysis after exposure to 10 μM of 3-NBA, ATR appeared to accumulate in apoptotic cells (Fig. 18).

H2AX phosphorylation plays a major role in nuclear events during meiosis and DNA double-strand breaks (DSBs). DSBs cause rapid phosphorylation of histone H2AX on large areas of chromatin flanking the breaks, thus making H2AX a reliable marker for DSBs (Marti et al. 2006, Ward & Chen 2004). In the present study, H2AX was detected only after 3-NBA exposure. Western blotting analyzed the amount of phosphorylated protein with a molecular weight and antibody binding properties corresponding to γH2AX (i.e. phosphorylated H2AX), and suggested a large induction of γH2AX. Flow cytometry analysis showed a slight right shift in fluorescence of the whole peak, after staining for γH2AX, suggesting some increase in activated H2AX, for most cells. Immunocytochemistry confirmed the results obtained with western blotting and flow cytometry analysis, showing increased amounts of phosphorylated H2AX, γH2AX, in apoptotic cells (Fig 19).

Checkpoints Chk1/Chk2 are effector kinases activated by ATR/ATM, respectively. Chk1 is primarily induced by ATR in response to single strand breaks, while Chk2 is phosporylated by ATM after double strand breaks. Analysis of the DNA damage response showed that 3-NBA induced an increase in phosphorylation of Chk2 at threonine 68, while 2-NBA, 1-NP and B[*a*]P resulted in no marked Chk2 phosphorylation (Fig. 21). This phosphorylation, analyzed with the same western blotting procedure as elucidated above, is most probably a result of ATM-activation caused by the DNA damage formed. Meanwhile, no marked increase in the amount of Chk1 protein was observed (Fig.20). Judged by the large right shift in fluorescence of the whole peak, after probing with Chk2 antibody, seen with flow cytometry analysis, 3-NBA caused a strong activation of Chk2 in all cells. In contrast, Chk1 seemed to be less activated in BEAS-2B cells considering flow cytometry results. Finally, immunocytochemistry following 24 hrs of exposure to 3-NBA, after probing for Chk2, confirmed earlier findings achieved with western blotting and flow analysis, revealing a large increase in fluorescence from apoptotic cells, while only a slight accumulation of nuclei with condensated DNA was observed after staining with Chk1 antibody. All three methods correlated well, which supports further each finding.

p53 is considered to be a key regulator of signaling pathways involved in cell death, and plays a pivotal role in apoptosis. Following DNA damage, p53 is phosphorylated by enzymes including Chk1 and Chk2, and thereafter translocates to the nucleus where it may result in cell cycle arrest, allowing time for DNA repair, or trigger apoptosis (Efeyan & Serrano 2007). In order to examine p53's role, cells were exposed to different test compounds,

including 3-NBA, 2-NBA, 1-NP and B[*a*]P. Western blotting analysis of cell lysates following 24 hrs of exposure revealed that 3-NBA resulted in a prominent p53 phosphorylation (p-p53) at serine 15, while no changes in levels of p-p53 after 2-NBA, 1 – NP and B[*a*]P exposure were observed (Fig. 22A). Already at the lowest concentration of 3-NBA tested, an increase in p53 phosphorylation could be measured, and the amount of phosphorylation increased with higher concentrations. The same trend was seen at 12 hrs of exposure (data not shown). Flow cytometry analysis provided the same results obtained with western blotting, showing that 3-NBA exposure resulted in a large right shift in fluorescence of the whole peak, due to an increase in phosphorylated p53, for a subpopulation of the cells (Fig. 22B). This was further supported by translocation of p53 to the nucleus following 3-NBA exposure, observed with immunocytochemistry (Fig 22C). Western blotting further revealed that the total amount of p53 increased after exposure to 3-NBA and B[*a*]P. This marked activation of the p53-pathway is suggested to trigger the apoptotic process.

p21 is a cell cycle regulator, and is known to be a downstream target of p53, which inhibits various cyclin-dependent kinases and initiates a cell cycle arrest at the G_1 to S phase transition, as well as between the G_2 phase and mitosis (Luch 2002). In the present study we found a down-regulation in levels of p21 protein after 3-NBA exposure, measured by western blotting, flow cytometry and immunocytochemistry analysis (Fig. 23). Western blotting analysis revealed a decrease in the amount of p21, while flow cytometry analysis showed a left shift in fluorescence of the whole peak, which could indicate a decrease in p21 in the cells. No accumulation of p21 was observed with immunocytochemistry, which strengthens the results suggesting that 3-NBA does not activate p21.

Inhibitor of cell death; Pifithrin-α

Pifithrin-alpha (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, and is therefore thought to be a specific inhibitor of signaling by the tumor suppressor protein p53 (Komarova et al. 2003). Fluorescence microscopy analysis following Hoechst 33342 and PI staining revealed that PFT-α drastically inhibited 3-NBA induced cell death (Fig. 24), suggesting a role of p53 in the apoptotic signaling pathway.

DISCUSSION

Several studies have investigated the genotoxic properties of nitro-PAHs, however, little is known concerning their effects on cell signaling pathways involved in cell death, proliferation and inflammation. In the present study, we have shown that various nitro- and amino-PAHs induce quantitatively and qualitatively different effects on cell death, cytokine release, cell cycle, DNA damage, as well as DNA damage signaling pathways. The most striking findings were that 3-NBA exposure resulted in extensive DNA adducts formation, DNA strand breaks, accumulation of cells in S-phase, and a massive apoptotic response. The apoptotic response was most probably caused by DNA damage, resulting in activation of ATM, Chk2 and p53. In comparison, only minor toxic effects were observed following exposure to the isomer 2-NBA. With respect to cytokine release, the nitro-PAHs, and in particular 1-NP and 3-NF, predominantly induced CXCL8, while their corresponding amine forms, 1-AP and 3-AF, induced CCL5. Interestingly, 3-NBA induced NF-κB activation, but gave considerably less CXCL8 release than 1-NP and 3-NF.

Several studies have revealed the induction of apoptosis and necrosis after exposure to different PAHs (Holme and others 2007, Landvik and others 2007, Solhaug and others 2004). In this study, nitro- and amino-PAHs were capable of causing different types of cell death to various extents in BEAS-2B cells. 3-NBA, 1-NP, 3-ABA and 3-NF induced apoptosis and necrosis, judged by fluorescence microscopy and flow cytometry analysis, whereas 2-NBA induced neither apoptosis nor necrosis in any of the concentrations tested. With regard to 3-NBA, the most potent cytotoxic compound, a significant increase in apoptosis was detected by both fluorescence microscopy and flow cytometry, already at concentrations as low as 1 μM.

In the present study, we wanted to investigate signals connecting the DNA damage with the activation of p53. p53 may trigger apoptosis by increasing transcription of Bax, or by changing its localization from the cytosol to the mitochondria (Holme and others 2007, Moll et al. 2005, Solhaug and others 2004), resulting in release of cytochrome c and further activation of caspases. Caspases are often found to be essential for the activation of apoptotic pathways. Caspase 3 is an effector caspase and its activation cleaves a number of structural and regulatory proteins, including PARP, which is mainly involved in DNA repair and

apoptosis. The activation of caspases is responsible for the characteristic morphology of apoptotic cells, including nuclear condensation and fragmentation (Earnshaw, Martins, & Kaufmann 1999, Roos & Kaina 2006). In this study we observed that exposure to 3-NBA resulted in cleavage of both caspase 3 and PARP. This is in agreement with the induced apoptosis observed by fluorescence microscopy. The necrosis observed alongside the apoptosis after exposure to 3-NF, 3-NBA, 3-ABA and 1-NP, is commonly seen following exposure to various toxic chemicals. This might be due to an incomplete apoptotic process, and a switch from induced apoptosis to necrosis, probably due to inhibition of mitochondrial respiration or inactivation of caspases (Nicotera, Leist, & Ferrando-May 1999, Solhaug and others 2004).

Additionally, the compounds seemed to affect cell morphology somewhat differently. The 2-NBA-induced vacuolization could possibly be due to compromised intracellular organelles. Staining with Mito Tracker®, which accumulates in functional mitochondria, however, indicated that there was no major injury to the mitochondria after PAHs treatment. Thus, further investigations are needed to explore this finding.

Cytokines are key regulators of inflammation, with both pro- and anti-inflammatory functions, and can also modulate tumor growth and progression (Hussain & Harris 2007). When acute inflammation manifests for a short period of time, it may have therapeutic benefits with regard to limiting bacterial/viral infections. However, when inflammation becomes chronic, it can often prove harmful and may lead to serious diseases, including cancer (Aggarwal and others 2006). The various test compounds may react with the cells in different ways, explaining the induction of different cytokines. 1-NP-/3-NF induced the most CXCL8, which is a cytokine that may contribute to human cancer progression (Aggarwal and others 2006, Oppenheim and others 1997, Xie 2001). In comparison, CCL5, which was mostly induced by 1-AP/3-AF, may have tumor-suppressive effects (Moran and others 2002). In the present study, we observed no effects on TNF-α, which is an important pro-inflammatory cytokine that regulates expression of other cytokines (Barksby et al. 2007, Grivennikov et al. 2006). However, further studies may be needed to verify whether TNF-α may be induced at earlier time-points. The cytokine release induced by some nitro-PAHs, such as 1-NP, may contribute to their carcinogenic potency, as these compounds have been found to be both mutagenic and carcinogenic (Rosenkranz & Mermelstein 1983, Salmeen and others 1982, Tokiwa & Ohnishi 1986).

In the present study, 3-NBA and B[*a*]P were by far the most potent compounds with regard to causing significant accumulation of cells in S-phase. Moreover, a study using human lung epithelial A549 cells (Hansen, Seidel, & Borlak 2007b), and a recent study using Hepa1c1c7 cells (Landvik and others 2009), both found that exposure to 3-NBA leads to an accumulation in S-phase. The accumulation was probably due to the large amount of DNA adducts and SSBs, which in turn probably resulted in delayed DNA replication. The higher amount of DNA damage formed after exposure to 3-NBA compared to its amine metabolite suggest that nitro reduction is more efficient than *N*-oxidation in the Hepa1c1c7 and BEAS-2B cells. After 72 hrs of 2-NBA exposure, an accumulation of cells in S-phase was detected. This indicates that also 2-NBA may induce DNA damage, but its response is lower and delayed, suggesting a slower metabolic rate when compared to 3-NBA. However, this assumption needs further investigation (detection of DNA adduct formation and oxidative DNA damage after 72 hrs of exposure) to be substantiated.

p21 is a cell cycle inhibitor, that can result in a G1-arrest (Gartel & Radhakrishnan 2005). A deregulation of cell checkpoints and a change in the balance between cell survival and cell death, may have implications for mutation frequency and the development of cancer. Some PAHs have been shown to damage DNA, but result in less G1 arrest than expected (Khan et al. 1998, Luch 2002). This has been found to be due to a lack of p53-induced p21 response (Gartel 2005, Gartel & Radhakrishnan 2005). Accordingly, we found that 3-NBA-induced p53 did not increase levels of p21, possibly explaining the apparent lack of G1 arrest and rather an accumulation of cells in S-phase.

In accordance with previous findings, in A549 lung cells (Nagy et al. 2006), and in Hepa1c1c7 cells (Landvik and others 2009), we found that 3-NBA induced SSBs in DNA as well as DNA adduct formation in BEAS-2B cells. Regarding DNA adduct formation, 3-NBA gave a massive increase compared to the other compounds. Reduction reactions of nitro-PAHs will generate ROS, and several studies done with different cell lines have documented formation of oxygen radicals after 1-NP exposure (Cherng et al. 2006, Kim et al. 2005, Park & Park 2009b). In our study, 1-NP and 3-NBA induced low levels of oxidative DNA-damage, whereas B[*a*]P induced oxidative damage to a higher degree. Since B[*a*]P induced relatively low cytokine production, this supports the notion that oxidative stress often results in cytotoxicity, rather than stimulating cytokine responses in BEAS-2B cells (Oslund et al. 2004, Ovrevik et al. 2008). It is noteworthy that a variance in type of radical species, or the

cellular site for the ROS formation, could play a central role for the final outcome of oxidative stress.

PAHs may induce apoptosis as a result of DNA damage, and/or cytosolic damage (Asare and others 2009b, Holme and others 2007, Huc et al. 2007, Solhaug and others 2004, Solhaug and others 2005, Solhaug, Refsnes, & Holme 2004). With regard to 3-NBA-induced apoptosis, which is main focus of this study, we wanted to investigate more in depth the mechanisms and pathways behind the induction of this type of cell death. DNA damage initiates cell signaling pathways, which may result in the activation of cell cycle checkpoints and the delay of cell cycle progression, which provides time to coordinate DNA repair, or the induction of senescence or cell death. Extensive characterization of various signaling routes that are involved in DNA damage detection, and connect DNA damage to p53, has identified a cascade consisting of serine/threonine kinases, including ATM and ATR. ATM and ATR become activated and sequentially activate the checkpoint kinases Chk2 and Chk1 respectively; both may result in the activation of p53. This DNA damage detection system is crucial for sustaining the genome stability in cells (Efeyan & Serrano 2007, Ljungman 2005, Zgheib and others 2005). In this study, we observed a marked increase in the activation of ATM, Chk2, H2AX and p53, in 3-NBA-exposed cells. In contrast, 2-NBA treatment did not induce any activation of proteins involved in DNA damage response pathways. Notably, a stronger activation of Chk2, judged by increased phosporylated protein and translocation of the protein to the nucleus, was observed. While Chk1 gave no increase in phosphorylation, it gave some nucleus localization. Regarding Chk1 activation, the data obtained in this study is not reliable, and further investigation is necessary to clarify any possible role of Chk1 in 3-NBA-induced apoptosis. Considering the results obtained after 1-NP and B[*a*]P treatment, no clear evidence of activation of the different proteins investigated was obtained, due to large variances between the independent experiments.

