Sink- og arsen-indusert kjemokinfrigjøring og cytotoksisitet i humane bronkiale epitelceller

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FORORD

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Anders Thormodsæter

INNHOLD

SAMMENDRAG

Svevestøv eller partikulært materiale (PM) er assosiert med helseskader og utvikling og forverring av lungesykdommer som kronisk obstruktiv lungesykdom (KOLS), astma og lungekreft. I dette studiet har vi undersøkt eksponering for sink og arsen i en human epitelial cellelinje. Disse metallene har blitt vist å indusere helseskader hos mennesker både i form av komponenter i PM, og som metaller alene. Bronkiale epitelceller, BEAS-2B, ble eksponert for sink (Zn^{2+}), arsenitt og arsenat. Cytotoksisitets-data etter farging med propidium iodid (PI) og Hoechst 33342 og analyse ved hjelp av fluorescensmikroskopi, viste at arsenitt induserte nekrose og apoptose ved lavere konsentrasjoner enn sink og arsenat. Videre induserte eksponering for arsenitt en opphopning av celler i mitose analysert ved fluorescensmikroskopi og flowcytometri. Dette indikerer en rolle for arsenitt i mitotisk arrest. Eksponering for arsenat ga også opphopning av celler i mitose, men ved høyere konsentrasjoner enn arsenitt. Sink, arsenitt og arsenat induserte en økning i CXCL8-frigjøring ved lavere konsentrasjoner enn induksjon av celledød. Både sink og arsenitt så ut til å aktivere mitogen-aktiverte protein kinaser (MAPK) fordi nivåene av fosforylerte ekstracellulær signal-regulert kinase (ERK) og p38 økte ved eksponering for metallene. Videre ga hemming av p38 en reduksjon av den metall-induserte CXCL8-frigjøringen til basale nivåer, mens hemming av ERK ga bare en delvis reduksjon. Dette indikerer en viktig rolle for MAPK i produksjon av CXCL8. Transkripsjonsfaktoren nukleær faktor kappa B (NF-κB) ble aktivert av begge metaller. Både sink og arsenitt induserte oksidativt stress målt ved økte nivåer av H_2O_2 (DCFD-HA), og O_2^- (DHE), samt induksjon av heme oxygenase-1 (HO-1). Dette ble målt ved tilsvarende konsentrasjoner som ga maksimal CXCL8-frigjøring. Forbehandling med antioksidanten N-acetyl-L-cystein (NAC) reduserte CXCL8-frigjøringen indusert av sink og arsenitt. Dessuten ble også opphopningen av celler i mitose observert for arsenitt redusert. Difenylen iodonium (DPI), en NAD(P)H oxidase inhibitor, reduserte ikke CXCL8-frigjøringen, men reduserte opphopning av celler i mitose. En mulig additiv effekt ble observert ved kombinert eksponering av sink og arsenitt på CXCL8-frigjøring og cytotoksisitet. Kort oppsummert induserte eksponering for både sink og arsenitt inflammatoriske responser inkludert oksidativt stress, cytotoksisitet og CXCL8-frigjøring i bronkiale epiteliale lungeceller. Dette

indikerer en mulig rolle for metallene i negative helseeffekter indusert av PM. Dannelse av frie oksidative radikaler, aktivering av MAPK og NF-κB er trolig involvert i inflammasjonsresponsene.

FORKORTELSER

AD, aerodynamisk diameter; AP-1, aktivatorprotein 1; BEAS-2B, humane bronkiale epitelceller; CXCL8, IL-8 (Interleukin 8); DCFH-DA, 2',7'-dichlorfluorescein-diacetate; DHE, dihydroetidium; DPI, difenyl iodonium ERK, ekstracellulær signalregulert kinase;, ROS, frie radikaler (reactive oxygen species); HO-1, heme oksygenase-1; H_2O_2, hydrogenperoksid; I- κB,, inhibitor av kappa B; JNK, C-jun aminoterminal kinase; CO, karbonmonoksid. KOLS, kronisk obstruktiv lungesykdom; MAPK, mitogen-aktiverte protein kinaser; NAC, n-acetyl-cystein; NF-κB, NO_x, nitrogenoksider; nukleær faktor kappa B; $O2^-$, superoksid anion, O_3, ozon; $^{\bullet}OH$, hydroksyl radikal; PAH, polysykliske aromatiske hydrokarboner; PI, propidium iodid; PM, partikulært materiale; $ROO^{\bullet}$, peroksyl radikal; SO_2, svoveldioksid; TNF-α, tumor nekrose faktor alfa.

1. BAKGRUNN

1.1 Introduksjon

Helseskader forbundet med luftforurensning er ikke noe nytt fenomen. Allerede så tidlig som i 1775 fant Sir Percival Pott en sammenheng mellom soteksponering og kreft (Brown 1957). Men den hendelsen som i sterkest grad etablerte en sammenheng var London-tåken som inntraff 5. – 9. desember i 1952. Sterk kulde og medfølgende økt vedfyring og biltrafikk endte i 4000 estimerte dødsfall som følge av dårlig luftkvalitet, etterfulgt av ytterligere 8000 de neste ti ukene. Svoveldioksidverdier ble målt opptil 10 ganger høyere enn normalt (Wilkins 1954).

Luftforurensning forbindes i dag med en lang rekke helseeffekter, særlig hjertekar- og luftveissykdommer (Martin 2010). Det er en kompleks blanding av komponenter som varierer med både region og årstid. De viktigste komponentene i luftforurensning er svevestøv, svoveldioksid (SO_2), nitrogenoksider (NO_x), karbonmonoksid (CO) og ozon (O_3) (Brook et al. 2004)

1.2 Svevestøv

Svevestøv eller partikulært materiale (PM) betegner mikroskopiske partikler som kan bli værende i lufta over lengre tid. PM er i stor grad relatert til luftforurensning, blant annet er utslipp fra trafikk og industri viktige kilder. Dessuten inneholder PM komponenter som en ikke forbinder med luftforurensning, blant annet pollen, sporer og endotoksiner (SFT 2007).

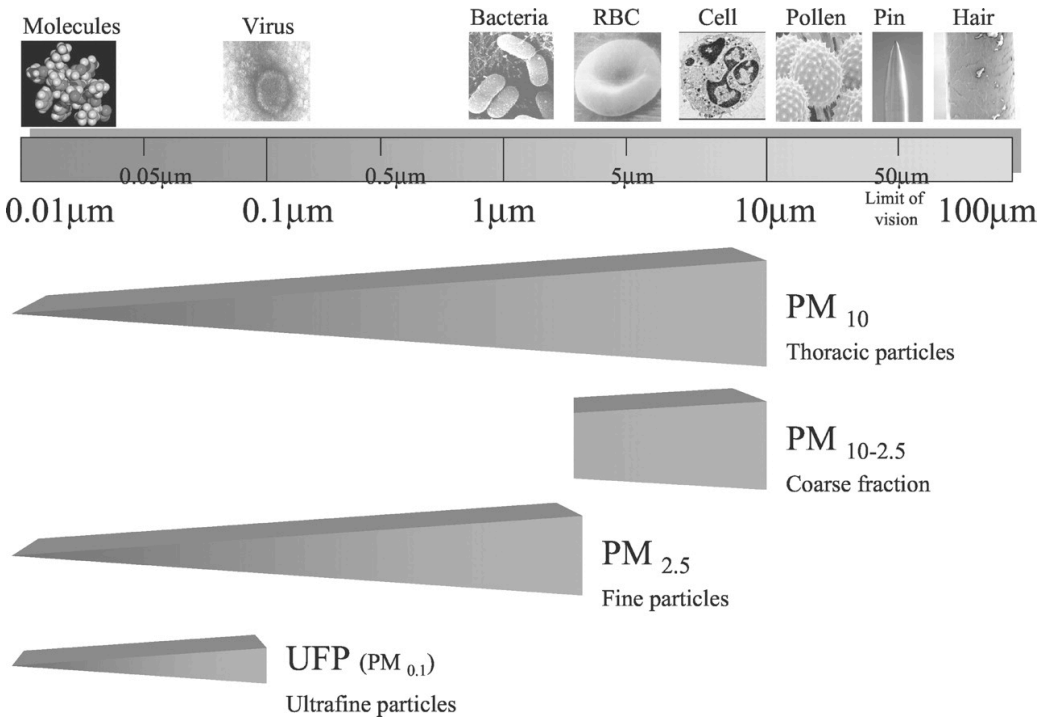
1.2.1 Helseeffekter

Undersøkelser har vist at en reduksjon i PM-nivåer også har gitt reduksjon i helsepåvirkning fra luftforurensning (Clancy et al. 2002; Pope et al. 2009). Verdens helseorganisasjon (WHO) rapporterte i 2002 at anslagsvis 800.000 dødsfall i verden var relatert til svevestøv per år (WHO 2002). Omfanget og typen av helseskader påført av inhalert PM avgjøres av deres fysiske egenskaper og kjemiske komposisjon.

Det kan være vanskelig å si nøyaktig hva i svevestøvet som utløser toksisitet, men innholdet av metaller, polysykliske aromatiske hydrokarboner (PAH) og muligens også NO_x har blitt vist å være avgjørende for utløsning av inflammasjon og organ-, vevs- eller celleskade. (Gualtieri et al. 2010; Molinelli et al. 2002; SFT 2007) Det har dessuten blitt vist at adsorberte endotoksiner i noen tilfeller kan være viktige bidragsyter til inflammasjon (Becker et al. 2005).

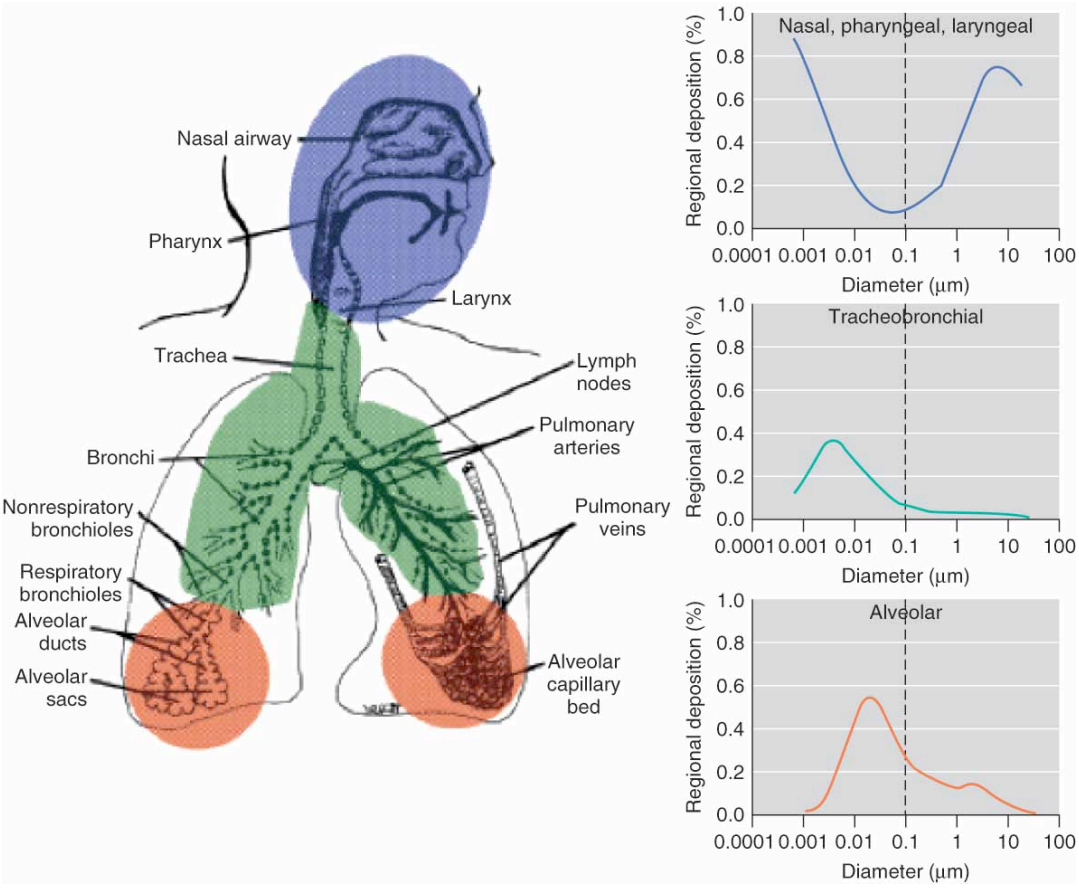
1.2.2 Størrelsesfraksjoner og deponering

En deler svevestøv inn i fraksjoner ut i fra aerodynamisk diameter (AD) på partiklene. Dette er et uttrykk for den teoretiske diameteren til en sfærisk partikkel med samme aerodynamiske oppførsel som den faktiske partikkelen. Den grove PM-fraksjonen (PM10-PM2.5) betegner alle partikler med AD fra 10 μm – 2.5 μm. Denne fraksjonen skiller seg fra de andre fraksjonene særlig ved at den inneholder det meste av ikke-forbrent PM, erosjonspartikler og slitasjepartikler. Her finner en hoveddelen av mineralpartiklene fra jordskorpen samt adsorberte elementer som endotoksiner (SFT 2007). Den fine PM-fraksjonen (PM2.5) inneholder alle partikler med AD mindre enn 2.5 μm. Denne fraksjonen er i større grad dominert av forbrenningspartikler (Marcazzan et al. 2001). Det samme gjelder den ultrafine fraksjonen (PM0.1) som betegner de aller minste partiklene med AD mindre enn 0.1 μm (SFT 2007).