ATM signaling, which was prominent in 3-NBA-exposed cells, is often found to be triggered following DSBs in DNA. However, this increase in ATM levels may also be caused by larger chromatin changes, caused by DNA adducts (Tanaka and others 2007). There was a dramatic increase in levels of γH2AX, probably due to secondary DSBs formation, as a consequence of stalled replication/repair, as well as ATM activation. There is also the possibility that γH2AX formation is an early chromatin modification, following the initiation of DNA fragmentation during apoptosis (Rogakou et al. 2000), or that the γH2AX is present in

senescent cells and suggested to mark non-repairable DNA lesions (Sedelnikova et al. 2004). The DNA damaging signals after 3-NBA treatment via Chk2, could be triggered by a combination of DNA adducts and increased levels of SSBs, resulting in stalled DNA repair and DNA replication forks, possibly activating ATR. Immunocytochemistry revealed some nucleus localization after probing with ATR, but several analyses are needed to confirm ATR activation. The activation of Chk2 kinase could most probably explain the increased phosphorylation of p53. In accordance, only 3-NBA led to the stabilization and accumulation of p53 in the nucleus, which previously has been suggested to be important for triggering apoptosis (Landvik and others 2007, Solhaug and others 2004, Vousden & Lu 2002). Furthermore, inhibition of p53 with pifithrin-α (PFT-α), reduced 3-NBA-induced cell death. This corroborated that p53 is involved in the 3-NBA-induced apoptosis, and that this is triggered by the DNA damage response via ATM/Chk2 pathway, as suggested by this study.

To further characterize the mechanisms behind cell death, cytokine release and cell cycle alterations, we wanted to investigate the importance of the AhR/ARNT pathway. AhR is an intracellular receptor and transcription factor, which plays a pivotal role in mediating the toxicity of a variety of PAHs, as well as other hydrocarbons (Denison & Nagy 2003, Safe 2001). The most well-studied AhR responsive genes are CYP1A1 and CYP1B1, which are enzymes involved in the metabolic activation of several AhR ligands, including PAHs like B[*a*]P. CYP-enzymes metabolize PAHs into highly reactive electrophilic metabolites (Casarett 2001). In the present study, we found that only B[*a*]P, which failed to induce cytokine responses, induced increased expression of the AhR/ARNT-regulated genes, namely AhRR, CYP1A1 and CYP1B1. In corroboration with our findings, Iwanari and colleagues (2002) have shown that 1-NP, 1-AP and 3-NF, in general, are considerably weaker inducers of CYP enzymes, compared to B[*a*]P, in a variety of different carcinoma cell lines. Thus, it appears that neither 1-NP/3-NF nor 1-AP/3-AF induced their characteristic cytokine profiles through activation of the classical AhR/ARNT-pathway. However, this does not exclude a role of AhR in these responses, as the AhR may activate different sets of genes depending on the activating ligand (Quintana et al. 2008). It is interesting to note that increasing evidence suggests that AhR also plays an important role in the regulation of several cytokine genes (Haarmann-Stemmann, Bothe, & Abel 2009b). Recent studies have shown that AhR after ligation may bind directly to the transcription factor RelB, a NF-κB family member. This AhR/RelB-complex appears to be involved in regulating the

transcription of several cytokine genes, including CXCL8 (Vogel & Matsumura 2009). This hypothesis is currently the subject of further investigation within our research group.

DNA damage, cytokine induction, and cytotoxicity, may all be triggered through common mechanisms, such as the formation of reactive metabolites and ROS (Boland et al. 2000, Nanavaty et al. 2002). On the other hand, the toxic effects of reactive metabolites may also cause cell death. It is possible that the massive apoptotic response induced by 3-NBA may have attenuated cytokine responses, explaining why this nitro-PAH induced only low levels of CXCL8 release, compared to 1-NP and 3-NF. However, DNA damage caused by 3-NBA also activated NF-κB, a key transcription factor involved in the regulation of a variety of cytokine genes after stress stimuli, including CXCL8 and CCL5 (Henriquet and others 2007). NF-κB is constitutively expressed in all cells and is inhibited by IκB in unstressed cells. During activation IκB is released and subsequently degraded. The free NF-κB dimer then translocates to the nucleus, and activates transcriptions of target genes. We observed reduced IκB protein levels in BEAS-2B cells exposed to 3-NBA, which suggests the activation of NF-κB. Surprisingly, 3-NBA was the only one that induced IκB-degradation, but still resulted in considerably less cytokine release than 1-NP and 3-NF. However, genotoxic stress has recently been reported to counteract NF-κB effects on cytokine production, through the activation of p53. NF-κB appears to activate another set of genes upon DNA damage than after traditional NF-kB activation (Janssens & Tschopp 2006). This probably explains why 3-NBA induced much lower cytokine levels than the other nitro-PAHs. Notably, diesel exhaust particles (DEPs) may have attached various PAHs with different properties. Some PAHs stimulate cytokine production, whereas others rather induce DNA damage. When found together, the PAHs inducing DNA damage may suppress the effect of cytokine producing PAHs, thereby giving DEPs a lower inflammatory potential than expected from of the individual components. This suggests that the combined effects of co-existing PAHs cannot be predicted by simple linear addition, and that further evaluation is needed.

In the present study, we observed that different PAHs induced various cellular effects. The qualitative effects appears to be related to whether a nitro- or amino-group is attached to the PAHs, while some quantitative effects appear to be determined by the ring structure of the compound. One explanation could be that different enzymes are involved in the metabolism

of nitro-compounds, than those metabolizing amino-compounds. Furthermore, the ring structures possibly affect the affinity of the compounds to various metabolic enzymes. Comparing 2-NBA to 3-NBA indicates that the orientation of the nitro substituent with respect to the aromatic ring can predict the compound's pattern of metabolism (Fu and others 1994), which influences its level of cytotoxicity. Exposure to 3-NBA caused a massive apoptotic response compared to the other nitro- and amino-PAHs, and B[*a*]P. For that reason, 3-NBA-induced apoptosis became the main subject of this thesis. No such dramatic differences were observed with regard to necrosis, however, the nitro-PAHs induced a slightly stronger necrotic response than the corresponding amines ($P<0.05$). Considering all this, it is interesting to note that a rather small change in the molecular structure of PAHs, dramatically alters their toxic potential.

In conclusion, the present study showed that the different PAHs had very different toxic potentials (cell death and cytokines), and that small changes in the molecular structures, such as a change of the nitro-group from position 3 to 2 or a substitution of a nitro- with an amino-group, had dramatic effects on the cellular response. The most striking observation was 3-NBA's high potency for causing DNA damage, followed by the triggering of DDR signals, characterized by the activation of ATM/Chk2/p53, resulting in cell death as the final outcome.

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FIGURE LEGENDS

Figure 1. Chemical structures of the test compounds.

Figure 2. Morphology changes determined by light microscopy. BEAS-2B cells were exposed to 15 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA or 2-NBA, for 24 hrs. Original magnification; 100X and 200X.

Figure 3. Effects of PAHs on cell morphology, determined by fluorescence microscopy. BEAS-2B cells were treated with B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, 2-NBA, or DMSO (control), for 24 hrs. The concentration used was 30 μM. The figure shows only B[*a*]P, 1-NP, 3-NBA, 2-NBA, and DMSO. The cells are stained with Hoechst 33342 and propidium iodide (PI). The nucleus of Hoechst stained DNA appears fluorescent blue. Hoechst penetrates all plasma membranes. PI is red fluorescent dye, which only stains cells that have disrupted membranes. V: viable cells. Nec: necrotic cells. Ap: apoptotic cells. Ap-nec: apoptotic-necrotic cells. Original magnification; 400X.

Figure 4. Cell death determined by flow cytometry and fluorescence microscopy.

(A) BEAS-2B cells were exposed to 15 or 30 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, or 2-NBA, for 24 hrs. Control cells were treated with DMSO only. Cells were exposed and trypsinated, stained with Hoechst 33258, and analyzed for apoptosis using flow cytometry. (B) BEAS-2B cells were trypsinated and stained with PI/Hoechst 33342, and subsequently analysed for apoptosis and necrosis using fluorescence microscopy. The data presents mean ± SEM of independent experiments (n = 3−6). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 5. 3-NBA-induced cell death, determined by fluorescence microscopy and flow cytometry. (A) BEAS-2B cells were exposed to various low concentrations of 3-NBA or DMSO, for 24 hrs, trypsinated and stained with PI/Hoechst 33342, and subsequently analysed for apoptosis and necrosis with fluorescence microscopy. The data presents mean ± SEM of independent experiments (n = 3-5). * Significantly different from DMSO-treated controls ($p < 0.05$). (B) BEAS-2B cells were exposed and trypsinated, stained with Hoechst 33258, and analyzed for apoptosis using flow cytometry. The data presents mean ± SEM of independent experiments (n = 6-8). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 6. Comparisons of 3-NBA- and 2-NBA-induced cell death, determined by fluorescence microscopy and flow cytometry. (A) BEAS-2B cells were exposed to 0.3, 1 or 3 μM of 3-NBA and 3, 10 or 15 μM of 2-NBA, or DMSO, for 72 hrs, trypsinated and stained with PI/Hoechst 33342, and subsequently analysed for apoptosis and necrosis with fluorescence microscopy. (B) BEAS-2B cells were exposed and trypsinated, stained with Hoechst 33258 and analyzed for apoptosis with flow cytometry. The data presents mean ± SEM of independent experiments (n = 2-3). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 7. Effects of PAHs on cleaved caspase 3 and PARP, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of cleaved PARP and cleaved caspase 3 were analyzed by Western blotting, after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of two separate experimental set-ups. (B) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of caspase 3 was measured by flow cytometry using specific antibodies directly against the cleaved form of the protein, after 24 hrs of exposure. Data is shown for one representative experiment of two separate incubations. (C) Cleaved caspase 3, sub-cellular localization, analyzed by

immunofluorescence, after 24 hrs of exposure to 10 μM of 3-NBA. The picture shows representative cells from one experiment. Original magnification; 1000X.

Figure 8. Mitochondrial changes induced by 3-NBA and 2-NBA, measured by fluorescence microscopy. Mito Tracker® red staining of mitochondria, and DAPI staining of nuclei, analyzed after 24 hrs of exposure to 10 μM of 3-NBA, 2-NBA, or DMSO, in BEAS-2B cells. Pictures show one representative experiments out of two. Original magnification; 400X.

Figure 9. CXCL8 and CCL5, determined by ELISA. BEAS-2B cells were exposed to 15 or 30 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, 2-NBA, or DMSO for 24 hrs, and the media were subsequently analysed. The data presents mean ± SEM of independent experiments (n = 4). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 10. PAHs effects on NF-κB, determined by Western blotting. BEAS-2B cells were exposed to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO, for 24 hrs and analyzed for IκB expression. The picture is representative for one experiment with lysat.

Figure 11. AhR, ARNT, AhRR, CYP1A1 and CYP1B1 gene expression, measured by real-time RT-PCR. BEAS-2B cells were exposed to 15 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, or 2-NBA, for 10 hrs. Data are presented as relative change (fold) compared to control cells (DMSO). Values represents mean ± SEM of independent experiments (n = 3).

Figure 12. CYP1A1 and CYP1B1 gene expression, measured by real-time RT-PCR. BEAS-2B cells were exposed to 15 μM of 1-NP for 8 and 12 hrs, and to 15 μM of B[*a*]P for

24 hrs. Data is presented as relative change (fold) compared to control cells (DMSO). Values represents mean ± SEM of independent experiments (n = 3).

Figure 13. Cell cycle distributions measured by flow cytometry. BEAS-2B cells were exposed to 15 or 30 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, 2-NBA, or DMSO, for 24 hrs, trypsinated and stained with Hoechst 33258. Data is presented as the relative proportion of cells (%) in the different cell cycle phases. Each bar represents mean ± SEM of independent experiments (n = 3–5).

Figure 14. 3-NBA-induced cell cycle distributions, measured by flow cytometry. BEAS-2B cells were exposed to various low concentrations of 3-NBA, or DMSO, for 24 hrs, trypsinated and stained with Hoechst 33258. Data is presented as the relative proportion of cells (%) in the different cell cycle phases. Each bar represents mean ± SEM of independent experiments (n = 3-5).

Figure 15. 3-NBA- and 2-NBA-induced cell cycle distributions, measured by flow cytometry. BEAS-2B cells were exposed to 0.3, 1 or 3 μM of 3-NBA and 3, 10 or 15 μM of 2-NBA, or DMSO, for 72 hrs, trypsinated and stained with Hoechst 33258. Data is presented as the relative proportion of cells (%) in the different cell cycle phases. Each bar represents mean ± SEM of independent experiments (n = 2-3).

Figure 16. DNA adduct formation determined by ^{32}P-postlabelling. BEAS-2B cells were exposed to 15 μM of B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, 2-NBA, or DMSO, for 15 hrs. (A) Autoradiographic profiles of DNA adducts by using the nuclease P1 digestion (*lower panel*) or butanol extraction (*upper panel*) enrichment versions of the ^{32}P-postlabelling assay. (B) Total DNA adduct levels (RAL, relative adduct labelling). Values represent mean ± SEM of separate incubations (n = 3); each DNA sample was determined by two independent analyses. 2-NBA was none-detected.

Figure 17. DNA damage measured by comet assay. BEAS-2B cells were exposed to 15 μM B[*a*]P, 1-NP, 1-AP, 3-NF, 3-AF, 3-NBA, 3-ABA, 2-NBA, or DMSO, for 15 hrs. DNA strand breaks were measured without enzyme treatment, and oxidative damage to DNA was measured after treatment with FPG or Endo III enzymes. Values (% tail DNA) represent mean ± SEM of independent experiments (n = 3). * Significantly different from DMSO-treated controls ($p < 0.05$).

Figure 18. Effects of PAHs on ATM and ATR, determined by flow cytometry and immunocytochemistry. In BEAS-2B cells: (A-B) ATM and ATR, sub-cellular localization, were analyzed by immunofluorescence after 24 hrs of exposure to 10 μM of 3-NBA, or DMSO. Cells were stained with specific antibodies directly against the phosphorylated form of the protein, and with DAPI that stains nucleus. The pictures show representative cells from one experimental set-up. Original magnification; 1000X. (C) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of ATM was measured by flow cytometry using specific antibodies directly against the cleaved form of the protein, after 24 hrs of exposure Data is shown for one experimental set-up.

Figure 19. Effects of PAHs on histone H2AX, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of phosphorylated H2AX (γH2AX) were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of two separate experimental set-ups. (B) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of H2AX was measured by flow cytometry using specific antibodies directly against phosphorylated form of the protein, after 24 hrs of exposure. Data is shown for one experimental set-up. (C) γH2AX sub-cellular localization, analyzed by immunofluorescence, after 24 hrs of exposure to 10 μM of 3-NBA. The picture show representative cells from one experimental set-up. Original magnification; 1000X.

Figure 20. Effects of PAHs on Chk1, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of phosphorylated Chk1 (pChk1) were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of three separate experimental set-ups. (B) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of Chk1 was measured by flow cytometry using specific antibodies directly against the phosphorylated form of the protein, after 24 hrs of exposure. Data is shown for one experimental set-up. (C) p-Chk1 sub-cellular localization, was analyzed by immunofluorescence, after 24 hrs of exposure to 10 μM of 3-NBA. The picture shows representative cells from one experimental set-up. Original magnification; 1000X.

Figure 21. Effects of PAHs on Chk2, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of phosphorylated Chk2 (pChk2) were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of four separate experimental set-ups. (B) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of Chk2 was measured by flow cytometry using specific antibodies directly against the phosphorylated form of the protein, after 24 hrs of exposure. Data is shown for one experimental set-up. (C) p-Chk2 sub-cellular localization, was analyzed by immunofluorescence, after 24 hrs of exposure to10 μM of 3-NBA. The picture shows representative cells from one experimental set-up. Original magnification; 1000X.

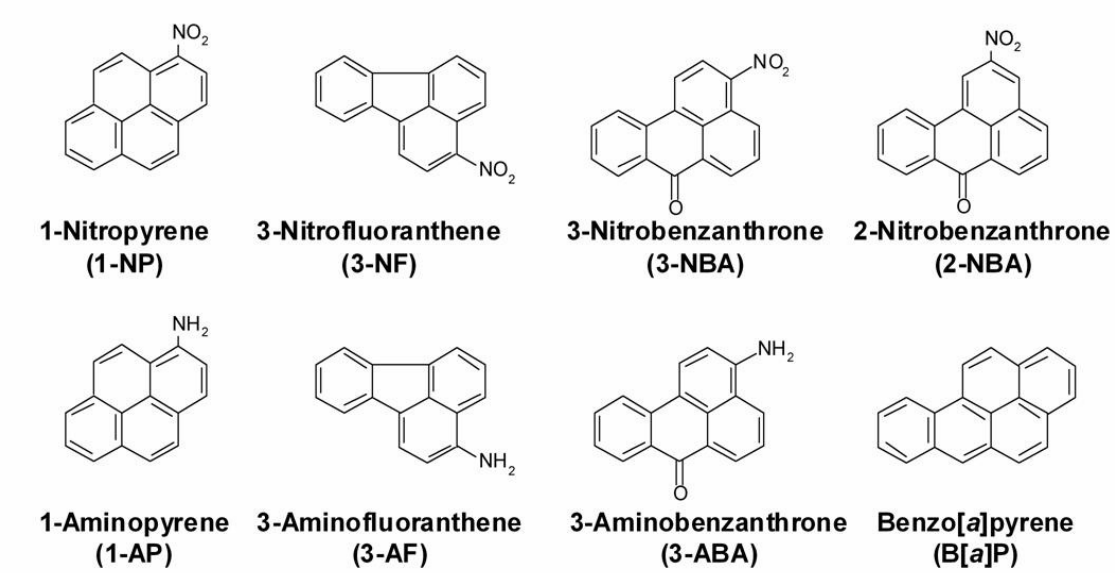
Figure 22. Effects of PAHs on total p53 and phosphorylated p53, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of total p53 and phosphorylated p53 (p-p53) were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of four separate experimental set-ups. (B) Various

concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of p53 was measured by flow cytometry using specific antibodies directly against the phosphorylated form of the protein, after 24 hrs of exposure. Data is shown for one experimental set-up. (C) p53 sub-cellular localization, was analyzed by immunofluorescence, after 24 hrs of exposure to 10 μM of 3-NBA. The picture shows representative cells from one experimental set-up. Original magnification; 1000X.

Figure 23. Effects of PAHs on p21, determined by Western blotting, flow cytometry and immunocytochemistry. In BEAS-2B cells: (A) Levels of p21 were analyzed by Western blotting after 24 hrs of exposure to various concentrations of 2-NBA, 3-NBA, 1-NP, B[*a*]P, or DMSO. The picture is representative of two separate experimental set-ups. (B) Various concentrations of 3-NBA and 2-NBA induced activation of DNA damage related kinases. Activation of p21 was measured by flow cytometry using specific antibodies directly against the protein, after 24 hrs of exposure. Data is shown for one experimental set-up. (C) p21 sub-cellular localization, was analyzed by immunofluorescence, after 24 hrs of exposure to 10 μM of 3-NBA. The picture shows representative cells from one experimental set-up. Original magnification; 1000X.

Figure 24. 3-NBA-induced p53 activation and involvement in cell death, determined by fluorescence microscopy. Microscopic examination, following PI/Hoechst 33342 staining, of the effect of the p53 inhibitor pifithrin-α (PFT-α) on 3-NBA-induced cell death (apoptosis and necrosis). The BEAS-2B cells were pre-treated for 1 h with PFT-α (10 μM) followed by co-exposure to 3-NBA (1, 3 or 10 μM), or DMSO, for 24 hrs. Percentage of cell death was estimated by fluorescence microscopy counts. Data is only acquired from one experimental set-up. (A) Effect of PFT-α on living cells exposed to 3-NBA. (B) Effect of PFT-α on apoptotic and necrotic cells exposed to 3-NBA.

Figure 1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(1-NP)
NO2
3-Nitrofluoranthene
(3-NF)
NO2
O
3-Nitrobenzanthrone
(3-NBA)
NO2
O
2-Nitrobenzanthrone
(2-NBA)
NH2
1-Aminopyrene
(1-AP)
NH2
3-Aminofluoranthene
(3-AF)
NH2
O
3-Aminobenzanthrone
(3-ABA)
Benzo[a]pyrene
(B[a]P)