Figur 1. Inndeling av PM i forskjellige fraksjoner, sammenliknet med utvalgte biologiske materialer (Brook et al. 2004).

Respirasjonssystemet deles inn i øvre og nedre luftveier. I det øvre finner vi neseganger, munnhule, svelget og strupehodet. Det nedre består av luftrøret som deles i bronkier, som igjen deles videre til bronkioler og ender i alveoler hvor gassutveksling foregår. Store partikler (AD >10 µm) deponeres generelt i de øvre luftveiene. Partiklene med AD i finfraksjonen deponeres i større grad i de nedre luftveiene, i bronkioler og alveoler (Oberdörster et al. 2005). De aller minste partiklene kan deponeres både i gassutvekslingssonen, men også i de øvre luftveiene hvis partiklene er svært små (< 10 nm) . Størrelse har dessuten blitt vist å ha betydning for graden av skadevirkninger. De minste partiklene induserer generelt mer toksisitet enn større partikler av samme type forbindelse. Små partikler har relativt sett større overflate enn større partikler og ved å justere for dette har forskjeller i respons til dels forsvunnet (Schwarze et al. 2010). Partikkeloverflate har derfor blitt ansett å ha stor betydning for toksisitet (Oberdörster 1996).



Figur 2. Forventet deponering av inhalerte partikler basert på størrelse (Oberdörster et al. 2005).

1.2.3 Metaller i svevestøv

En rekke metaller finnes i PM. En måling av PM i 13 steder i USA viste at jern etterfulgt av aluminium og sink var de vanligste metallene i PM2.5 (Chen and Lippmann 2009). Metaller i PM har mange mulige kilder som opphav. Blant de viktigste antropogene kildene har vi forbrenning av fossilt brensel, metallsmelteverk og gruvedrift. Trafikkrelaterte kilder som slitasje av veidekke, dekk og bremser bidrar dessuten i stor grad. (SFT 2007).

Metaller utgjør en liten volummessig del av det totale PM, men mange studier viser at metaller bidrar til en vesentlig del av PM-indusert inflammasjon, celleresponser og -død (Chen and Lippmann 2009; Molinelli et al. 2002; Schaumann et al. 2004). Et eksempel er fra Utah Valley, der luftprøver i nærheten av et metallsmelteverk ble

samlet inn ved drift, etter driftstans og like etter gjenåpning av verket et år senere. Cytokinresponsen i lungeskyllevæsken hos frivillig eksponerte ble vist å være markert høyere etter 24 t eksponering for prøvene samlet under drift av verket enn ved driftstans, noe som indikerte induksjon av inflammatorisk respons (Ghio and Devlin 2001). Videre ble det foretatt undersøkelser hvor metaller i PM fra luftprøvene ble inaktivert ved hjelp av den chelatbindende agenten chelex. Dette fjernet responsen tidligere observert ved drift, noe som tydet på at metaller var viktigste faktor i økt cytokindannelse (Molinelli et al. 2002).

Studier har vist at mange metaller kan endre intracellulær redoks-tilstand, og en rekke metaller har blitt koblet til dannelse av frie radikaler (ROS) og derav indusert inflammatorisk respons (Leonard et al. 2004). Det har videre blitt funnet sammenheng mellom metalleksponering i PM og endringer i aktiviteten til en rekke transkripsjonsfaktorer, blant annet nukleær faktor kappa B (NF-κB) og aktivatorprotein 1 (AP-1) (Chen and Lippmann 2009). Videre har det blitt vist at en del mitogen-aktiverte protein kinaser (MAPK) kan bli indusert av metaller til å uttrykke inflammasjonsproteiner (Samet et al. 1998).

1.2.3.1 Sink

Sink er et metall med atomnummer 30 og symbolet Zn. Det har vanligvis oksidasjonstall +2. Viktige antropogene kilder til sink i PM er metallsmelteverk, fossilt brensel samt slitasjepartikler fra bildekk (Councell et al. 2004; WHO 2005). Sink er et essensielt metall for mennesker. Mangel på sink berører nesten to milliarder mennesker, hovedsakelig i utviklingsland, og kan gi tilstander som veksthemming, forstyrrelser i immunsystemet og kognitiv hemming (Prasad 2009b). Sink er et intracellulært signalmolekyl i blant annet monocytter, dendrittiske celler og makrofager og spiller en sentral rolle både i immunfunksjoner og oksidativt stress. Det er dessuten ansett å være både en antioksidant og en anti-inflammatorisk forbindelse (Jomova and Valko 2011; Prasad 2009a)

Til tross for at sink er essensielt for mennesker viser undersøkelser at sink både i PM og alene kan indusere negative helseeffekter (Adamson et al. 2000; Gordon et al. 1992; Iitaka et al. 2001). Adamson et. al (2000) viste at sink var den enkeltfaktoren

blant metaller i en luftprøve (EHC-93) fra urbane strøk som induserte sterkest toksisitet i lungevev hos mus (Adamson et al. 2000). Videre har det blitt vist at sink kan indusere metallstøvfeber, en sykdom som forekommer hos sveisere (Gordon and Fine 1993). Sink har også blitt relatert til en økning i inflammatorisk respons kjennetegnet ved økt CXCL8-produksjon, apoptose, aktivering av MAPK og transkripsjonsfaktorer samt induksjon av ROS (Samet et al. 1999; Iitaka et al. 2001; Kim et al. 2006; Maret 2009). Mekanismen for hvordan sink induserer slike skadevirkninger er uklar, men jeg vil i følgende avsnitt kort skissere to mulige mekanismer som kan være aktuelle for sink-induserte inflammasjonsresponser.

Sink har i likhet med noen andre metaller hemmende egenskaper på fosfataser (Samet et al. 1999). Fosfataser er involvert i reversibel fjerning av fosfatgrupper fra proteiner. Fosforylering er sentral i aktivering av en rekke celleresponser, blant annet aktivering av transkripsjonsfaktorer som påvirker ekspresjon av proteiner involvert i inflammatorisk respons (Stoker 2005) Sink har blitt vist å aktivere både MAPK og transkripsjonsfaktorer i BEAS-celler , og dette har blitt koblet til hemming av fosfataser (Samet et al. 1999).

Til tross for at sink er et overgangsmetall, ansees det å være relativt stabilt og inngår derfor hovedsakelig ikke i redoksreaksjoner. Videre kontrolleres sinknivåer i cellen nøye av blant annet metallotionin (MT) (Maret 2000). Men det har blitt vist at høye konsentrasjoner av sink kan mette MT og andre proteiners evne til å binde opp sink slik at frie sink-ioner opphopes i cellen. Dette kan gi toksisitet blant annet ved indirekte ROS-dannelse ved at tiol-grupper på proteiner oksideres (Maret 2009).

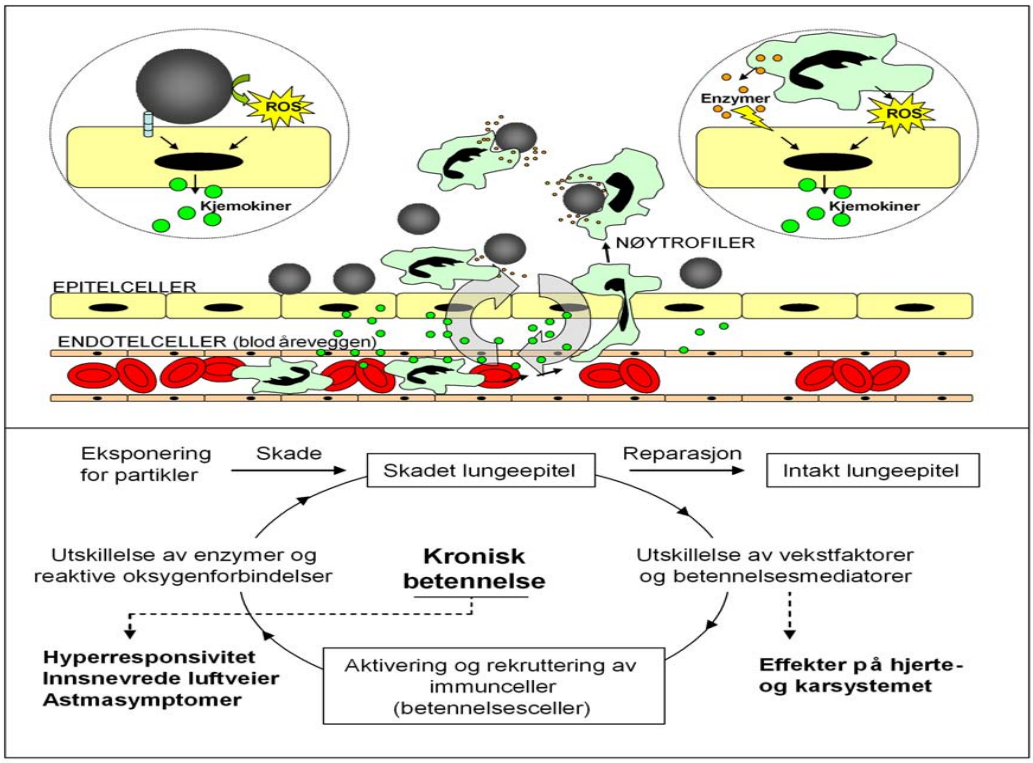
1.2.3.2 Arsen

Arsen er et metalloid, men benevnes gjerne som et metall. Det har atomnummer 33 og symbolet As. Arsen har vanligvis oksidasjonstall +5, +3 eller -3 og har opphav i PM fra forbrenning av fossilt materiale (Jomova et al. 2011). Arsen har som sink blitt vist å gi skadevirkninger både som faktor i PM og også alene, men trolig er det delvis andre mekanismer som ligger bak toksisiteten til arsen. Blant annet har ikke arsen i samme grad blitt koblet til inhibering av fosfataser (Samet et al. 1999). Arsenitt (+3) er den varianten av arsen som i størst grad har blitt vist å indusere toksisitet hos

mennesker. Arsenat (+5), en annen variant påvist i PM gir mindre skadevirkninger enn arsenitt blant annet fordi den i liten grad tas opp av celler og de skadevirkninger som er påvist induseres via overgang til arsenitt (Jomova et al. 2011). Eksponering til arsen har blitt koblet til flere typer kreft, blant annet lungekreft, kardiovaskulære sykdommer, nevrologiske skader og hudrelaterte skader (Cohen et al. 2006; Miller et al. 2002; Navas-Acien et al. 2005; Vahidnia et al. 2007).

Mange studier har vist at arsen kan indusere ROS-dannelse til tross for at det ikke er et overgangsmetal (Valko et al. 2005). Blant annet har økt dannelse av peroksyl radikaler ($ROO^{\bullet}$), superoksid anion (O_2^-), hydroksyl radikal ($^{\bullet}OH$) og hydrogen peroksid (H_2O_2) blitt observert ved eksponering til arsen hos mennesker (Flora et al. 2007). Videre har arsen blitt vist å påvirke antioksidant-nivåer. Glutathion (GSH) er en effektiv antioksidant som spiller en viktig rolle i å opprettholde cellens redoks-tilstand, GSH-nivået er derfor en god markør for oksidativt stress. (Rahman and MacNee 2000). Flere studier har rapportert en senkning i nivåer av GSH etter eksponering til arsen, noe som indikerer at arsen hemmer GSH-dannelse (Jomova et al. 2011) Dette i kombinasjon med økt dannelse av ROS gjør arsen til en potent oksidant.