Figure 2

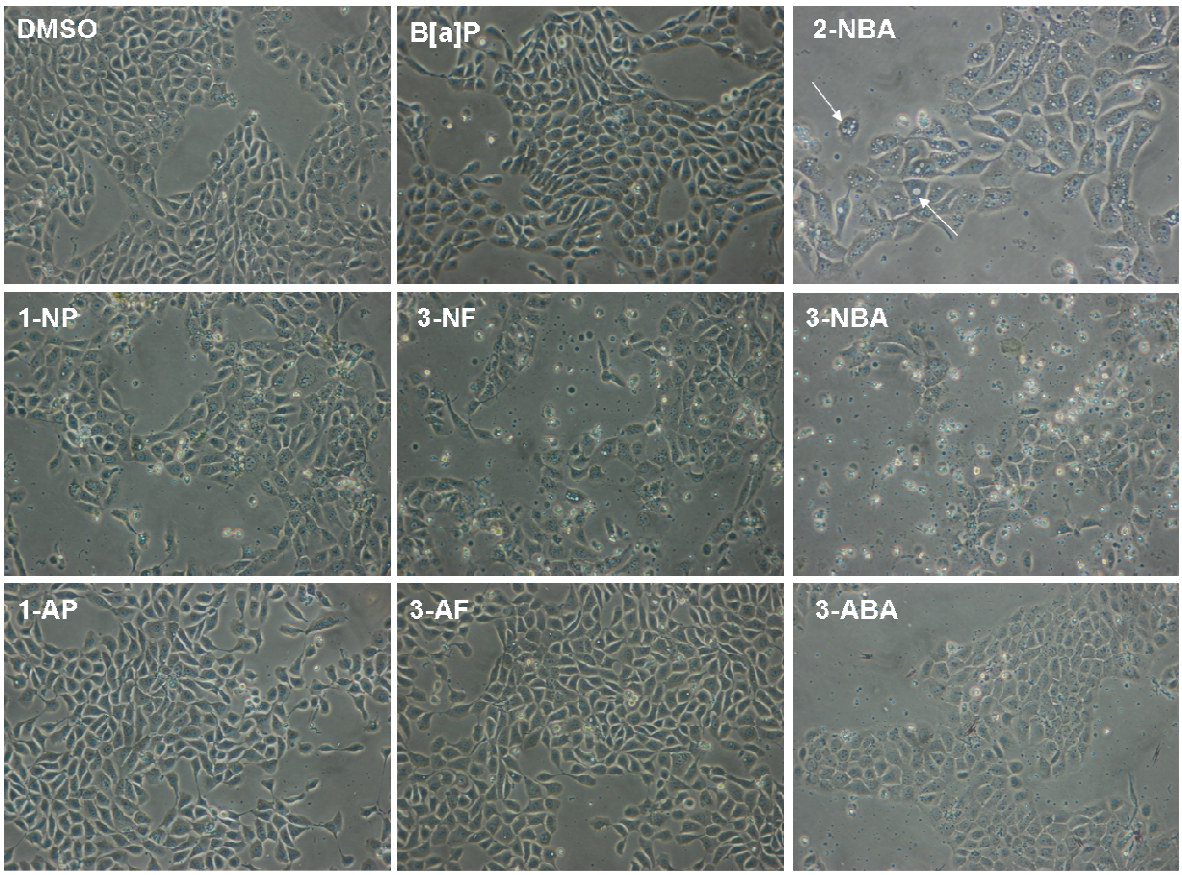


Figure 3

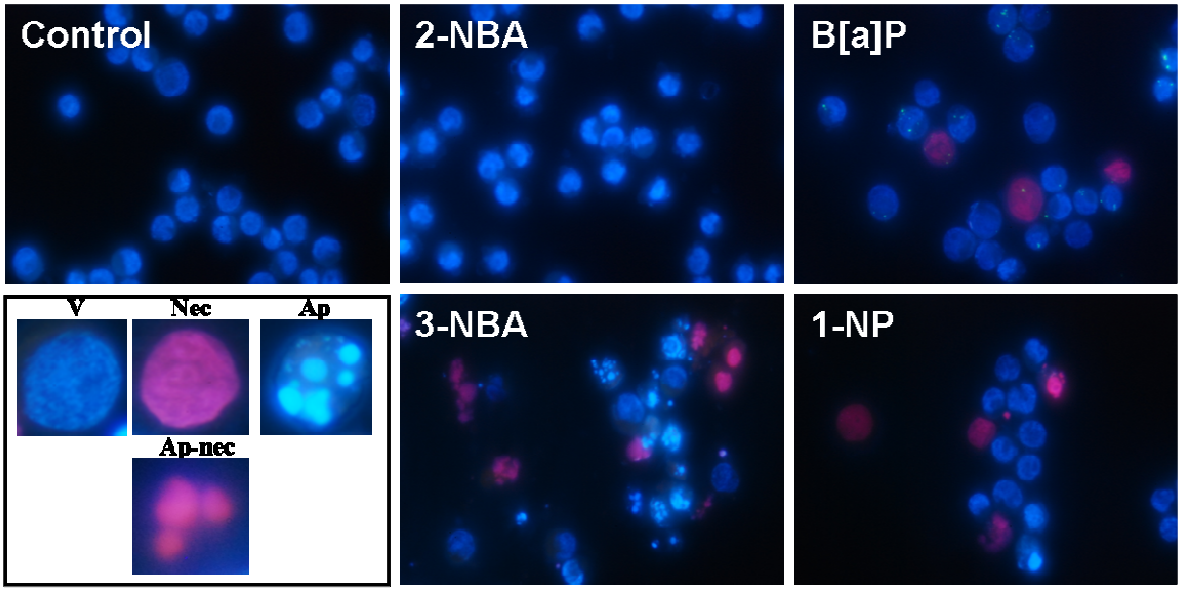


Figure 4

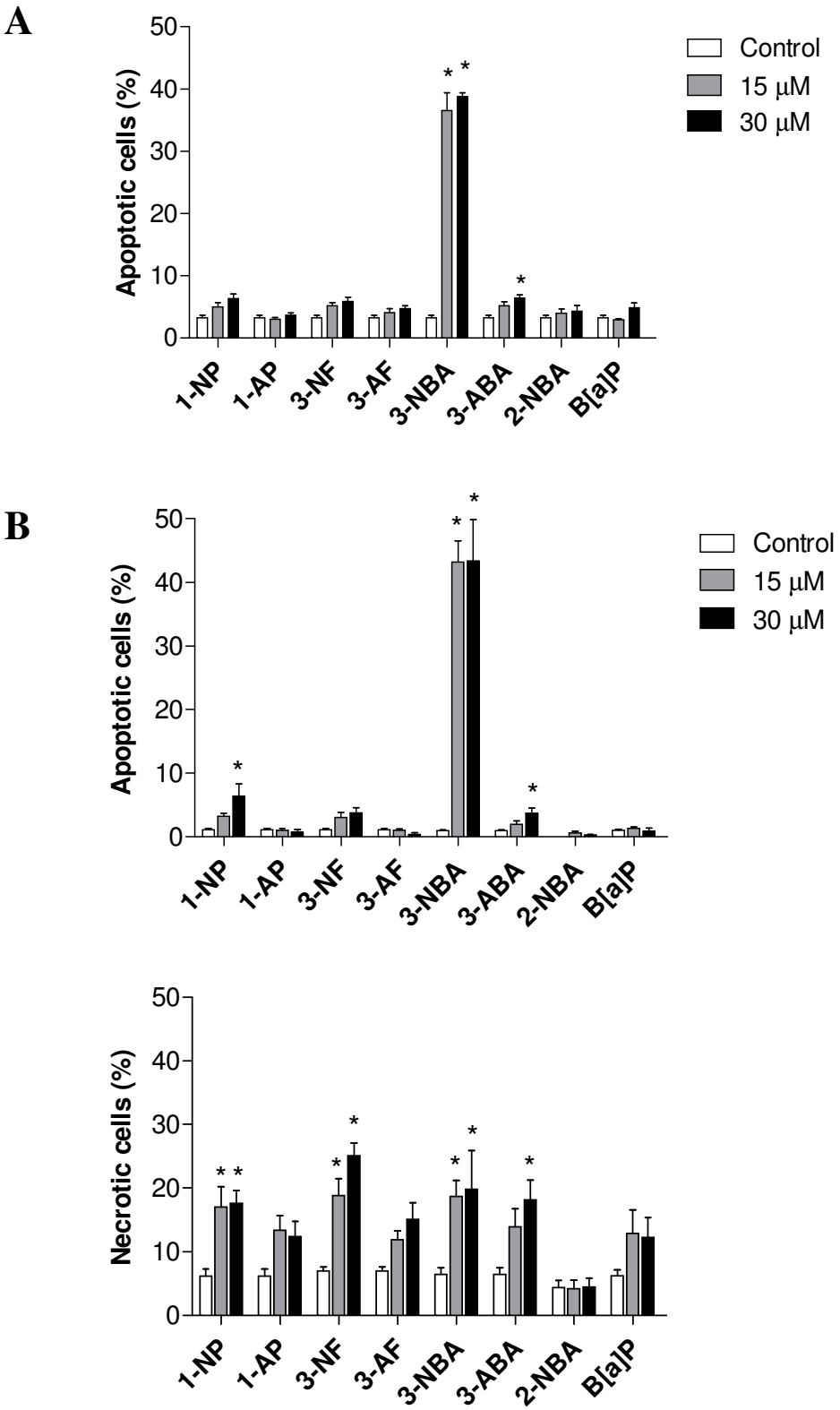

A
Apoptotic cells (%)
Control
15 μM
30 μM
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P
B
Apoptotic cells (%)
Necrotic cells (%)

Figure 5

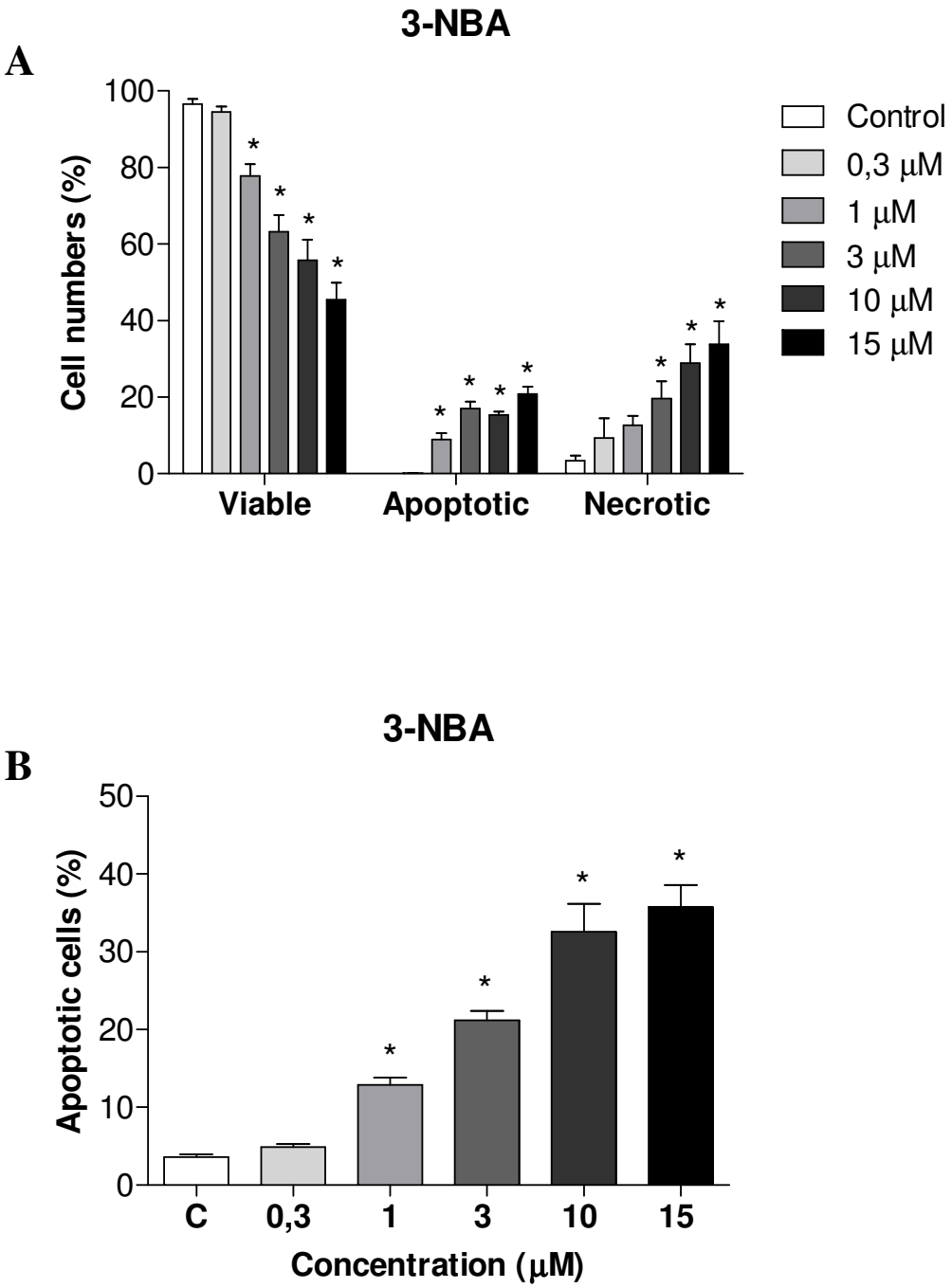

A
3-NBA
Cell numbers (%)
100
80
60
40
20
0
Viable
Apoptotic
Necrotic
Control
0,3 μM
1 μM
3 μM
10 μM
15 μM
B
3-NBA
Apoptotic cells (%)
50
40
30
20
10
0
C
0,3
1
3
10
15
Concentration (μM)

Figure 6

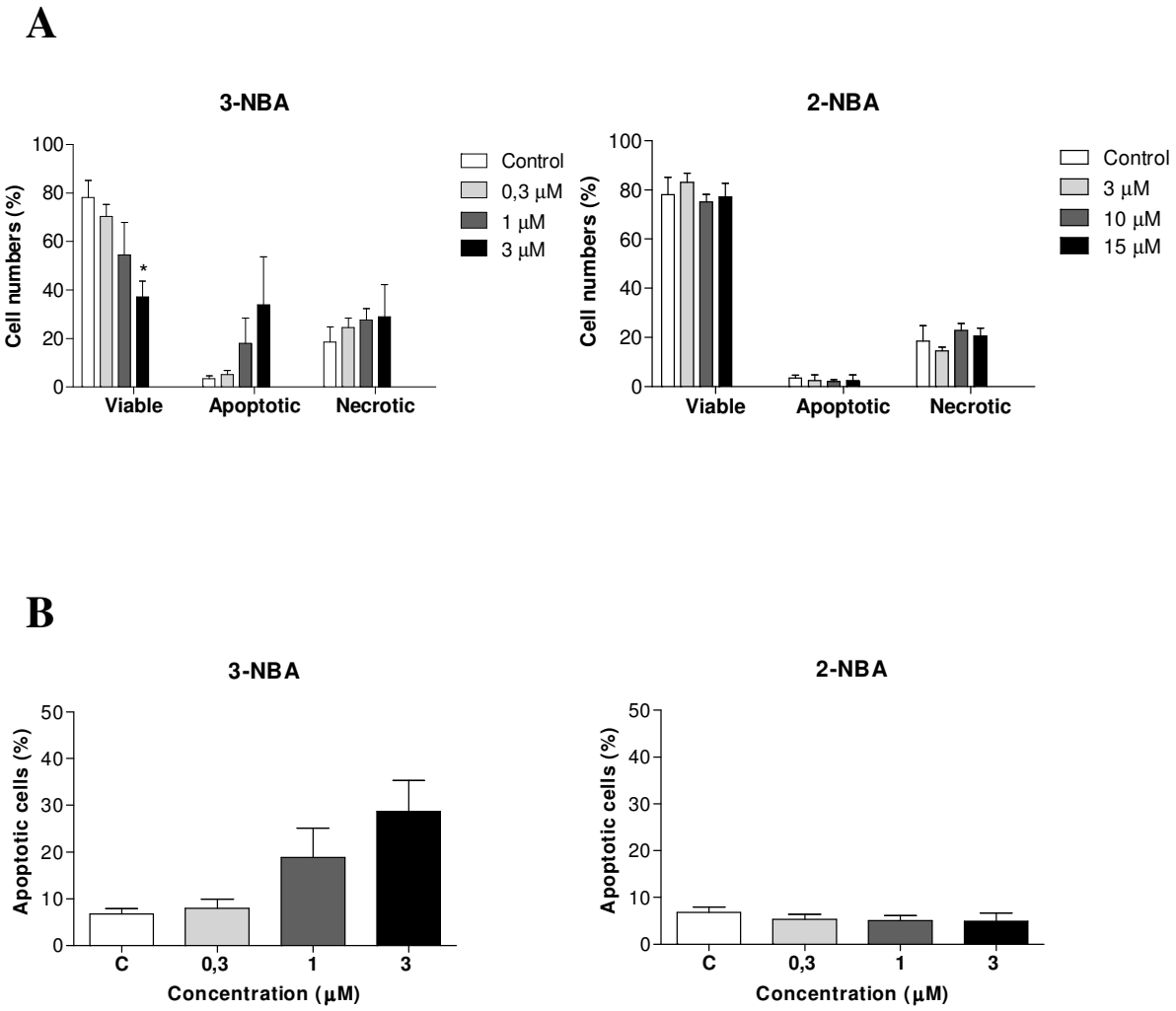

A
3-NBA
Cell numbers (%)
100
80
60
40
20
0
*
Viable
Apoptotic
Necrotic
Control
0,3 µM
1 µM
3 µM
2-NBA
Cell numbers (%)
Viable
Apoptotic
Necrotic
Control
3 µM
10 µM
15 µM
B
3-NBA
Apoptotic cells (%)
50
40
30
20
10
0
C
0,3
1
3
Concentration (µM)
2-NBA
Apoptotic cells (%)
C
0,3
1
3
Concentration (µM)