1.3 Inflammasjon



Figur 3. Mekanismer for inflammasjon i lungene. (Figuren er gjengitt med tillatelse fra J. Øvrevik, Nasjonalt Folkehelseinstitutt)

Inflammasjon er en viktig beskyttelsesprosess for kroppen og har normalt terapeutisk virkning. Men kronisk inflammasjon kan gi ugunstige helseeeffekter og i luftveiene kan dette føre til lungesykdommer som astma, kronisk obstruktiv lungesykdom (KOLS) og lungekreft (Aggarwal et al. 2006; Rahman and Adcock 2006; Yang and Omaye 2009). PM og metaller i PM har blitt vist å være faktorer som kan utløse kronisk inflammasjon i lungene med påfølgende utvikling eller forverring av sykdommer (Ling and van Eeden 2009; Schaumann et al. 2004). I påfølgende avsnitt vil jeg skissere opp noen viktige mekanismer involvert i inflammasjon relatert til mitt studie. Det er viktig å huske på at disse mekanismene ikke er uavhengige, men i stor grad interagerer med hverandre og også andre mekanismer i det komplekse maskineriet som regulerer inflammatoriske responser.

1.3.1 Cytotoksisitet

Celledød ble tidligere inndelt i apoptose, autofagisk celledød og nekrose (Lockshin and Zakeri 2001). Per i dag er det en rekke måter å karakterisere celledød på, og mange nye klassifiseringer har kommet til (Tang et al. 2008). Jeg vil her konsentrere meg om de som har størst relevans for mitt studie. Jeg har valgt å benevne dette under betegnelsen cytotoksisitet i stedet for celledød fordi jeg her også inkluderer mitotisk arrest. Som nevnt nedenfor er utfallet av mitotisk arrest ofte, men ikke alltid celledød.

1.3.1.1 Nekrose

Nekrotisk celledød eller nekrose er karakterisert ved økning i cellevolum (oncosis), degradering av organeller og oppløst plasmamembran (Kroemer et al. 2009). Ved at potensielt toksiske cellekomponenter frigis som følge av dette kan ytterligere skade skje i nærliggende vev, og inflammatoriske reaksjoner oppstå.

1.3.1.2 Apoptse

I mosetning til nekrose er apoptose en essensiell mekanisme for å opprettholde homeostase i organismer (Tang et al. 2008). Apoptose kjennetegnes ved en reduksjon i cellevolum (pyknosis), kromatinkondensasjon og fragmentering av nukleus. Membranen oppløses ikke og apoptotiske celler blir fagocytert og gir dermed ikke potensiell inflammasjon som nekrose (Kroemer et al. 2009).

1.3.1.3 Mitotisk arrest

Ved skader på DNA før selve mitosen eller ved skader på spindelapparatet kan mitotisk arrest oppstå (Blagosklonny 2007). Mitotisk arrest er ingen endelig skjebne. Cellen vil derfor enten dø, som oftest ved apoptose, eller i sjeldne tilfeller unnslippe mitotisk arrest og fortsette normal funksjon eller eventuell påfølgende arrest i G_1 (Blagosklonny 2007).

1.3.2 Cytokiner

Tidlig i en inflammatorisk respons frigjør immunceller, blant annet epitelceller, en rekke signalmolekyler (Schwarze et al. 2010). Blant disse finner en cytokiner, små proteiner som virker som signalmolekyler mellom celler. Cytokinene inngår i reaksjoner ment å regulere og minimere skade. Kjemokiner er en type cytokiner som har som funksjon å tiltrekke immunologiske celler til skadestedet (Mukaida 2000).

CXCL8 er en av de viktigste kjemokinene, og kan produseres av leukocytter samt endotelceller, fibroblaster og epitelceller (Mukaida 2000). CXCL8-produksjon induseres gjerne av proinflammatoriske cytokiner som IL-1 og tumor nekrose faktor alfa (TNF-α), men kan også induseres direkte av bakterier og (Mukaida 2000). Dessuten har miljøfaktorer blitt vist å kunne indusere CXCL8-produksjon, blant annet gjennom ROS-dannelse. Dette kan aktivere NF-κB,, som sammen med AP-1 trolig er involvert i det det meste av gentranskripsjonen til CXCL8 (Janssen-Heininger et al. 2000). Etter frigjøring fra cellen vil CXCL8 fungerer som en potent kjemo-attraktant for nøytrofiler (Mukaida 2000).

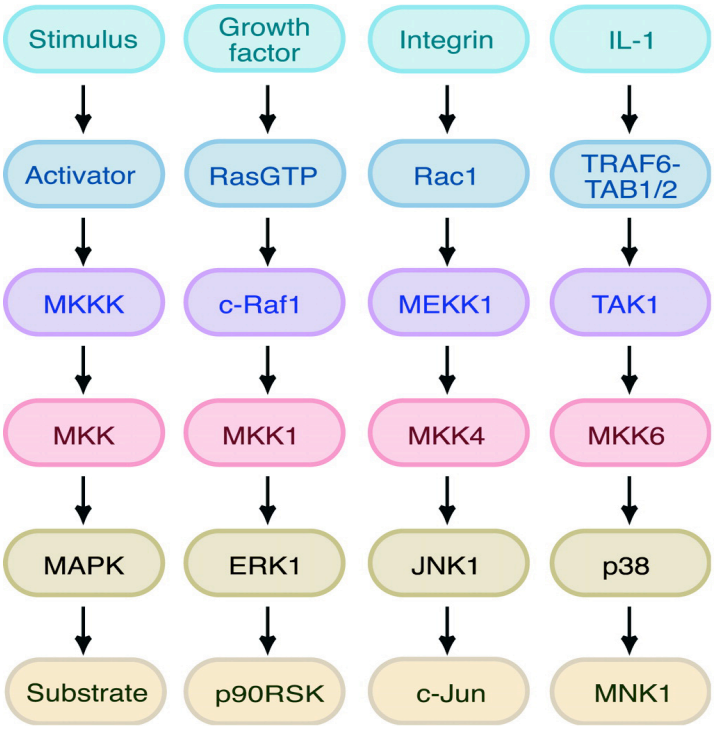
Økte nivåer av CXCL8 har blitt assosiert med dannelse og forverring av lungesykdommer som KOLS og bronkitt, samt utvikling av astma. Det har også blitt foreslått at CXCL8 har en rolle i utvikling av visse typer kreft (Mukaida 2003).

1.3.3 MAPK

Protein kinaser er enzymer som binder fosfat til sidekjeden til serin, threonin eller tyrosin hos spesifikke intracellulære proteiner. MAPK responderer på ekstracellulære stimuli, blant annet vekstfaktorsignaler, stress og proinflammatoriske cytokiner, og fosforylerer en rekke faktorer som blant annet inngår i regulering av ekspresjon, proliferasjon, metabolisme og apoptose (Johnson and Lapadat 2002).

Det er tre godt beskrevne subfamilier av MAPK, ekstracellulære signalregulerte kinaser (ERK (1 og 2)), c-jun aminoterminalkinaser (JNK (1,2 og 3)) og p38 (α, β, γ

og δ) (Johnson and Lapadat 2002). Det bør nevnes at flere andre er funnet, og at mulige kandidater også studeres.



Figur 4. Illustrasjon av prinsippene bak MAPK-signalveien og en forenklet visning av signalveiene til de tre best beskrevne subfamiliene (Johnson and Lapadat 2002).

ERK1 og ERK2 er involvert i regulering av meiose, mitose og postmitotiske funksjoner. Aktivering av ERK er vanligvis knyttet til celleoverlevelse og proliferasjon, men ERK kan også indusere apoptose i noen celletyper (Zhuang and Schnellmann 2006). ERK har blitt knyttet til mulig kreftutvikling og hemmere av denne er potensiell agent mot kreft (Johnson and Lapadat 2002). JNK1, JNK2 og JNK3 fosforylerer c-Jun som er en del av AP-1 komplekset og involvert i regulering av genekspresjon, blant annet for cytokiner. JNK er dessuten viktig i kontroll av apoptose (Johnson and Lapadat 2002). P38 dekker fire forskjellige kinaser: α, β, γ, and δ . P38-α er den vanligste og uttrykkes i en rekke typer celler. P38 blir aktivert av inflammatoriske cytokiner og er viktig i aktivering av immunresponsen, blant annet ved å indusere økt cytokinekspresjon og aktivering av NF-κB (Johnson and Lapadat 2002). Videre har p38 blitt vist å være en viktig mediator i apoptose (Chen et al. 2000).

MAPK har en sentral rolle ved inflammasjon, og både ERK, JNK og p38 har blitt knyttet til økt CXCL8-frigjøring (Samet et al. 1998).

1.3.4 NF-κB

Transkripsjonsfaktorer er proteiner som binder seg til spesifikke DNA-sekvenser og regulerer transkripsjonen. NF-κB er blant de mest sentrale transkripsjonsfaktorene hos eukaryoter og er en viktig mediator i inflammasjonsresponsen. NF-κB er vanligvis en hetereodimer bestående av p50 og p65. I celleplasma er NF-κB bundet til I-κB (inhibitor av κB) i inaktiv form. Ved aktivering blir I-κB degradert via ubiquitinylering, fosforylering av NF-κB og påfølgende translokasjon til cellekjernen. Her binder NF-κB seg til κB elementer i promotor hos målgener og regulerer transkripsjon (Leonard et al. 2004)

Mange stimuli forbundet med inflammasjon kan aktivere NF-κB, blant annet MAPK signalisering, cytokiner og ROS. NF-κB induserer videre ekspresjon av gener involvert i immunforsvaret, blant annet mediatorer av inflammasjon, karsinogenese og antiapoptotiske reaksjoner. (Leonard et al. 2004).

1.3.5 Oksidativt stress

Biologiske systemer blir kontinuerlig eksponert for oksidanter enten generert endogent eller påført eksogent. Endogen dannelse kan skje ved metaboliske reaksjoner, som ved elektrontransportkjeden i mitokondrier eller ved aktivering av fagocytter. Eksogene kilder til oksidanter kan være eksponering for blant annet luftforurensning og sigarettrøyk. Lungene befinner seg i et oksygenrikt miljø og kombinert med et stort overflateareal er de sårbare for skader fra ROS (Rahman 2002). Vanligvis er cellen i stand til å regulere ROS-nivåer via antioksidanter og enzymer, men ved stress forårsaket av blant annet partikler involvert i redoks-reaksjoner og inflammatorisk respons kan ROS-nivået i cellen stige dramatisk og gi en tilstand av oksidativt stress (Lawless et al. 2010).

Oksidanter og inflammatoriske mediatorer aktiverer transkripsjonsfaktorer som NF-κB og AP-1 som videre induserer ekspresjon av pro-inflammatoriske gener (Rahman 2002). Endogen ROS er også vist å kunne hemme fosfatase-aktivitet og dermed indusere aktivering av MAPK (Lee and Esselman 2002). Dannelsen av ROS har blitt antydet å være en viktig initieringsfaktor for partikkelindusert inflammasjon, og helseproblemer assosiert med høye nivåer av PM har blitt satt i sammenheng med endringer i oksidativt stress (Donaldson et al. 2003; Risom et al. 2005; Yang and Omaye 2009). Oksidativt stress har blitt koblet til patogenesen til flere inflammatoriske lungesykdommer (Klaudia Jomova and Valko 2011; Lawless et al. 2010; Rahman and Adcock 2006)..

2. MÅLSETNINGER

Målsetningen med denne oppgaven var å kartlegge det inflammatoriske potensialet og toksisiteten til sink (Zn^{2+}) og arsen (arsenitt og arsenat) i den humane bronkiale epitelcellelinjen BEAS-2B. Vi fokuserte på hvordan metallene førte til frigjøring av kjemokinet CXCL8, forskjellige former for celledød og endringer i cellesyklus.

Delmål:

- Å undersøke cytotoksisiteten til metallene; å studere i hvilken grad de bidrar til nekrose, apoptose og endringer i cellesyklus.
- Å undersøke om metallene fører til cytokinfrigjøring, målt ved CXCL8-frigjøring.
- Å undersøke hvorvidt sink og arsenitt aktiverer MAPK og videre kartlegge eventuelle sammenhenger mellom spesifikke MAPK og frigjøring av metall-indusert CXCL8.
- Å undersøke i hvilken grad metallene induserer oksidativt stress og hvorvidt dette påvirker CXCL8-frigjøring, viabilitet og cellesyklus ved å bruke velkjente antioksidanter.
- Å undersøke forskjellige kombinasjoner av sink og arsenitt med hensyn på frigjøring av CXCL8 og viabilitet.

3. KONKLUSJON

- BEAS-2B celler eksponert for sink, arsenitt og arsenat ga både økt nekrose, apoptose og økt andel celler i mitose. Arsenitt induserte cytotoksisitet ved lavere konsentrasjoner enn sink og arsenat. Sink ga en en massiv økning i nekrose ved konsentrasjoner over 100 μM, noe som kan tyde på at den normale sink-homeostasen er ødelagt med en påfølgende stor økning i frie sink-ioner. Arsenitt og arsenat induserte en sterk økning av mitotiske celler, noe som kanskje indikerer mitotisk arrest.
- Økt CXCL8-frigjøring ble observert ved eksponering for både sink, arsenitt og arsenat, noe som indikerer en rolle for metallene i inflammatorisk respons. Begge metallene induserte dette ved lavere konsentrasjoner enn for cytotoksisitet.
- Både eksponering for sink og arsenitt ga hurtig fosforylering av MAP-kinasene p38 og ERK. Videre ga hemming av p38 og ERK nedgang i CXCL8-frigjøring indusert av sink og arsenitt. Hemming av p38 ga en reduksjon ned til basalnivåer, mens hemming av ERK ga bare en delvis reduksjon av CXCL8-frigjøring. Resultatene antyder at MAPK er involvert i induksjon av CXCL8-frigjøring.
- Både eksponering for sink og arsenitt aktiverte også NF-κB målt gjennom fosforylering av p65 og nedbrytning av den inhibitoriske enheten (I-kB), men på senere tidspunkt enn MAPK fosforylering. Arsenitt induserte den sterkeste økningen i fosforylert p65.
- Eksponering for både sink og arsenitt ga økning i oksidativt stress målt ved ROS-dannelse og induksjon av stress-proteinet HO-1. Sink ga raskere økning av HO-1, men etter 8 t ga arsenitt den sterkeste økningen av proteinnivået. Resultater etter eksponering for sink og arsenitt og forbehandling med antioksidanter ga indikasjoner på at ROS er involvert i skadevirkninger av sink og arsenitt. N-acetyl-cystein (NAC) ga sterk nedgang i CXCL8-frigjøring og nekrose indusert av metallene. Videre ga NAC nedgang i arsenitt-indusert mitoseopphoping, det samme gjorde difenylen iodonium (DPI).
- Kombinasjoner av sink og arsenitt i cellekulturene indikerte additive effekter både for CXCL8-frigjøring og viabilitet.

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Zinc- and arsenic-induced chemokine release and cytotoxicity in human bronchial epithelial cells

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KEYWORDS: Zinc, arsenic, epithelial lung cells, CXCL8, cell death.

ABBREVIATIONS

1-NP, 1-Nitro pyrene; AB; antibodies; APS, ammonium persulfate; BEAS-2B, human bronchial epithelial cells; BSA, bovine serum albumin; CXCL8, IL-8 (Interleukin 8); COX, Cyclooxygenase; DCFH-DA, 2',7'-dichlorfluorescein-diacetate; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; ELISA, Enzyme-Linked ImmunoSorbent Assay; ERK, extracellular signal–regulated kinase; FCS, fetal calf serum; GSH, glutathione; HO-1, heme oxygenase-1, H_2O_2, hydrogen peroxide; I-κB, inhibitor of kappa B; JNK, c-Jun amino-terminal kinase; MAPK, mitogen-activated protein kinase; MT, Methallothionein; NAC, N-acetyl-L-cysteine; NF-κB nuclear factor kappa B; PFA, paraformaldehyde; PM, Particulate matter.

ABSTRACT

In the present study we have examined and compared effects of zinc- and arsenic compounds on epithelial lung cells. Both metals are present in particulate matter (PM) and have been demonstrated to exert adverse health effects on humans as components in PM and also as single elements. Human bronchial epithelial cells (BEAS-2B) were exposed to Zn^{2+}, arsenite and arsenate. Experiments on cytotoxicity, determined by propidium iodide (PI) and Hoechst 33342 staining and fluorescence microscopy analysis, revealed that arsenite induced necrosis and apoptosis at lower concentrations than Zn^{2+} and arsenate. Furthermore, arsenite exposure induced an increase of cells in mitosis as demonstrated by fluorescence microscopy and flow cytometry, possibly indicating roles of arsenite in mitotic arrest. This was also observed for arsenate, but at higher concentrations. Zn^{2+}, arsenite and arsenate induced an increase in CXCL8 release at lower concentrations than induction of cell death. Both Zn^{2+} and arsenite seemed to activate the mitogen-activated protein kinases (MAPKs), as the levels of phosphorylated extracellular signal–regulated kinase (ERK) and p38 were increased after the metal exposure. Furthermore, inhibition of p38 reduced the metal-induced CXCL8 release to basal level and inhibition of ERK partially reduced the CXCL8 release. These findings indicate roles of MAPKs in the release of CXCL8. Moreover, the transcription factor nuclear factor kappa B (NF-κB) was activated by both metals. Zn^{2+} and arsenite both induced oxidative stress, as determined by measures of levels of H_2O_2 (DCFD-HA), O_2^- (DHE) and induction of the stress-induced protein heme oxygenase-1 (HO-1) at concentrations that induced the maximum CXCL8 release. Pretreatment with the antioxidant N-acetyl cysteine (NAC) decreased the CXCL8 release induced by Zn^{2+} and arsenite and the number of cells in mitosis induced by arsenite. The NAD(P)H oxidase inhibitor diphenylene iodonium (DPI) did not reduce CXCL8 release, but decreased the number of cells in mitosis. Our data could also suggest an additive effect of the combined exposure of Zn^{2+} and arsenite on CXCL 8 release and cytotoxicity. In conclusion, both Zn^{2+} and arsenite induced inflammatory responses including oxidative stress, cytotoxicity and CXCL8 responses in epithelial lung cells, supporting a possible role of the metals in PM-induced adverse health effects. Formation of reactive oxygen species (ROS) and activation of MAPKs and NF-κB seemed to be involved.

INTRODUCTION

Particulate matter (PM) is a complex mixture of components with various sizes and contents dependent on the sources. Urban PM may contain different metals, such as zinc and arsenic (Laden et al. 2000; Sanchez de la Campa et al. 2011). Sources related to fossil fuel combustion are major contributors to metals in PM as well as mining and other industry-related emissions (WHO 2006). Tire-wear is also a contributor to zinc-laden particles in PM (Councell et al. 2004). It has been found that metals may be a major factor in lung injury caused by PM (Chen and Lippmann 2009; Costa and Dreher 1997; Molinelli et al. 2002).

Both zinc and arsenic in PM, and also as single elements, have been associated with lung inflammation and cellular toxicity (Adamson et al. 2000; Gordon et al. 1992; Jomova and Valko 2011). Zinc has been suggested as one of the main components in PM contributing to toxic effects (Adamson et al. 2000). However, zinc is also known to be an essential metal for humans, playing a central role in catalytic activity of many enzymes and in the regulation of transcriptional activity (Prasad 2009b). Impairment of zinc homeostasis may have serious physiological consequences (Nodera et al. 2001; Prasad 2009b). Exposure to arsenic has been associated with increased cancer incidence and potential toxicity includes oxidative stress, DNA damage and activation of certain signal transduction pathways (Huang et al. 2004; Jomova et al. 2011). Arsenic is able to form both inorganic and organic compounds in the environment and also within the human body (Hei and Filipic 2004). Inorganic arsenic is in general more toxic than organic arsenic and the trivalent form arsenite is considered the most toxic (Jomova et al. 2011).

When acute inflammation is manifested for a short period of time, it may have a therapeutic consequence. However, when inflammation becomes chronic or lasts too long, it can prove harmful and may lead to and exacerbate human pulmonary diseases like asthma, chronic obstructive pulmonary disease (COPD) and lung cancer (Aggarwal et al. 2006; Rahman and Adcock 2006; Yang and Omaye 2009). Cytotoxicity plays a central role in the inflammatory process and may lead to cell death. Necrosis is a type of cell death that is characterized by a degradation of the

organelles and swelling of the cytoplasm resulting in the loss of the membrane integrity. This may lead to additional toxicity by exposing the nearby cells to harmful cellular components and further trigger inflammatory processes (Kroemer et al. 2009). Apoptosis is characterized by rounding-up of the cell, reduction of cellular volume , chromatin condensation, nuclear fragmentation and finally engulfment by phagocytes (Kroemer et al. 2009). The plasma membrane in apoptotic cells is usually not ruptured, as opposed to necrotic cells. Hence, this particular type of cell death is usually considered less inflammatory, although apoptotic cells may in some cases become necrotic (Leist and Jaattela 2001; Nicotera et al. 1999). Higher concentrations of both zinc and arsenite are known to induce necrosis or apoptosis in several cell lines (Formigari et al. 2007; Iitaka et al. 2001). On the other hand, lower zinc concentrations have been shown to reduce apoptosis in zinc-deficient animals (Nodera et al. 2001). Another outcome of cytotoxicity, mitotic arrest, may be induced temporarily by influence on the spindle dynamics involved in mitosis or by DNA damage prior to or during mitosis (Blagosklonny 2007). The cell cannot be arrested in mitosis indefinitely, so ultimately it will either die by apoptosis or necrosis, or exit mitosis by mitotic slippage (Blagosklonny 2007). Arsenite has been linked to the induction of mitotic arrest in several cell types (Huang and Lee 1998; Yih et al. 2005).

Furthermore, inflammation involves a complex set of reactions such as formation of reactive oxygen species (ROS), activation of transcription factors and release of cytokines leading to the recruitment of immune- and inflammatory cells to the lungs. CXCL8 plays a central role in the inflammatory response and is released by cells of the immune system and also from epithelial cells (Mukaida 2003). The release of CXCL8 leads to recruitment of neutrophils among other cells and further intensification of the inflammatory response. These biological functions suggest that CXCL8 may have crucial roles in various pathological conditions such as chronic inflammation and cancer (Mukaida 2003). Both zinc and arsenite has been found to induce an increase in the inflammatory response as well as increase the CXCL8 release in airway epithelial cells (Jaspers et al. 1999; Kim et al. 2006).

Multicellular organisms have three well-characterized subfamilies of mitogen-activated protein kinases (MAPKs) that control a vast array of physiological processes, many of them important for inflammatory responses. Signaling through the

MAPK pathways may lead to a phosphorylation-dependent activation of a variety of transcription factors that modulate cytokine gene expression (Samet et al. 1998). The extracellular signal–regulated kinases (ERKs) are involved in the control of cell division. Thus, regulation of meiosis, mitosis as well as post-mitotic functions may be regulated by ERK (Johnson and Lapadat 2002). The c-Jun amino-terminal kinases (JNKs) are critical regulators of transcription and may also be involved in controlling apoptosis (Tournier et al. 2000). The p38 MAPKs have been demonstrated to regulate the expression of many cytokines (Johnson and Lapadat 2002). Furthermore, activation of p38 in immune cells by inflammatory cytokines appears to play a major part in human disease (Johnson and Lapadat 2002). Exposure to zinc and arsenic has been shown to cause activation of all three MAPKs mentioned in human airway epithelial cells (Samet et al. 1998).

A transcription factor, nuclear factor kappa B (NF-κB) has a key role in inflammatory processes. NF-κB may be activated by a variety of stimuli including cytokines, MAPK signaling and ROS (Leonard et al. 2004). It is recognized as an important regulatory factor mediating the co-ordinate expression of genes, which are part of the cellular machinery that functions to protect an organism against damage (de Martin et al. 1999). The role of NF-κB upon arsenite and zinc exposure is somewhat complicated, as these metals have been demonstrated to both induce and inhibit activation of NF-κB (Jomova and Valko 2011; Leonard et al. 2004).