Figure 7

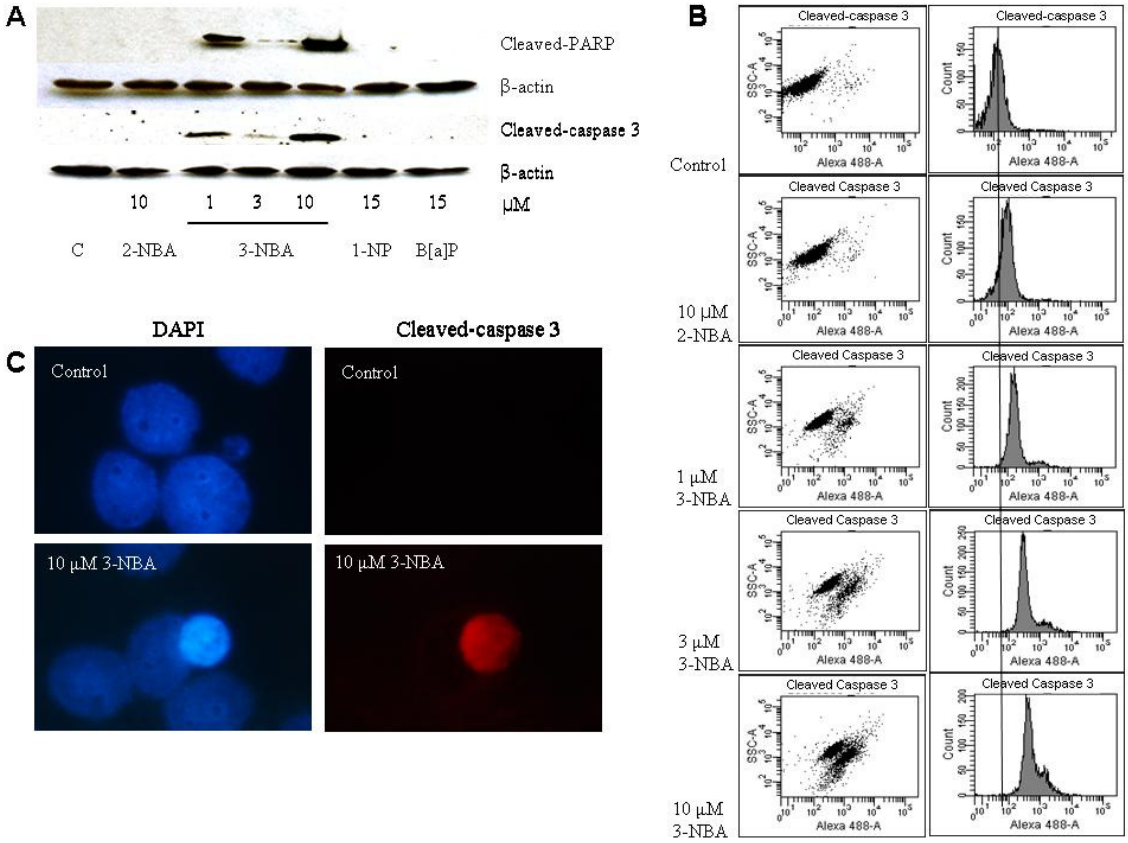

A
Cleaved-PARP
β-actin
Cleaved-caspase 3
β-actin
10 1 3 10 15 15 μM
C 2-NBA 3-NBA 1-NP B[a]P
B
Cleaved-caspase 3
Cleaved-caspase 3
SSC-A
Count
Alexa 488-A
Control
Cleaved Caspase 3
10 μM 2-NBA
1 μM 3-NBA
3 μM 3-NBA
10 μM 3-NBA
C
DAPI
Cleaved-caspase 3
Control
10 μM 3-NBA

Figure 8

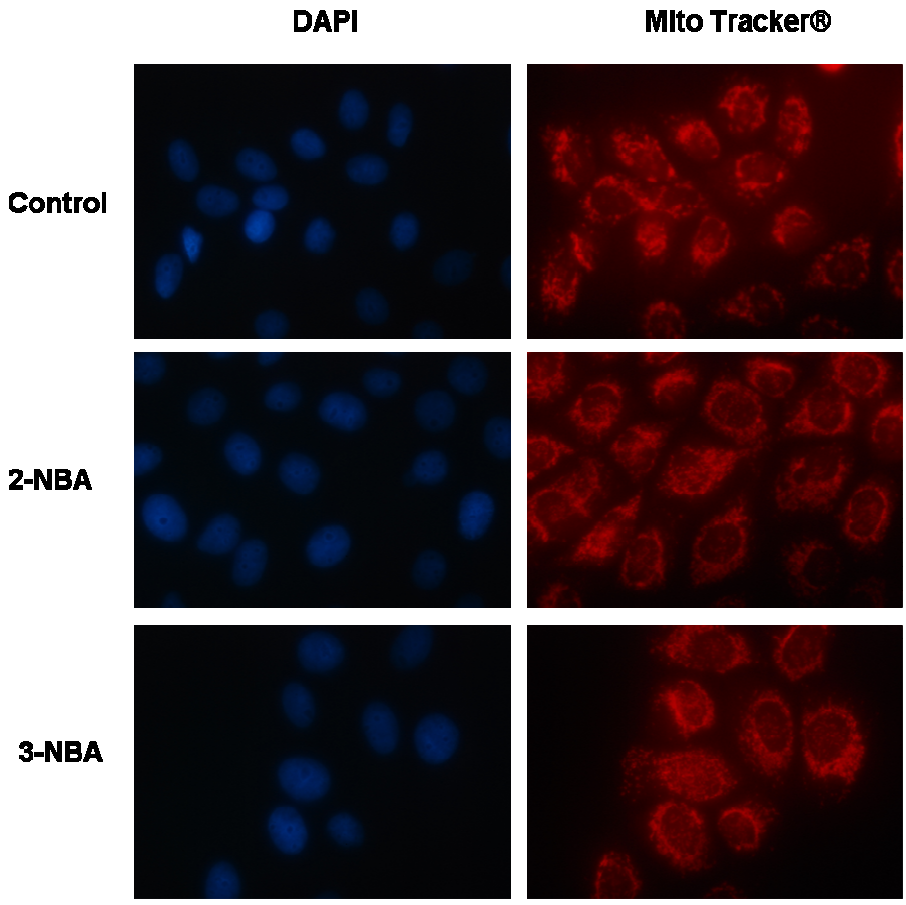


Figure 9

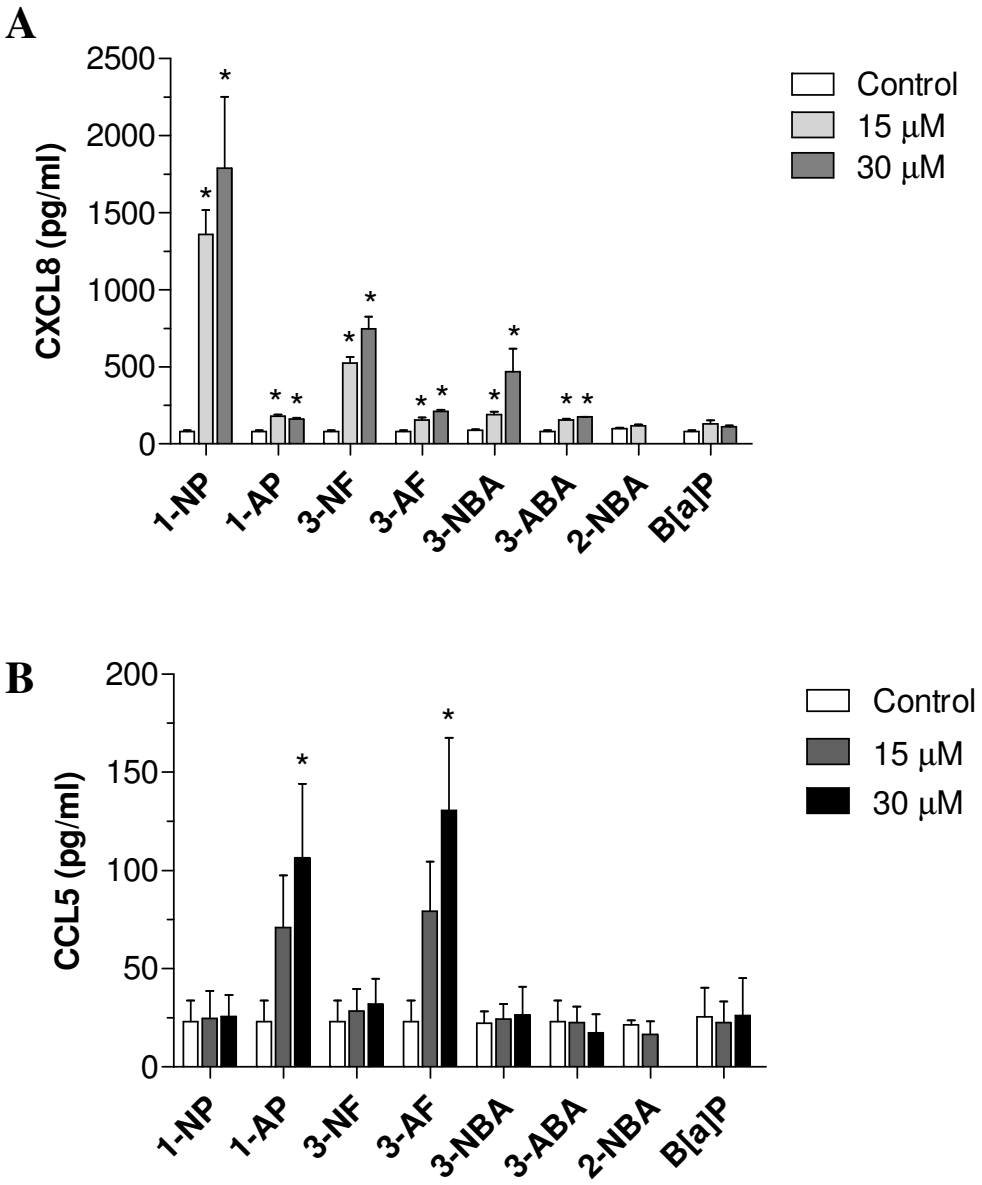


Figure 10

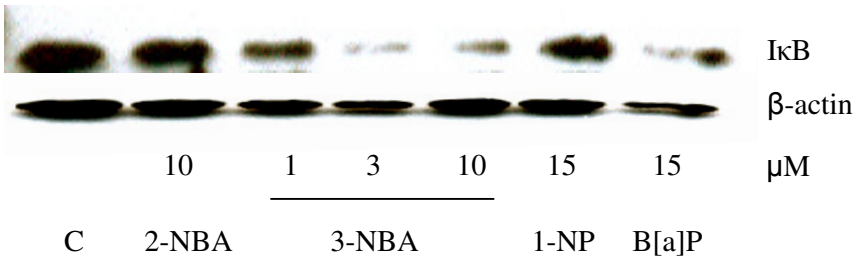


Figure 11

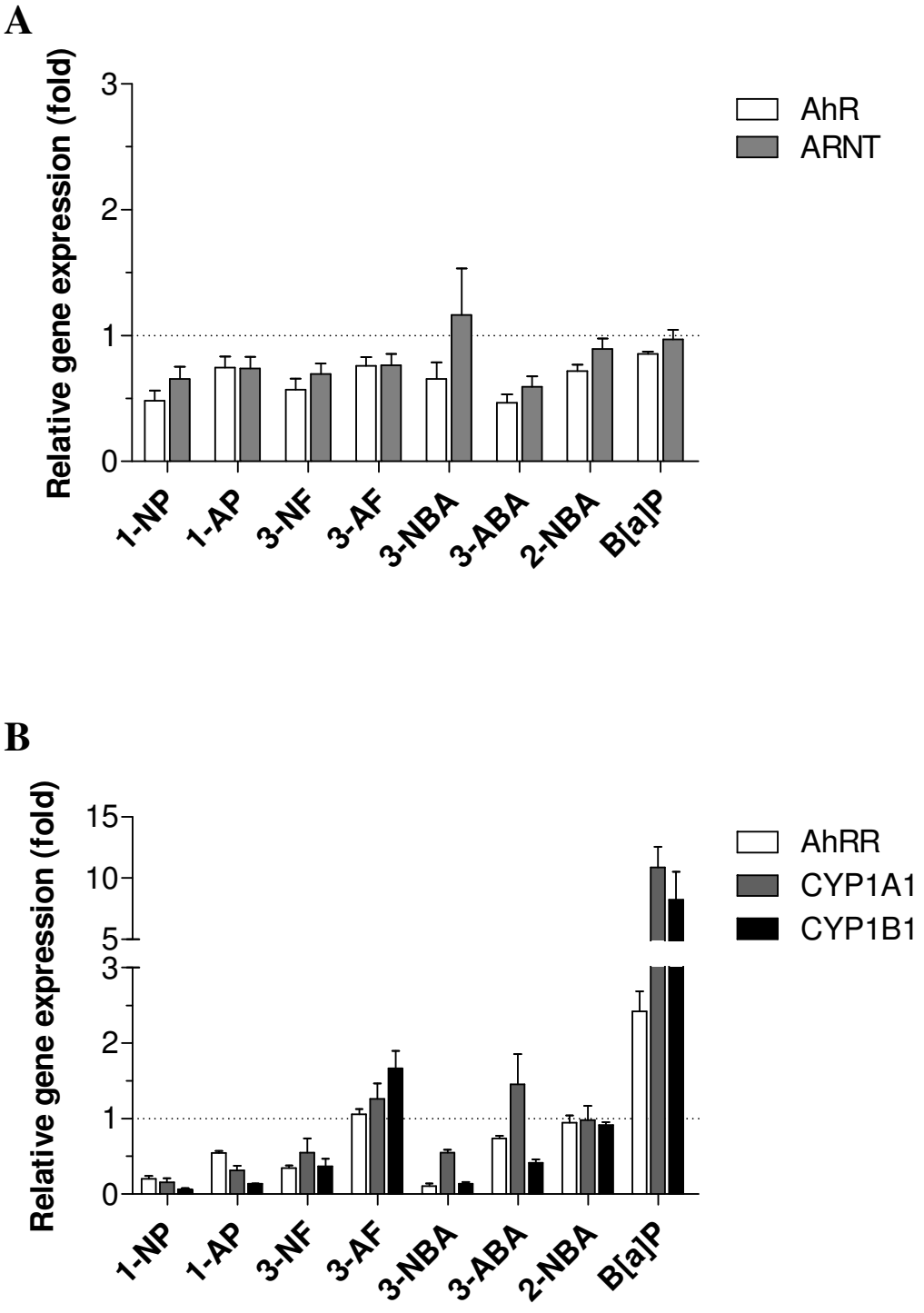

A
Relative gene expression (fold)
3
2
1
0
AhR
ARNT
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P
B
Relative gene expression (fold)
15
10
5
3
2
1
0
AhRR
CYP1A1
CYP1B1
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P