Numerous reports link oxidative stress to PM-induced adverse health effects. Increasing evidence is being collected that reactive oxygen species (ROS) and oxidative stress are involved in PM-mediated inflammation and injury (Xia et al. 2006). The oxidative capacity of PM has primarily been attributed to its transition metal constituents, as transition metals are able to catalyze one-electron reductions of oxygen necessary to generate ROS (Tao et al. 2003). Although arsenic is not considered a transition metal, many studies have confirmed the generation of various types of ROS during arsenite metabolism in cells (Jomova et al. 2011). Studies on the impact of zinc, however, have revealed different effects of exposure. Lower concentrations of zinc have been reported to reduce ROS levels because of its antioxidant properties (Prasad 2009a), however, it has been suggested that zinc has an

indirect role in the formation of ROS by oxidation of thiol groups on proteins (Maret 2009).

The aim of this study was to examine the potential of zinc (Zn^{2+}) and arsenite to induce CXCL8 responses, oxidative stress and cytotoxicity in the human epithelial cell line BEAS-2B. The cell death induced by the metals was characterized. Combinations of metals were also examined. Furthermore, the involvement of oxidative stress and the MAPKs ERK and p38 in CXCL8 release and cytotoxicity were studied.

MATERIALS AND METHODS

Chemicals

LHC-9 cell culture medium was supplied by Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) was supplied by Gibco BRL (Paisley, Scotland, UK). Zinc chloride ($ZnCl_2$) and sodium arsenate ($NaAs_3O_4$) were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Sodium arsenite ($NaAsO_2$) was supplied by Merck and Co, Inc (New Jersey, NJ, USA). CXCL8 (Human IL-8 Cytoset) for Enzyme-Linked ImmunoSorbent Assay (ELISA), 2',7'-dichlorfluorescein-diacetate (DCFH-DA) and dihydroethidium (DHE) was purchased from Invitrogen (Carlsbad, CA, USA). Acrylamide/bis solution, ammonium persulfate (APS), Immun-star Western C luminol/enhancer and peroxide solution, Precision plus protein dual extra standards, reagent A, B and S protein assay kit and tetramethylethylenediamine (temed) were supplied by Bio-Rad Laboratories, Inc (Hercules, CA, USA). Sulfur acid (H_2SO_4), glycerol and hydrogen peroxide (H_2O_2) were purchased from Merck and Co, Inc (New Jersey, NJ, USA). Re-blot plus mild and strong stripping solutions were supplied by Millipore (Billerica, MA, USA). 1-Nitropyrene (1-NP), sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), Hoechst 33258, Hoechst 33342, menadione, N-acetyl cysteine (NAC), diphenyl iodonium (DPI), thiourea, paraformaldehyde (PFA), Ponceau S, propidium iodide (PI), tetramethylbenzidine (TMB) and polyoxyethylene octyl phenyl ether (triton X-100) were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Antibodies (ABs) against: Cyclooxygenase (COX), Heme oxygenase (HO-1), inhibitor of kappa B (I-κB), c-Jun amino-terminal kinase (JNK), p-JNK, p-p65, p38 and p-p38 were supplied by Cell Signaling (Berverly, MA, USA). ABs against extracellular signal-related kinase (ERK) and p-ERK were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). AB against β-actin was from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Secondary ABs horseradish peroxidase-conjugated goat-anti-rabbit and horseradish peroxidase-conjugated rabbit anti-mouse was purchased from Dako (Glostrup, Denmark). AB against phospho-

histone H3 (pS28) was supplied by Invitrogen (Carlsbad, CA, USA). All other chemicals were purchased from commercial sources and were of analytical grade.

Cell culture

The BEAS-2B cells are a transformed bronchial epithelial cell line immortalized with SV40-adenovirus hybrid (Ad12SV40). The cell line was provided by Dr C.C. Harris (Reddel et al. 1988) and is commercially available from the European Collection of Cell Cultures (ECACC, Salisbury, UK). 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. Serum-free LHC-9 medium was used and the cells were grown on collagen (PureCol^{TM})-coated culture wells, dishes or flasks.

When splitting, the cells were washed with phosphate-buffered saline without Ca2+ and Mg2+ (Dulbecco´s PBS) before adding 0.0025% trypsin to re-suspend the cells that had adhered to the surface. The cells were then incubated for 3-5 minutes at 37°C. The trypsin was inactivated by adding LHC-9 medium containing 20% FCS. This was followed by centrifugation by 150 x g for 7 min at 4 ^oC , the supernatant was removed and fresh LHC-9 medium was added. Finally, the cells were transferred into new coated flasks and grown further.

Exposure

BEAS-2B cells were plated in wells (4.6 x 10^5 cells/well and 1 ml medium) or dishes (1.8 x 10^6 cells/well and 10 ml medium) and grown close to confluence. The medium was changed 24 h before exposure and also immediately before the exposure. When inhibitors were used, these were added 1 h before adding the metals. The cells were exposed to Zn^{2+} ($ZnCl_2$), arsenite ($NaAsO_2$), arsenate (Na3AsO4) or 1-NP for 0.5 h – 24 h. Selections of exposure time and metal-concentrations were calculated based on results from previous experiments conducted in the lab or by others. Dissolvents used were physiological salt solution (0.9 % NaCl), DMSO (>0.5% of final volume), Dulbecco´s PBS or LHC-9 medium depending on the solute.

Light microscopy

A Nikon TMS-F inverted light microscope was used to observe the morphology of the cells before exposure, after adding inhibitors and following metal exposure. Also, the light microscope was used for counting viable cells in the inhibition experiments before the metals were added to test whether the inhibitors themselves altered proliferation. The magnification used was 1000x.

Fluorescence microscopy

Following exposure for 24 h, the cells were centrifuged by 250 x g for 10 min at 4 ^{o}C. The supernatant was removed and kept at -40 ^{o}C before being analyzed by ELISA (see below). This was followed by treatment with trypsin on the remaining cells in the culture dish for 2 min and fresh LHC-9 medium with 20 % FCS was added to inactivate the trypsin. The suspension was centrifuged and the supernatant removed. Finally, this pellet (the attached cells) was added to the pellet from the medium (i.e. the floating cells) and 500 µl fresh LHC-9 medium. Cells were then analyzed by light microscopy or further treated for analysis by fluorescence microscopy.

Hoechst 33342 (5 µg/ml) and PI (10µg/ml) were added after exposure to stain the cells. After 30 min incubation in the dark at room temperature, the cells were centrifuged by 250 x g for 10 min at 4 ^{o}C before being resuspended with FCS and mounted on microscope slides. Hoechst 33342 (blue stain) is able to penetrate an intact plasma membrane and stain DNA, whereas PI (red stain) only stains DNA in cells with disrupted plasma membranes. The cells were categorized as viable, apoptotic, necrotic, mitotic or apoptotic-mitotic based on color and morphology by using a Nikon Eclipse E-400 with a UV-2A excitation filter and a magnification of 1000x. On each slide, 300 cells were counted.

Flow cytometry

By flow cytometry it is possible to analyze both the size and the optical properties of a suspension of cells, using a highly focused laser-beam. The resultant light-scattering

can be related to the attributes of the cells. Also, the absorption or emission of fluorescence from cell components stained with a fluorescent dye may be quantified (Otsuki et al. 2003).

Flow cytometry general preparation

Following exposure, the cells were prepared for flow cytometry by fixation in PFA (1% of total volume) for 15min. Triton X-100 (0.1% of total volume) was added to make the cells more permeable, followed by the addition of the dye. The flow cytometer used was BD LSRII (BD BioSciences, San Jose, CA, USA). Measurements were made with the BD Facs Diva software according to the manufacturer´s guidelines.

Cell cycle analysis by flow cytometry

By measuring the fluorescence of Hoechst 33258 this allowed us to get an indication of the amount of DNA in the cells, which helped determine the different cell cycle phases. Following exposure for 24 h and flow cytometry preparation, Hoechst 33258 (5 μg/ml) was added. This was followed by 30 min incubation in room temperature and a measurement of the Hoechst 33258 fluorescence. Additional measuring was performed using the Multicycle AV DNA analysis software for automatic determination of cell cycle phases.

Phospho-histone H3 measuring by flow cytometry

Another analysis performed by flow cytometry was the measurement of the amount of phospho-histone H3 in the cells (Goto et al. 1999). During mitosis, H3 is phosphorylated at serine 28 as well as other sites. Following exposure for 24 h and flow cytometry preparation, anti-phospho-histone H3 (pS28) (5 μg/ml) was added at the simultaneously as Hoechst 33258 followed by 30 min incubation in room temperature and measurements were taken of the anti-phospho-histone H3 (pS28) and Hoechst 33258 fluorescence.

Measuring of ROS-formation by flow cytometry

Flow cytometry may also be used for measuring ROS. In the presence of ROS, DCFH-DA is oxidized by H_2O_2. The resultant derivates will emit fluorescence, thus it may be used as an index to quantify the overall ROS in the cells (LeBel et al. 1992). DHE is able to freely permeate cell membranes and will form a red fluorescent product (2-hydroxyethidium) upon reaction with superoxide anions (O_2^-) (Amir et al. 2008). Following exposure for 2 h – 8 h and flow cytometry preparation, DCFH-DA (5 μM) and DHE (5 μM) were added, followed by 30 min incubation at room temperature and measurements of DCFH-DA and DHE fluorescence.

ELISA

ELISA is considered the standard cytokine measurement method and is widely used in clinical laboratories and biomedical research. The results are highly quantitative and generally reproducible (Leng et al. 2008). Exposure for 24 h was followed by centrifugation by 250 x g for 10 min at 4 ^oC and the supernatant was then stored at -40 ^oC. Cytokine CXCL8 (Human IL-8 Cytoset) levels were measured by "sandwich ELISA", i.e. a double binding to the antigene, which makes the method more specific. The capture AB was first added to the wells followed by the selected antigene and lastly the detecting AB. A substrate consisting of streptavidin and horse radish peroxidase (HRP) that binds to the AB-antigen-complex was used. Activation of HRP was performed by the addition of citrate-buffer, TMB and H_2O_2. The fluorescence emitted by HRP was measured and compared to a protein standard using Magellan V 2.22 software and the plate reader used was TECAN sunrise (Phoenix Research Products, Hayward, CA, USA).

Western blotting immunoassay

By Western blotting we may identify specific proteins according to their size by detecting them whem with ABs. Following exposure for 30 min – 8 h in dishes, the cells were washed twice with cold Dulbecco's PBS and the dishes with attached cells were stored at -70 ^oC. Later, the cells were thawed and lysed by a 200 μl lysate buffer (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) for 15 min. The solutions were then sonicated, centrifuged and the supernatant was collected. Protein concentration was measured using the Bio-Rad DC protein assay kit. The solutions were adjusted to equal protein concentrations using lysate-buffer, 5x SDS and 87% glycerol, followed by heating at 95^{o}C for 5 min. The samples were now ready for use by SDS polyachrylamide gel electrophoresis (SDS-PAGE).

After loading of the samples (10 μg protein) and precision plus protein dual extra standards (8 μl) into different wells, 10% SDS-PAGE was performed. Following this, the proteins were transferred by electroblotting to nitrocellulose membranes. Equal protein loading on the membrane was controlled by Ponceau S staining. Blocking of the membrane was done by adding a 3% dry milk solution. Primary antibody for the specific protein was then applied to the membrane, and after a 2 h incubation at room temperature, secondary antibody containing horseradish peroxidase (HRP) was added for 2 h. Finally, incubation of the membrane in a luminol/enhancer and peroxide buffer solution for 5 min was performed, followed by the processing of an image of the membrane with the desired protein using Bio-Rad ChemiDoc XRS+ processing hardware and Bio-Rad Imagelab software.

Statistical analysis

Statistical significance was evaluated by using analysis of variance (ANOVA) using the Bonferroni post-test for two-way ANOVA and the Dunnett post-test for one-way ANOVA. $P<0.05$ was considered a statistically significant difference. Values were presented as means ± SEM. All calculations were performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Cytotoxicity

To address the cytotoxicity of arsenic and zinc the BEAS-2B cells were exposed to 0-110 μM Zn^{2+}, 0-20 μM arsenite or 0-200 μM arsenate for 24 h. The cell morphology was observed by light microscopy and the number of dead cells (floating cells) was roughly estimated (Figure 1). It should be noted, however, that mitotic cells have a similar morphology as apoptotic and necrotic cells when seen in the light microscope. Exposure to 100 μM Zn^{2+} induced a toxicity shown by the presence of a large number of detached cells, whereas exposure to 20 μM arsenite appeared to result in less toxicity.

By staining with Hoechst 33342 and PI we were able to determine the cytotoxicity by fluorescence microscopy. As illustrated in Figure 2, after exposure for 24 h to the metals PI positive cells were classified as necrotic and cells with bright Hoechst and condensed or fragmented nuclei as apoptotic (also if PI positive). PI negative and Hoechst positive cells with clearly visible chromosome condensation were classified as mitotic and similar cells with brighter Hoechst indicating apoptosis, as apoptotic/mitotic.

Both exposure to Zn^{2+} and arsenite induced a significant increase in necrosis (Figure 3). For Zn^{2+}, a marked, significant increase occurred at 110 μM. For arsenite the necrosis increased gradually, with a significant increase from 15 μM. The increase of necrosis was, however, markedly higher for Zn^{2+} with a maximum of 48% compared to a 5% maximum at 20 μM for arsenite. Arsenate exposure showed a similar tendency as arsenite, but the increase was not significant. A significant increase in apoptosis was observed at exposure to 110 μM for Zn^{2+} and at 20 μM for arsenite, with 19 % and 4% at maximum, respectively (Figure 3). Again, arsenite exposure induced similar non-significant tendencies as arsenite. Necrosis and apoptosis further increased upon exposures to higher concentrations of arsenite (Appendix: Figure 15).

When the number of cells undergoing mitosis was analyzed, no significant changes were observed for exposure to Zn^{2+} (no data shown), while both arsenite and arsenate showed a significant increase from 1 μM and 50 μM, respectively (Figure 3). Such accumulation in mitosis may be due to mitotic arrest. For arsenite, the percentage of mitotic cells counted by fluorescence microscopy was approximately 10 % at maximum (20 μM) and for arsenate 15 % at maximum (150 μM). Similar results were seen for apoptosis/mitosis, with a significant increase occurring from 5 μM for arsenite and from 100 μM for arsenate. Maximal percentages of mitotic-apoptotic cells were approximately 5 % (20 μM) and 8 % (150 μM), respectively. Phosphorylation of histone H3 is linked to the initiation of chromosome condensation and is a marker for normal mitotic cells (Goto et al. 1999). Arsenite exposure induced a marked increase in phosphorylation of histone H3 by flow cytometry, a small increase was also observed for Zn^{2+} (Appendix: Figure 17).

Cell cycle changes

By flow cytometry we were able to determine differences in cell cycle phase (Figure 4). BEAS-2B cells were exposed for 24 h to 0-110 μM Zn^{2+} or 0- 20 μM arsenite and Hoechst 33258 added. Both exposure to Zn^{2+} and arsenite induced a significant decrease of cells in G_1 compared to unexposed control (Figure 5). A significant increase in G_2 or the mitotic phase (G_2/M) was found for both metals, with arsenite being the most potent. For arsenite, significant changes were observed from 10 μM for both G_1 and G_2/M with 15 μM inducing the largest difference. Exposure to Zn^{2+} showed significant changes for G_1 and G_2/M at 90 μM, however, higher concentrations induced a decrease of cells in G_2/M. The increase of cells in G_2/M was higher for arsenite than for Zn^{2+}. Neither Zn^{2+}, nor arsenite markedly affected the percentage of cells in the S-phase.

Cytokine release

To evaluate inflammatory potential, BEAS-2B cells were exposed to 0-110 μM Zn^{2+}, 0-20 μM arsenite or 0-200 μM arsenate for 24 h followed by measuring of CXCL8 release by ELISA. Both metals induced a significant increase in CXCL8 release (Figure 6). Zn^{2+} induced a significant increase in CXCL8 levels at 100 μM followed

by a decrease, possibly because of increased cell death. Arsenite and arsenate induced a significant increase from 10 μM and 150 μM, respectively. The magnitude of the maximal response was most pronounced for arsenite (approximately 8-fold at 20 μM) compared to Zn^{2+} and arsenate (approximately 3-fold at 100 μM and 200 μM, respectively).

Intracellular signaling

MAPKs

We wanted to examine to what extent Zn^{2+} and arsenite activated MAPKs and how this affected CXCL8 release and cell viability. Western analysis of cell lysates exposed to 100 μM Zn^{2+} or 20 μM arsenite (the concentrations that induced maximum CXCL8 increase) for 30 min – 4 h was performed. We found an early increase (30 min) in p38 and ERK phosphorylation (p-p38 and p-ERK, respectively) in arsenite-exposed cells (Figure 7A, 7B). Zn^{2+}-exposed cells also increased phosphorylation, albeit later (1 h). At 4 h a decrease in phosphorylation was observed for ERK after exposure to either metal, however, for p38 only exposure to Zn^{2+} showed a decrease of phosphorylation. An AB against phosphorylated JNK was also tested, but failed to show any proteins (data not shown) possibly indicating a non-functioning AB, as reported by others at FHI.

To further characterize the involvement of MAPKs in metal-induced CXCL8 release, we applied selective inhibitors of different MAPKs. BEAS-2B cells were pretreated with the p38-inhibitor SB202190 and the ERK-inhibitor PD98059 for 1 h before 100 μM Zn^{2+} and 20 μM arsenite were added. After 24 h a significant decrease in CXCL8 release measured by ELISA was found for both Zn^{2+}- and arsenite-exposed cells in the presence of the p38-inhibitor (Figure 8A) compared to no inhibitor. However, in the presence of the ERK-inhibitor only Zn^{2+}-exposed cells induced a significant decrease of CXCL8. No differences in viability were observed for cells pretreated with inhibitors and cells not inhibited (Figure 8B).

NF-κB

NF-κB is a heterodimer usually consisting of the p65 and p50 subunits and is kept in an inactive state by the binding to I-κB. The phosphorylation of the p65 subunit was measured by Western analysis of cell lysates following 30 min –4h exposure to 100 μM Zn^{2+} or 20 μM arsenite. Phosphorylation of p65 was observed already after 30 min both for Zn^{2+} and arsenite (Figure 7C). There was a marginal, further increase in phosphorylation with the exposure time. However, at 4 h a dramatic increase in the level of phosphorylated p65 was detected for arsenite. As expected, the suppressive protein I-κB was down-regulated both by exposure to Zn^{2+} and arsenite exposure (Figure 7D). Although, the observed difference between the metals at 4 h in p65 phosphorylation was not reflected in a similar difference with respect to I-κB levels. The roles of NF-κB in CXCL8-induction or cytotoxicity were not examined.

Oxidative stress

Flow cytometric measurements of ROS

To evaluate the potential for ROS-formation, BEAS-2B cells were pretreated with DCFH-DA or DHE for 1 h before 2-8 h exposure of 75 μM Zn^{2+}, 100 μM Zn^{2+}, 20 μM arsenite or 50 μM arsenite. DCFH-DA and DHE fluorescence indicates the presence of H_2O_2 and O_2^-, respectively and was measured by flow cytometry. Exposure to 75 μM Zn^{2+} induced a significant increase in DCFH-DA fluorescence at 4 h, whereas 100 μM Zn^{2+} significantly decreased the fluorescence at 8 h (Figure 9A). The decrease may be because of increased cell death. Arsenite increased fluorescence significantly, 20 μM arsenite had a maximum response at 4 h and 50 μM at 2 h (Figure 9B). DHE fluorescence was increased significantly at 4 h for exposure to both concentrations of Zn^{2+} (Figure 10A). A significant increase was observed for 20 μM arsenite at 8 h (Figure 10B).

HO-1 expression

To further characterize oxidative stress induced by Zn^{2+} and arsenite, Western analysis of HO-1 expression in cell lysates was performed following 4 h or 8 h

exposure to 75 μM Zn^{2+}, 100 μM Zn^{2+} or 20 μM arsenite. Figure 11 shows that exposure to 75 μM Zn^{2+} induced a marked increase of HO-1 at 4 h and further increased at 8 h. For 100 μM Zn^{2+} a markedly lower increase was observed. This may be accounted for by increased cell death. The increase of HO-1 by arsenite was delayed, however, at 8 h the increase in HO-1 levels was higher than for Zn^{2+}.

Effects of antioxidants on CXCL8 release

BEAS-2B cells were pretreated with the antioxidants DPI (1 μM) thiourea (20 μM) or NAC (40 mM) for 1 h before exposure to 100 μM Zn^{2+} or 20 μM arsenite and investigated for CXCL8 release and cytotoxicity after 24 h exposure to the metals. Only cells pretreated with NAC and exposed to 20 μM arsenite showed a significant decrease in CXCL8 release, however, NAC also tended to decrease the Zn^{2+}-induced CXCL8 levels (Figure 12A). Pretreatment with DPI and thiourea showed either a slight decrease or no changes of CXCL8 release. Furthermore, NAC pretreated cells showed a significantly higher viability when exposed to either zinc or arsenite (Figure 12B). Also, NAC induced a significantly lower necrosis upon Zn^{2+} exposure (Figure 12C) and a significantly lower mitosis upon arsenite exposure (Figure 12D). DPI also decreased the number of mitotic cells induced by arsenite significantly (Figure 12 D). Neither inhibitor changed apoptosis significantly (data not shown).

Combined exposure of zinc and arsenite

We wanted to clarify whether there were any combinatorial effects of exposures to combinations of arsenite and Zn^{2+}. BEAS-2B cells were exposed to either only 0-20 μM arsenite or 0-20 μM arsenite + 100 μM Zn^{2+} for 24 h. A significant increase was found in the CXCL8 release from 15 μM arsenite (Figure 13A). Also, a tendency to an increase in CXCL8 release in the combination of the metals up to 10 μM arsenite (+100 μM Zn^{2+}) compared to arsenite alone was observed. With higher concentrations of arsenite the CXCL8 levels decreased, which may be explained by increased cell death. The CXCL8 release after exposure to arsenite in combination with Zn^{2+} was higher than after exposure to the corresponding concentration of arsenite. When exposing to arsenite alone a significant decrease in viability was found

starting at 10 µM arsenite (Figure 13 B). For the combination of arsenite and Zn^{2+} the concentration curve showed lower viability than arsenite alone.

The concentration of arsenite that in combination with 100 µM Zn^{2+} gave the highest CXCL8 release (10 µM) was combined with 0 – 110 µM Zn^{2+}. Although no significant differences were observed, there was a tendency of CXCL8 release to increase for the combination of the metals up to 75 µM Zn^{2+} (+10µM arsenite) (Figure 14A). The CXCL8 release after exposure to Zn^{2+} in combination with arsenite was higher than after exposure to the corresponding concentration of Zn^{2+}. Generally, a lower viability was observed in the combination of metals compared to Zn^{2+} only (Figure 14B).

DISCUSSION

Metals in PM have been recognized in playing an important role in the mediation of adverse health effects both in the airways and the cardiovascular system (Chen and Lippmann 2009; Jomova and Valko 2011). In this study we observed zinc- (Zn^{2+}) and arsenic- (arsenite and arsenate) induced effects in the bronchial epithelial cell line BEAS-2B. Our most important findings include the ability of both metals to induce oxidative stress and CXCL8 release in BEAS-2B cells. Furthermore, the CXCL8 responses appeared to be mediated by the activation of p38 and to a lesser extent ERK. Exposure to arsenite induced oxidative stress, CXCL8 release, cytotoxicity and cell cycle changes at lower concentrations than Zn^{2+}. These responses have been linked to extensive pulmonary injury and induction and exacerbation of human diseases like COPD, cancer and asthma (Aggarwal et al. 2006; Dworski 2000; I. Rahman and Adcock 2006).