Figure 12

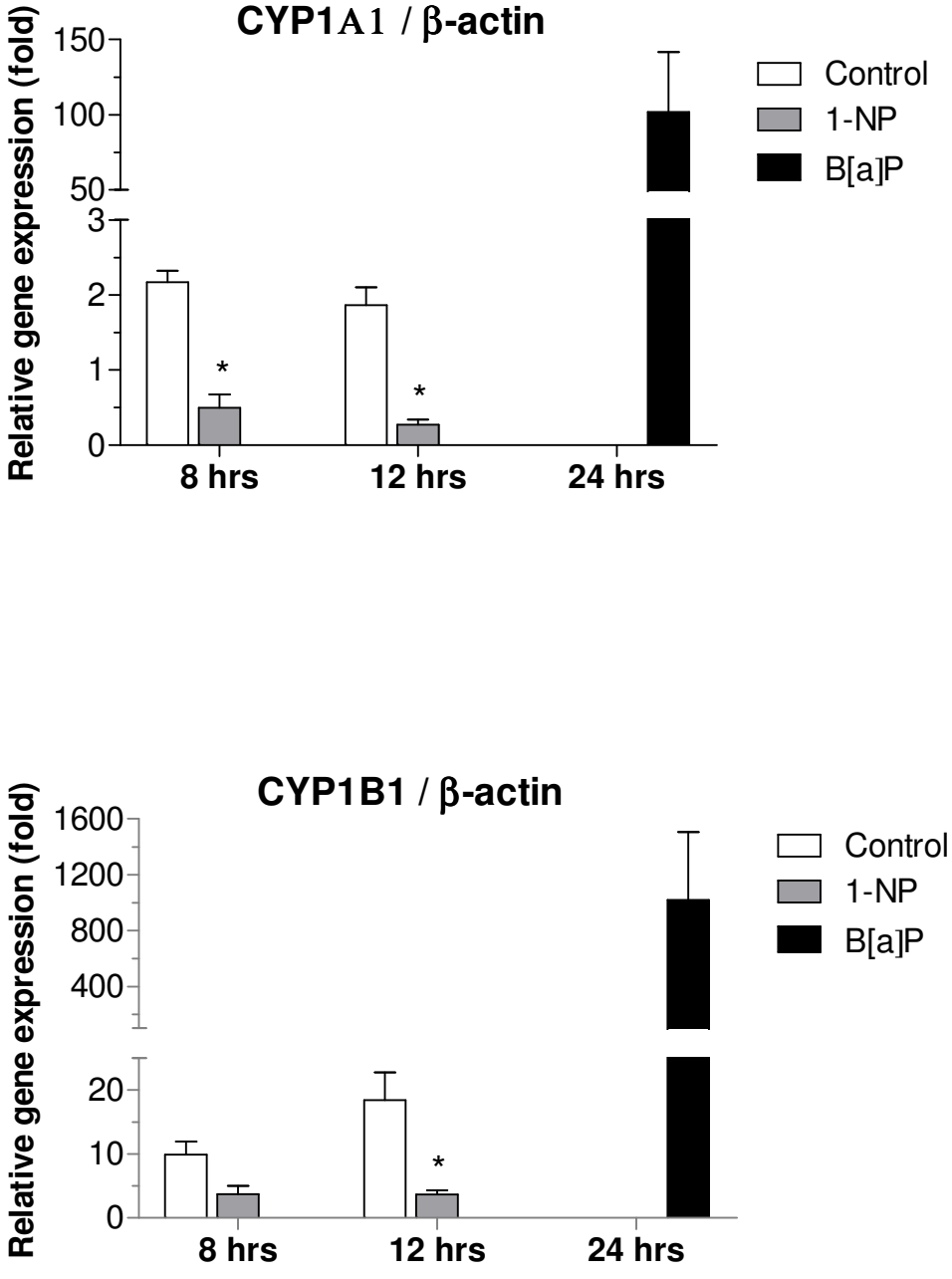

CYP1A1 / β-actin
Relative gene expression (fold)
150
100
50
3
2
1
0
*
*
8 hrs
12 hrs
24 hrs
Control
1-NP
B[a]P
CYP1B1 / β-actin
Relative gene expression (fold)
1600
1200
800
400
20
10
0
*
8 hrs
12 hrs
24 hrs
Control
1-NP
B[a]P

Figure 13

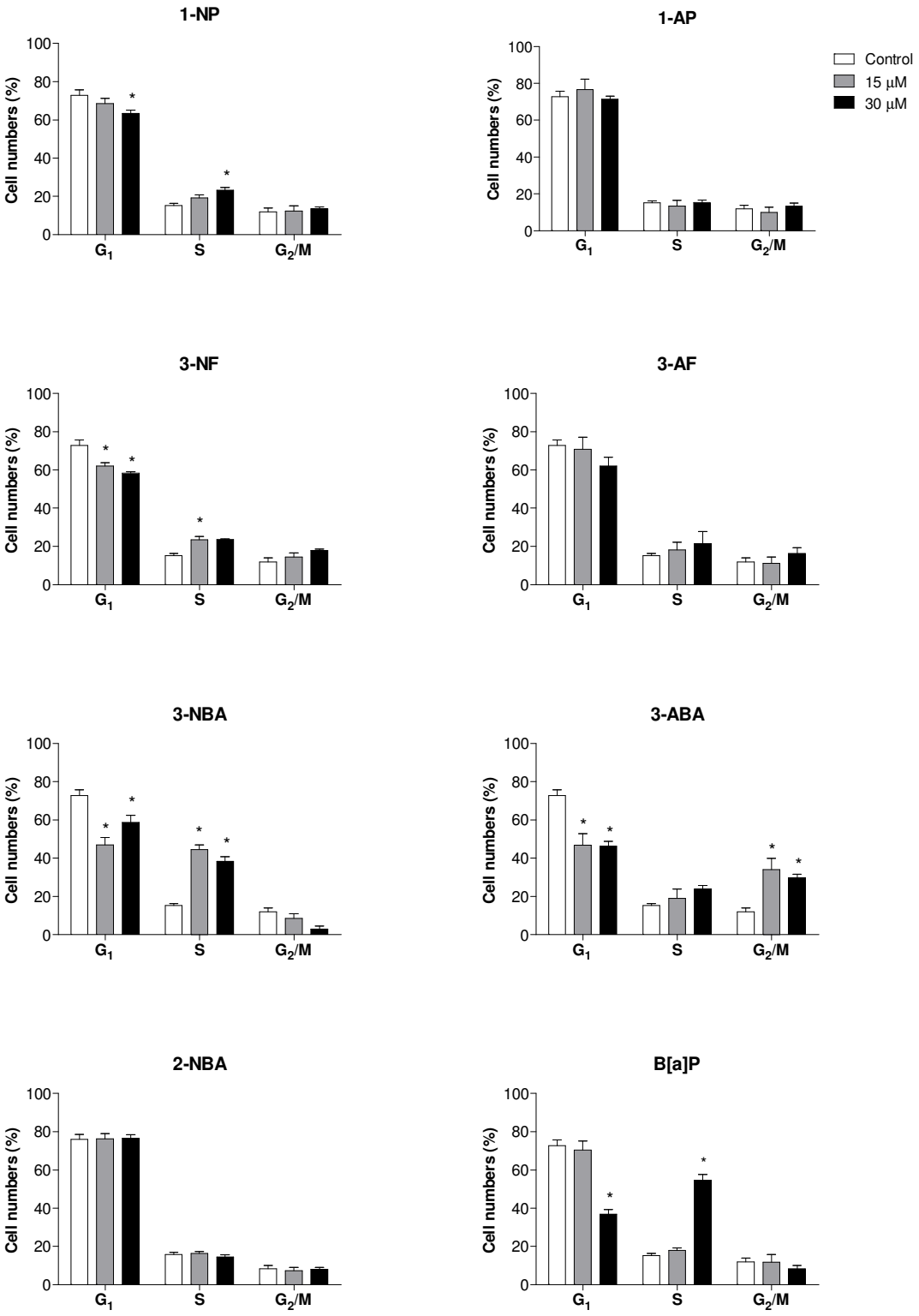

1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P
Control
15 µM
30 µM
Cell numbers (%)
0
20
40
60
80
100
G1
S
G2/M
*

Figure 14

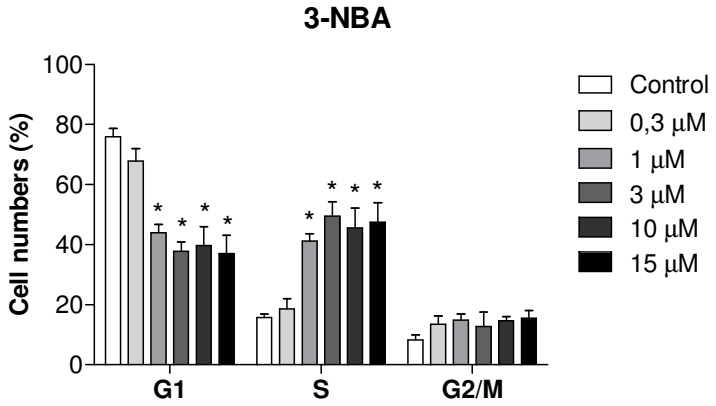
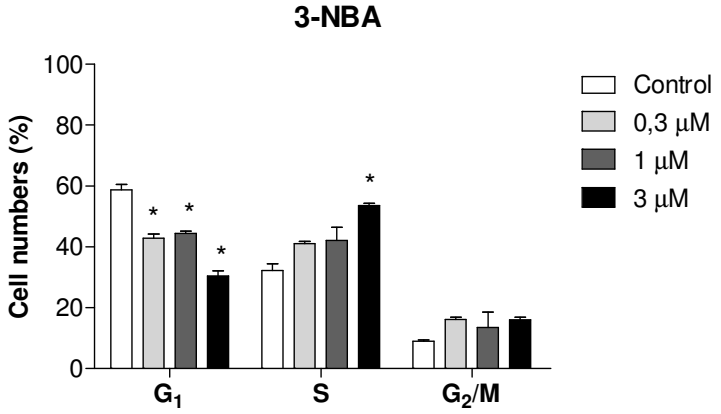