Inflammation has a key role in the development and exacerbation of lung diseases induced by PM. Both oxidative stress, cytokine release and cytotoxicity are important processes during such inflammatory responses. Several studies indicate a connection between the exposure to metals in PM and cytotoxicity (Jomova and Valko 2011; Molinelli et al. 2002). Zinc has previously been demonstrated to induce an increase in necrosis starting at around 100 μM in endothelial cells, similar to our observations (Szuster-Ciesielska et al. 2000). The intracellular concentration of zinc is closely regulated by metallothionein (MT) and other proteins. The zinc buffering capacity determines free zinc ion concentrations and a saturation of this capacity will lead to higher and potentially cytotoxic free zinc ion concentrations (Maret 2009). Our results indicate that a toxic concentration of free zinc ions is reached at concentrations of around 100 μM, thus explaining the marked increase in necrosis around this level. Concentrations of extracellular zinc exceeding the capacity of homeostatic control may also induce apoptosis (Beyersmann and Haase 2001). However, lower concentrations of zinc have been shown to protect cells from apoptosis induced by various agents (Jomova and Valko 2011). Our data also indicate that apoptosis is tending to decrease at lower zinc concentrations before significantly increasing at similar concentrations as necrosis.

Our microscopic analysis further showed that arsenite induced necrosis and apoptosis at lower concentrations than Zn^{2+} with a more linear concentration-effect curve. This might indicate that less cellular regulation is involved regarding to the exposure of arsenite. Furthermore, we observed a concentration-dependent increase of cells in mitosis and apoptosis/mitosis upon exposure to arsenite, possibly as a result of mitotic arrest. This was not observed for Zn^{2+} exposure. Also, by measuring cell cycle by flow cytometry, we found arsenite to induce a significant increase in G_2/M-levels and a decrease in G_1-levels, indicating a cell cycle arrest in G_2 or in the mitotic phase. Arsenite has previously been reported to induce cell-cycle arrest both in G_1, S, G_2 and the mitotic phase (Yih et al. 2005). How arsenite induces such effects is not known, however, arsenite has been linked to the attenuation of mitotic spindle dynamics in HeLa cells leading to mitotic arrest (Huang and Lee 1998). It has also been suggested that arsenite inhibits the activation of the G_2 DNA damage checkpoint in CGL2-cells and thereby allow cells with damaged DNA to proceed into mitosis before being arrested (Yih et al. 2005). This may be explanations for our observations, however, further studies on BEAS-2B cells need to be conducted to elucidate this.

In addition to cytotoxicity, cytokine release is important during an inflammatory response. An increase in CXCL8 –release has been observed for exposures to both Zn^{2+} and arsenite (Jaspers et al. 1999; Kim et al. 2006). We found that both metals increased CXCL8 release and that a significant increase was observed at lower concentrations than necrosis and apoptosis. Arsenite also induced CXCL8 release at lower concentrations (maximum at 20 μM) than Zn^{2+} (maximum at 100 μM), as well as a stronger fold-increase in CXCL8 release. Arsenate generally induced similar responses in cytotoxicity and CXCL8 levels as arsenite, but at higher concentrations. This is in line with other studies showing arsenite is the most toxic form of arsenic. Furthermore, only a small amount of arsenate can cross cell membranes and the toxicity of arsenate occurs via its reduction to arsenite (Jomova et al. 2011). Consequently, we chose to conduct further experiments without arsenate and instead focus on arsenite and Zn^{2+}.

A wide range of cellular responses involved in inflammation, immune activation, proliferation, differentiation an apoptosis are regulated by MAPKs. Furthermore,

MAPK signaling has been shown to act as key regulatory pathways for the synthesis of a number of cytokines (Puddicombe and Davies 2000). Both zinc and arsenite have been demonstrated to activate different MAPKs (Samet et al. 1998). The increased phosphorylation of MAPKs induced by zinc has been suggested to be mediated by inhibition of cytosolic phosphatases (Samet et al. 1999). We wanted to investigate whether the MAPKs p38 and ERK were activated by exposure to Zn^{2+} or arsenite and if these MAPKs were involved in CXCL8 release in lung epithelial cells. Exposure to the concentrations of which arsenite and Zn^{2+} induced the maximum CXCL8 release resulted in phosphorylation of both ERK and p38 already at 30 min (arsenite) and 1 h (Zn^{2+}). A similar time course for phosphorylation of p38 and ERK has been demonstrated by others (Kim et al. 2006; Liu et al. 1996; Samet et al. 1998). The activation of p38 was transient for Zn^{2+}, as the maximal response was followed by a decrease in the phosphorylation.

By using different inhibitors we further demonstrated a possible link between the metal-induced MAPK-phosphorylation and CXCL8 release, although the ERK-inhibitor did not induce a significant effect upon arsenite exposure. These results are supported by Samet et. al (1999), suggesting MAPKs may mediate zinc- and arsenite-induced release of CXCL8 leading to the expression of inflammatory proteins in human bronchial epithelial cells (Samet et al. 1999). A study by Kim et. al (2006) supported the role of ERK in CXCL8 release upon zinc exposure, however, in that study the inhibition of p38 was demonstrated to not prevent CXCL8 release in BEAS-2B cells (Kim et al. 2006). The explanation for this discrepancy is unclear, but lower zinc-concentrations and a different p38-inhibitor were used in the study of Kim and coworkers.

The activation of NF-κB is also central in the synthesis of cytokines as well as in cell proliferation and survival (de Martin et al. 1999). Degradation of I-κB and phosphorylation of p65 is often used experimentally as indications of NF-κB activation. Exposure to the concentration of which arsenite induced the maximum CXCL8 release degraded I-κB and increased phosphorylation of p65, and after 4 h the changes were substantial. Previous experiments with arsenite in different cell types show different effects on NF-κB, with some reporting arsenite being an inducer (Barchowsky et al. 1996) and others as an inhibitor of NF-κB-activation (Hershko et

al. 2002). The concentrations of arsenite, durations of exposure and cell type involved have been shown to be key factors that may be of importance for the differential effects (Leonard et al. 2004). Also, exposure to the concentrations of which Zn^{2+} induced the maximum CXCL8 release gave activation of p65, although less than for arsenite at 4 h. To our knowledge the relationship between zinc and NF-κB has been little studied. Further studies should address the role in zink- and arsenite-induced NF-κB-induction and subsequent CXCL8 release.

The induction of oxidative stress may have extensive influence on DNA-damage, cytokine release and cytotoxicity, which may all be triggered by the formation of reactive metabolites and ROS (Nanavaty et al. 2002). Oxidative stress has been extensively linked to arsenite and it has been shown to mediate the formation of both H_2O_2 and O_2^- (Jomova et al. 2011; Yamanaka and Okada 1994). Also, the inhibitoring of the antioxidant GSH has been shown to be induced by arsenite, further leading to oxidative stress (Jomova et al. 2011). However, the exact mechanisms responsible for the generation of the reactive species is not yet clear. Some studies have proposed the formation of intermediary arsine species, as arsenic has different oxidative levels (-3, +3 and +5), possibly leading to redox-reactions (Jomova et al. 2011).

Zinc, however, has been reported to exert dual effects on the ROS-system. It has been associated with antioxidant properties and may in some cases also inhibit the creation of ROS (Jomova and Valko 2011), although higher exposures have been shown to induce oxidative stress by indirect mechanisms (Maret 2009). We found that both metals induced oxidative stress by the formation of H_2O_2 or O_2. Both Zn^{2+} and arsenite markedly increased the levels of the stress protein HO-1, with arsenite giving a somewhat slower, but even stronger effect than Zn^{2+} at concentrations that induced the maximum CXCL8 release. An up-regulation of this protein has been demonstrated to be a general response to a variety of oxidative challenges and arsenite has been shown to induce HO-1 expression in several cell lines (Elbirt et al. 1998; Keyse and Tyrrell 1989; Ruiz-Ramos et al. 2009). Furthermore, both ERK and p38 have been suggested to be involved in the induction of HO-1 (Elbirt et al. 1998).

We also explored the possible involvement of oxidative stress in CXCL8 release. Three agents known to inhibit ROS by different mechanisms were added to Zn^{2+}- and

arsenite-exposed cells. DPI is widely used as an inhibitor of flavoenzymes, particularly NAD(P)H oxidase (Riganti et al. 2004). Thiourea, on the other hand, is a free radical scavenging agent (DeForge et al. 1993), whereas NAC is a precursor of glutathione and an anti-oxidant demonstrated to be central to redox defense during oxidative stress (Biswas and Rahman 2009). DPI did not markedly decrease CXCL8 release for either metal. However, as NAD(P)H oxidase has been proposed to account for the majority of superoxide anions in vascular endothelial cells, our findings was not expected (Barchowsky et al. 1999). On the other hand, DPI did reduce arsenite-induced increases of mitotic cells, significantly. This may indicate that NAD(P)H oxidase is involved in the mitotic arrest induced by arsenite and not in the cytokine release. NAC also decreased arsenite-induced mitosis significantly and was the only antioxidant decreasing CXCL8 release markedly for both metals. These results indicate that the formation of ROS is involved in the cytotoxic effects exerted by Zn^{2+} and arsenite. However, NAC may bind metals directly by its sulfhydryl-groups and the effects observed for NAC might be due to this binding. With respect to ROS and intracellular signaling, ROS has previously been demonstrated to activate NF-κB and this may be a possible explanation for the strong NF-κB activation that we observed (Leonard et al. 2004).

Several metals may be included in PM, thus it was of interest to investigate whether there where any interactions between combinations of the metals (Chen and Lippmann 2009; Laden et al. 2000). Although not significant, our findings indicated at least an additive effect in the combination of both metals compared to arsenite or Zn^{2+} exposure only, when measuring CXCL8 release. Furthermore, an additive effect for the combination of metals on decrease in viability compared to arsenite or Zn^{2+} only was observed. Conceivably, such effects of metal combinations are of importance to explain the contribution of metals in PM-induced inflammatory effects.

The level of zinc and arsenic present in many combustion-derived PM is highly variable, however, the amount of leachable zinc may correlate to micromolar levels in equivalent cell cultures (Riley et al. 2003). The BEAS-2B cell line used in this study was derived by transforming human bronchial cells, but previous studies have shown functional and mechanistic correlation to primary cultures of human airway epithelial cells (Samet et al. 1992). Nonetheless, it is possible that the responses of BEAS-2B

cells to metal exposure differ from those of the human lung and studies determining to which extent the effects observed in vitro are representative of cellular responses in vivo are needed.

In conclusion, the present study demonstrated that both Zn^{2+} and arsenite had toxic potential regarding to cytotoxicity, inflammatory responses and oxidative stress on BEAS-2B-cells, which potentially may contribute to PM-induced inflammatory effects. Both ROS and MAP-kinase activation seem to be involved in the triggering of the metal-induced CXCL8 release.

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FIGURE LEGENDS

Figure 1. Effects of Zn^{2+} and arsenite on cell morphology.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} or 0-20 μM arsenite. Representative pictures by light microscopy of control, cells exposed to 100 μM Zn^{2+} or 20 μM are shown to illustrate differences in morphology. Magnification: 1000x.

Figure 2. Effects of $Zn^{2+}$and arsenite on cell nucleus morphology.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} or 0-20 μM arsenite for 24 h, trypsinated and stained with PI (red) and Hoechst 33342 (blue). Representative examples of different cell nuclei morphology determined by fluorescence microscopy are shown. Magnification: 1000x.

Figure 3. Cytotoxic effects of Zn^{2+}, arsenite and arsenate.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} (A), 0-20 μM arsenite (B) or 0-200 μM arsenate (C) for 24 h, trypsinated and stained with PI and Hoechst 33342. Analysis of viability, necrosis, apoptosis, mitosis and apoptosis/mitosis was performed by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 4. Effects of Zn^{2+} and arsenite on cell cycle distributions.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} or 0-20 μM arsenite for 24 h, trypsinated, fixed by PFA and stained with Hoechst 33258 before measuring Hoechst 33258 fluorescence by flow cytometry. Un-exposed control, 100 μM Zn^{2+} and 25 μM arsenite exposure are shown to illustrate the differences cell cycle distributions. A minimum of 5000 cells were analyzed for each exposure. (A) Multi-cycle software was used to automatically distribute the cells between the different cell phases. **(**B**)** Distribution between the different cell phases were set manually in FACS-Diva software.

Figure 5. Effects of Zn^{2+} and arsenite on cell cycle distributions.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} (A) or 0-20 μM arsenite (B) for 24 h, trypsinated, fixed by PFA and stained with Hoechst 33258 before measuring Hoechst 33258 fluorescence by flow cytometry. Graphs are based on cell cycle distributions manually set in FACS-Diva software. A minimum of 5000 cells were analyzed for each exposure. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$).