Figure 15



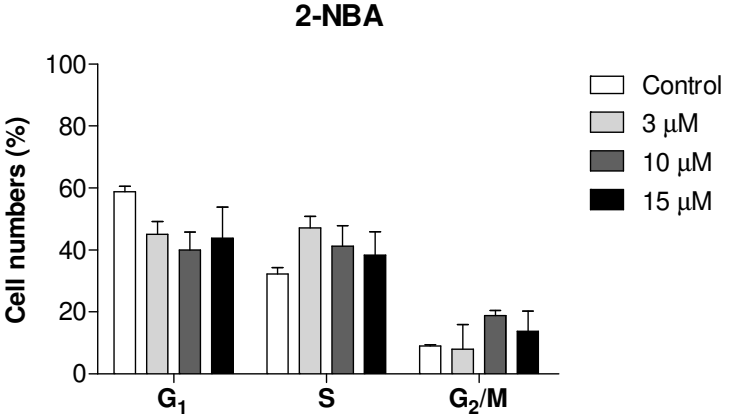


Figure 16

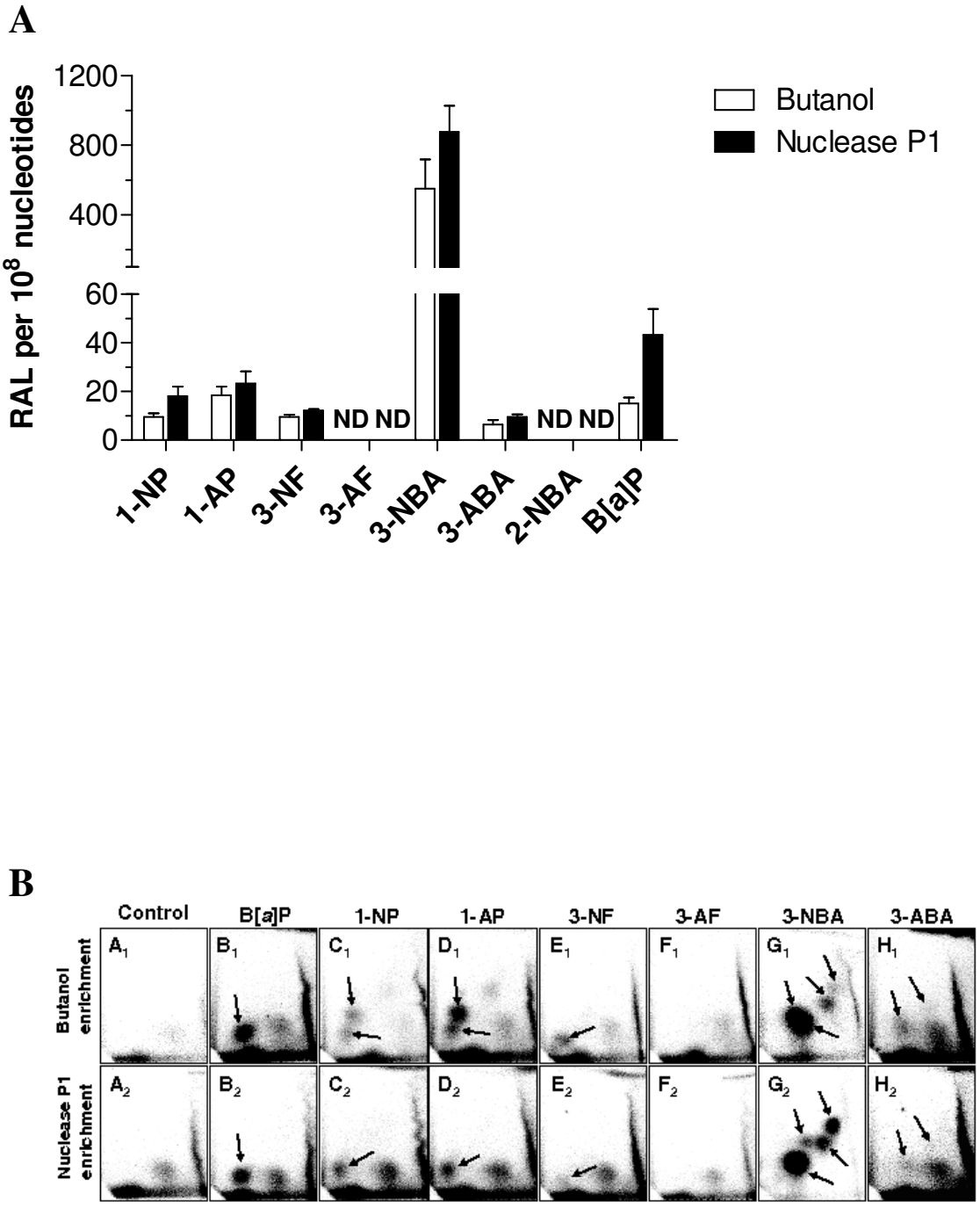

A
RAL per 10^8 nucleotides
1200
800
400
60
40
20
0
Butanol
Nuclease P1
ND ND
ND ND
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P
B
Control
B[a]P
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
Butanol enrichment
Nuclease P1 enrichment
A1
B1
C1
D1
E1
F1
G1
H1
A2
B2
C2
D2
E2
F2
G2
H2

Figure 17

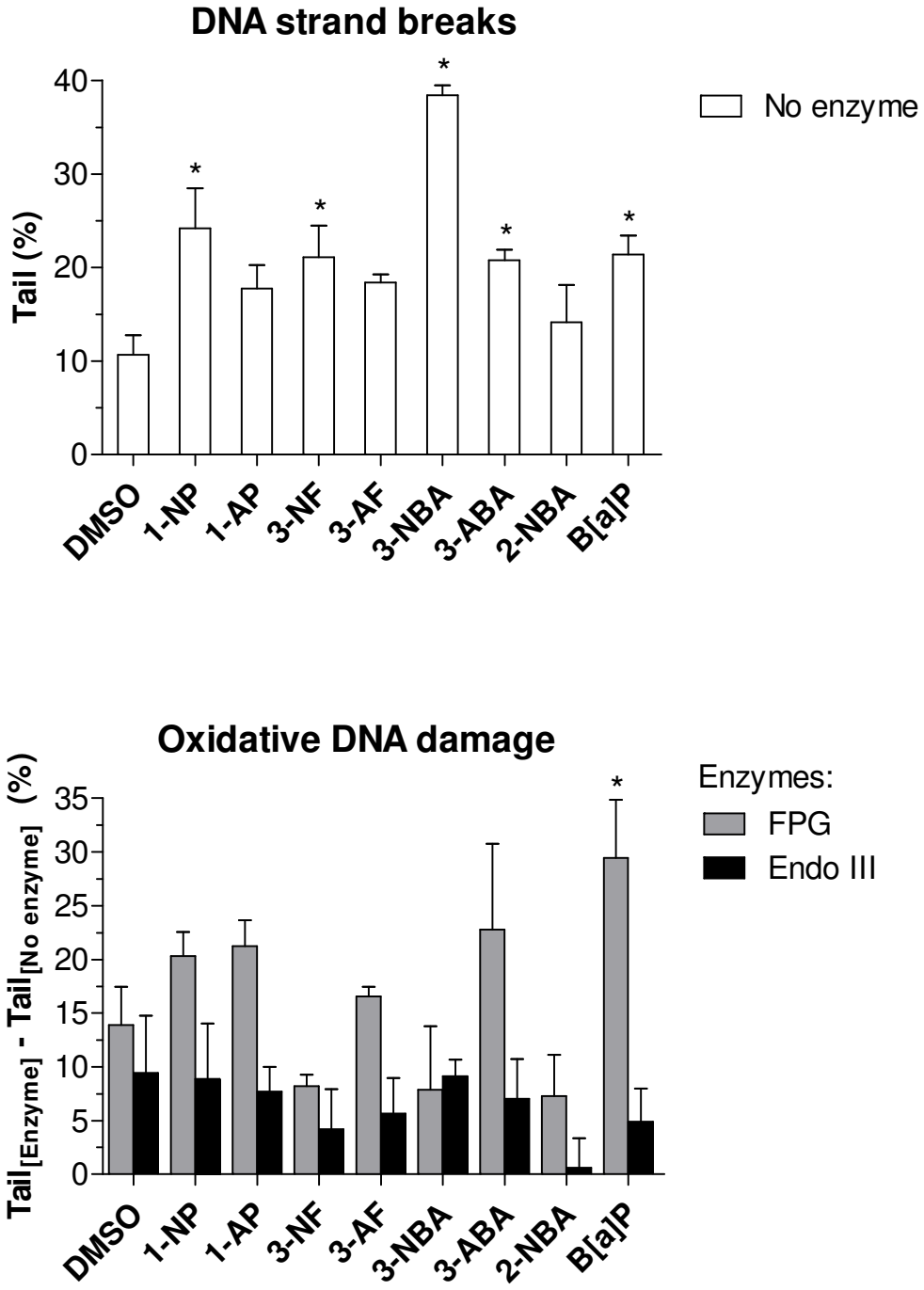

DNA strand breaks
Tail (%)
40
30
20
10
0
No enzyme
DMSO
1-NP
1-AP
3-NF
3-AF
3-NBA
3-ABA
2-NBA
B[a]P
Oxidative DNA damage
Tail[Enzyme] - Tail[No enzyme] (%)
35
30
25
20
15
10
5
0
Enzymes:
FPG
Endo III

Figure 18

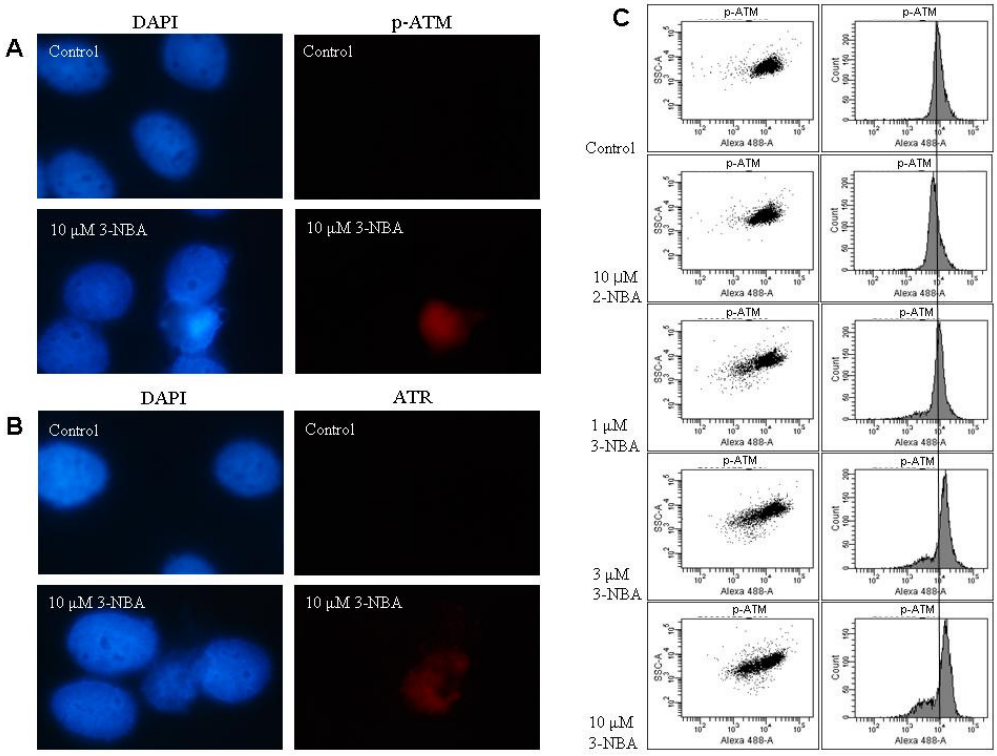


Figure 19

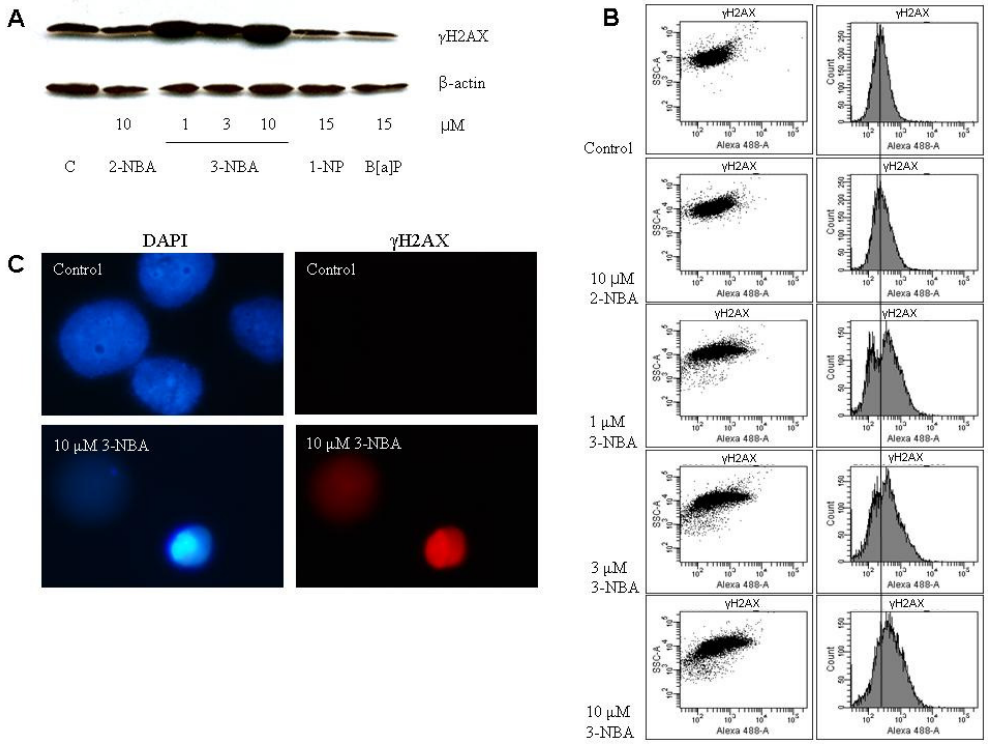


Figure 20

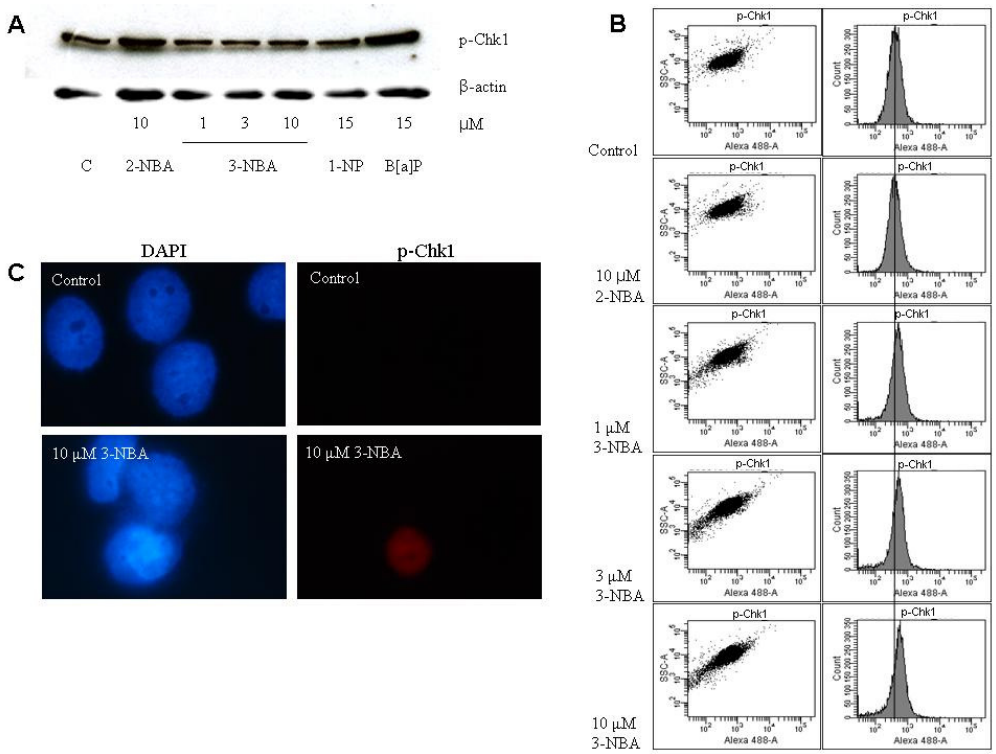


Figure 21

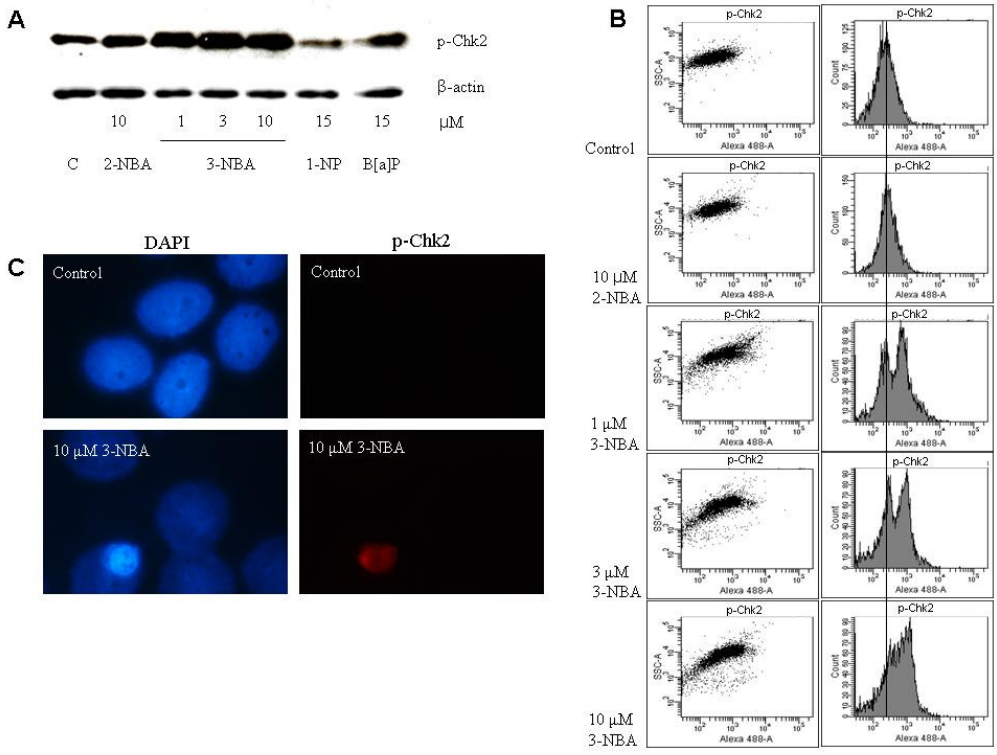


Figure 22

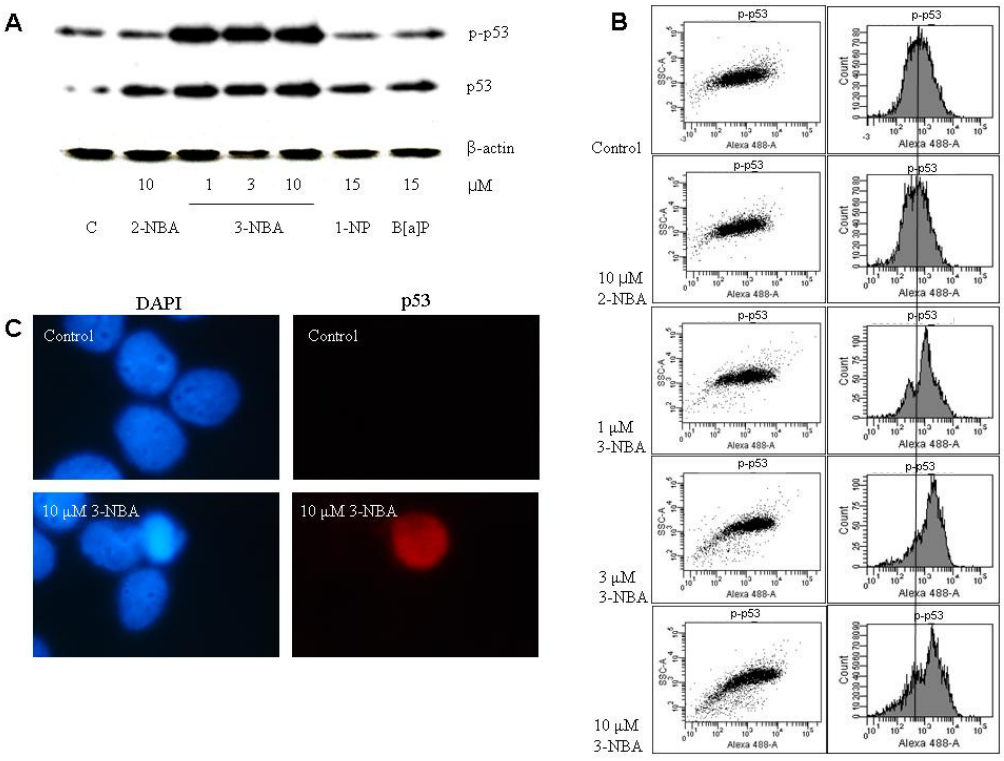


Figure 23

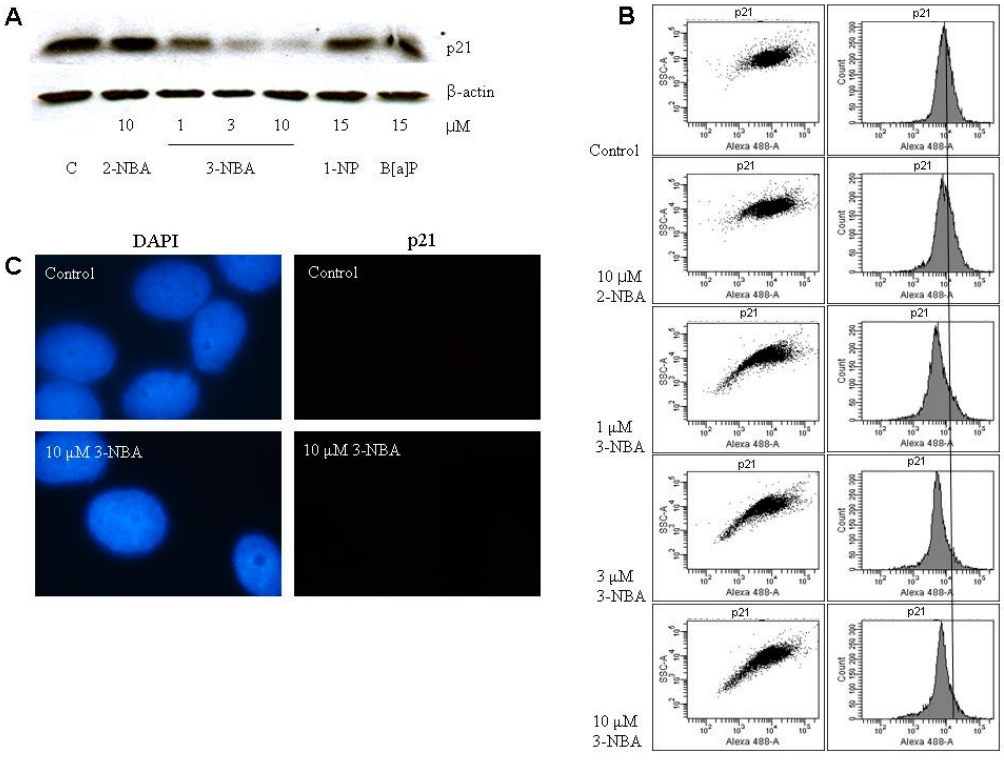
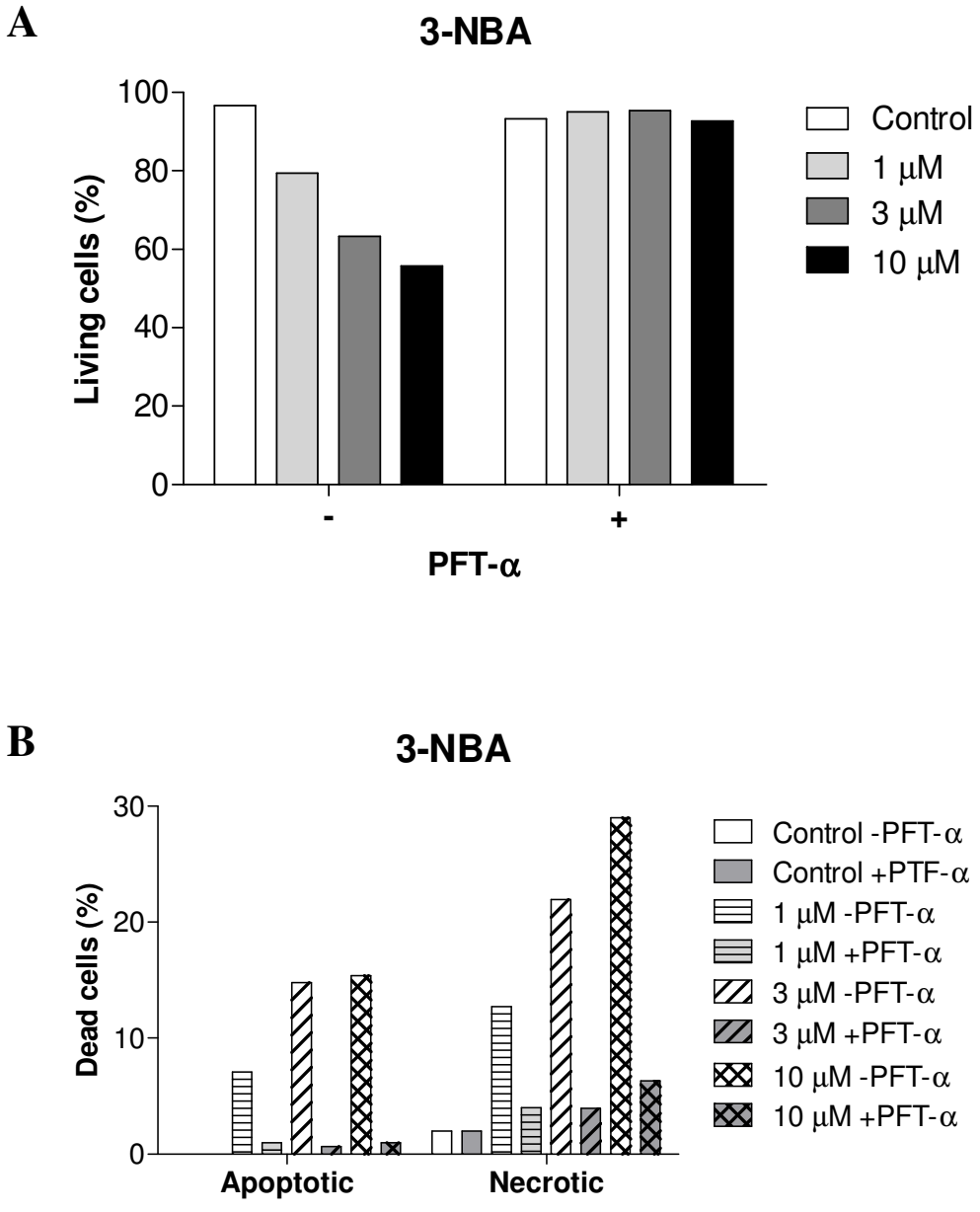


Figure 24


A
3-NBA
Living cells (%)
100
80
60
40
20
0
-
+
PFT-α
Control
1 μM
3 μM
10 μM
B
3-NBA
Dead cells (%)
30
20
10
0
Apoptotic
Necrotic
Control -PFT-α
Control +PTF-α
1 μM -PFT-α
1 μM +PFT-α
3 μM -PFT-α
3 μM +PFT-α
10 μM -PFT-α
10 μM +PFT-α