Figure 6. Effects of Zn^{2+}, arsenite and arsenate on CXCL8 release.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} (A), 0-20 μM arsenite (B) or 0-200 μM arsenate (C) for 24 h and the CXCL8 levels in the culture media were measured by ELISA. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$).

Figure 7. Effects of Zn^{2+} and arsenite on intracellular signaling.

BEAS-2B cells were exposed to 100 μM Zn^{2+} (Zn) or 20 μM arsenite (As) for 30 min-4 h, trypsinated, lysed and analyzed for expression of p-p38 (A), p-ERK (B), p-p65 (C) or I-κB (D) expression by Western blotting. As loading controls p38 (A), ERK (B), p65 (C) or β-actin (D) expression are used. These data are representative for one of a minimum of three independent experiments.

Figure 8. Involvement of intracellular signaling on CXCL8 release (A) and cell viability (B) induced by Zn^{2+} or arsenite.

BEAS-2B cells were pretreated for 1 h with SB202190 and PD98059 before being exposed to 100 μM Zn^{2+} or 20 μM arsenite for 24 h. The CXCL8-levels in the culture media were measured by ELISA. Cell viability was determined after cells were trypsinated and stained with PI and Hoechst 33342 by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 9. Effects of Zn^{2+} (A) and arsenite (B) on ROS-formation by DCFH-DA fluorescence.

BEAS-2B cells were exposed to 75 μM Zn^{2+}, 100 μM Zn^{2+}, 20 μM arsenite or 50 μM arsenite for 2-8 h and, trypsinated, and stained with DCFH-DA before measuring DCFH-DA fluorescence by flow cytometry. A minimum of 5000 cells were analyzed for each exposure. The graphs show normalized mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$) by one-sample T-test.

Figure 10. Effects of Zn^{2+} (A) and arsenite (B) on ROS-formation by DHE fluorescence

BEAS-2B cells were exposed to 75 μM Zn^{2+}, 100 μM Zn^{2+}, 20 μM arsenite or 50 μM arsenite for 2-8 h, trypsinated and stained with DHE before measuring DHE fluorescence by flow cytometry. A minimum of 5000 cells were analyzed for each exposure. The graphs show normalized mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$) by one-sample T-test.

Figure 11. Effects of Zn^{2+} and arsenite on heme oxygenase-1 expression.

BEAS-2B cells were exposed to 75 μM Zn^{2+} (in figure zn), 100 μM Zn^{2+} (in figure zn) or 20 μM arsenite (in figure As) for 4 or 8 h, trypsinated, lysed and analyzed for HO-1 expression by Western blotting. β-actin expression was used as loading control. These data are representative for one of a minimum of three independent experiments.

Figure 12. Involvement of ROS in CXCL8-release (A), cell viabiliy (B), necrosis (C) and mitosis (D) induced by Zn^{2+} and arsenite.

BEAS-2B cells were pretreated with thiourea, DPI or NAC for 1 h before being exposed to 75 μM Zn^{2+}, 100 μM Zn^{2+} or 20 μM arsenite for 24 h. The CXCL8-levels in the culture media were measured by ELISA. Cell viability, necrosis and mitosis was determined after cells were trypsinated and stained with PI and Hoechst 33342 by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 13. CXCL8-release (A) and cell viability (B) after combined exposure to Zn^{2+} and arsenite.

A: BEAS-2B cells were exposed to 0-20 μM arsenite with or without 100 μM Zn^{2+} for 24 h and the CXCL8-levels in the culture media were measured by ELISA. Cell viability was determined after cells were trypsinated and stained with PI and Hoechst 33342 by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 14. CXCL8-release (A) and cell viability (B) after combined exposure to arsenite and Zn^{2+}.

BEAS-2B cells were exposed to 0-110 μM Zn^{2+} with or without 10 μM arsenite for 24 h. The CXCL8 levels in the culture media were measured by ELISA. Cell viability was determined after cells were trypsinated and stained with PI and Hoechst 33342 by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

FIGURES

Figure 1

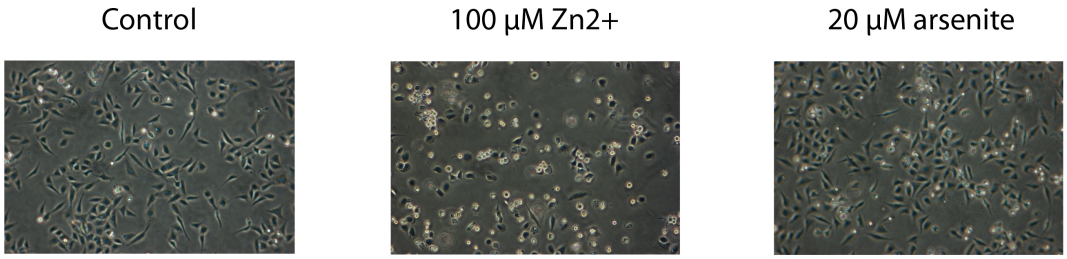


Figure 2

Figure 3

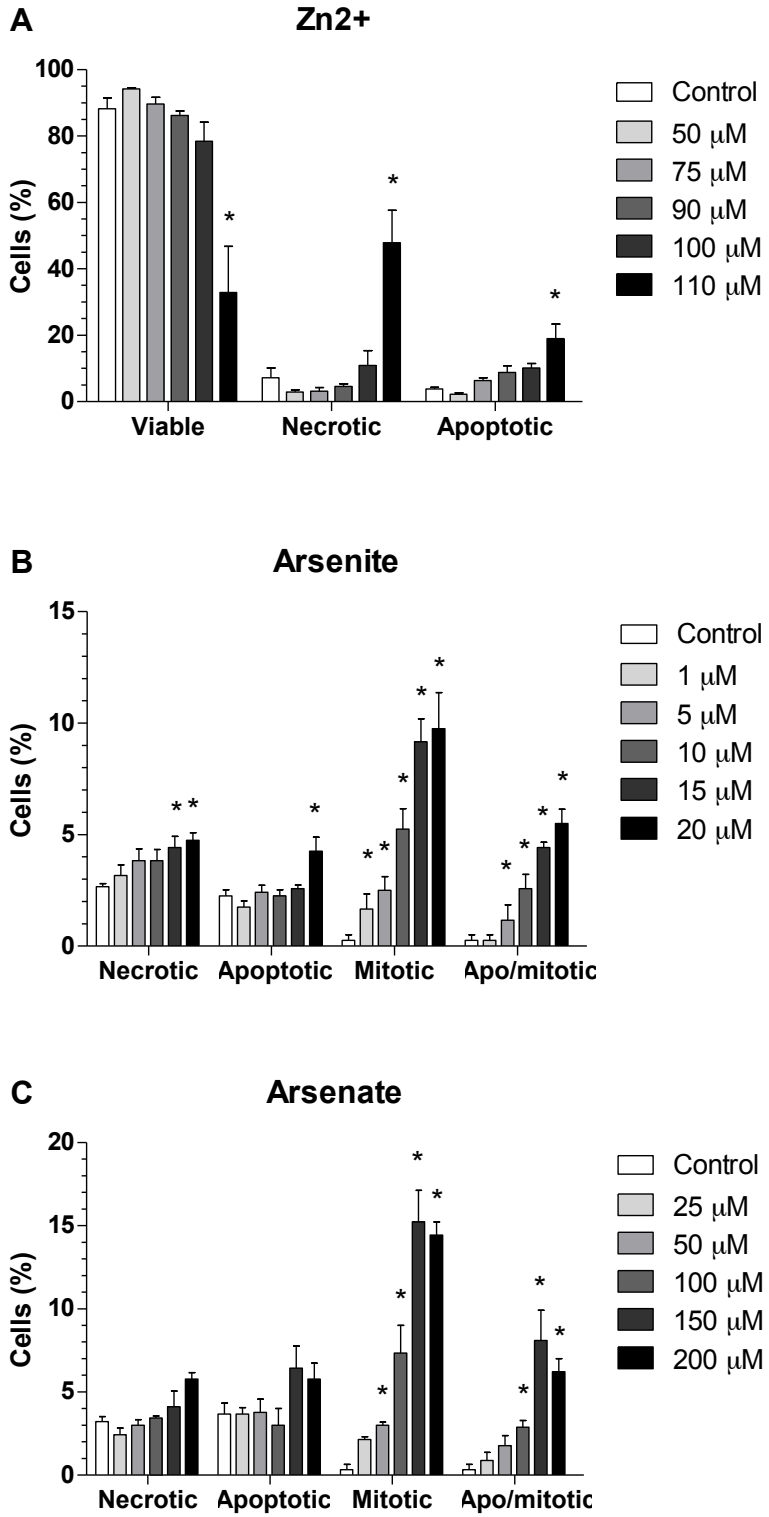
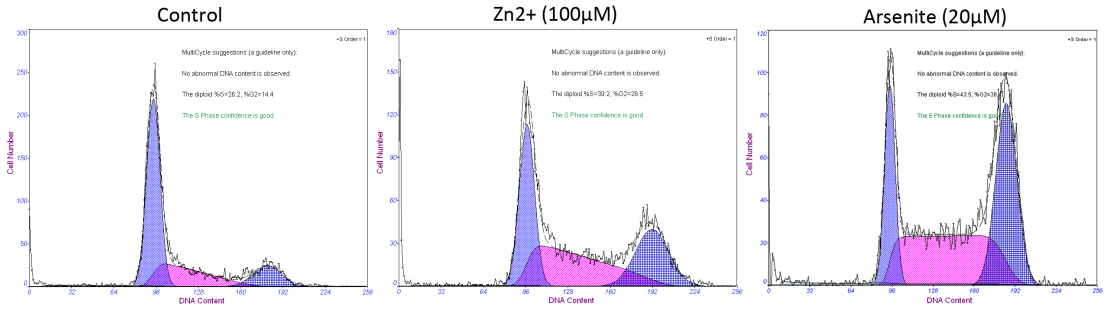
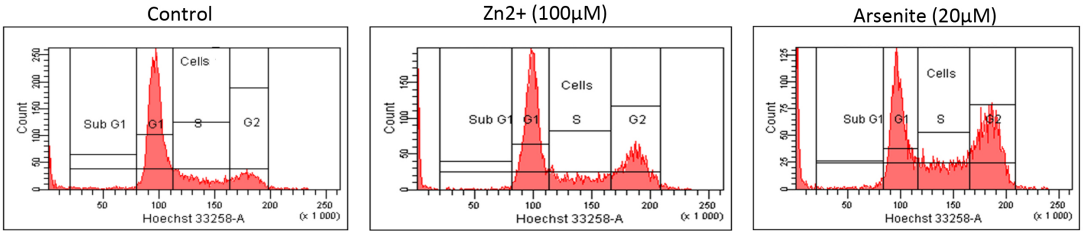

A
Zn2+
Cells (%)
100
80
60
40
20
0
Viable
Necrotic
Apoptotic
Control
50 μM
75 μM
90 μM
100 μM
110 μM
B
Arsenite
Cells (%)
15
10
5
0
Necrotic
Apoptotic
Mitotic
Apo/mitotic
Control
1 μM
5 μM
10 μM
15 μM
20 μM
C
Arsenate
Cells (%)
20
15
10
5
0
Necrotic
Apoptotic
Mitotic
Apo/mitotic
Control
25 μM
50 μM
100 μM
150 μM
200 μM

Figure 4

A



B



Population	#Events	%Parent	%Total
All Events	6 473	####	100,0
Cells	5 598	86,5	86,5
Sub G1	5 330	95,2	82,3
G1	132	2,5	2,0
S	3 497	65,6	54,0
G2	981	18,4	15,2
	720	13,5	11,1

Population	#Events	%Parent	%Total
All Events	6 107	####	100,0
Cells	5 257	86,1	86,1
Sub G1	4 892	93,1	80,1
G1	66	1,3	1,1
S	2 568	52,5	42,1
G2	849	17,4	13,9
	1 409	28,8	23,1

Population	#Events	%Parent	%Total
All Events	6 471	####	100,0
Cells	5 388	83,3	83,3
Sub G1	4 889	90,7	75,6
G1	99	2,0	1,5
S	1 862	38,1	28,8
G2	1 094	22,4	16,9
	1 834	37,5	28,3

Figure 5

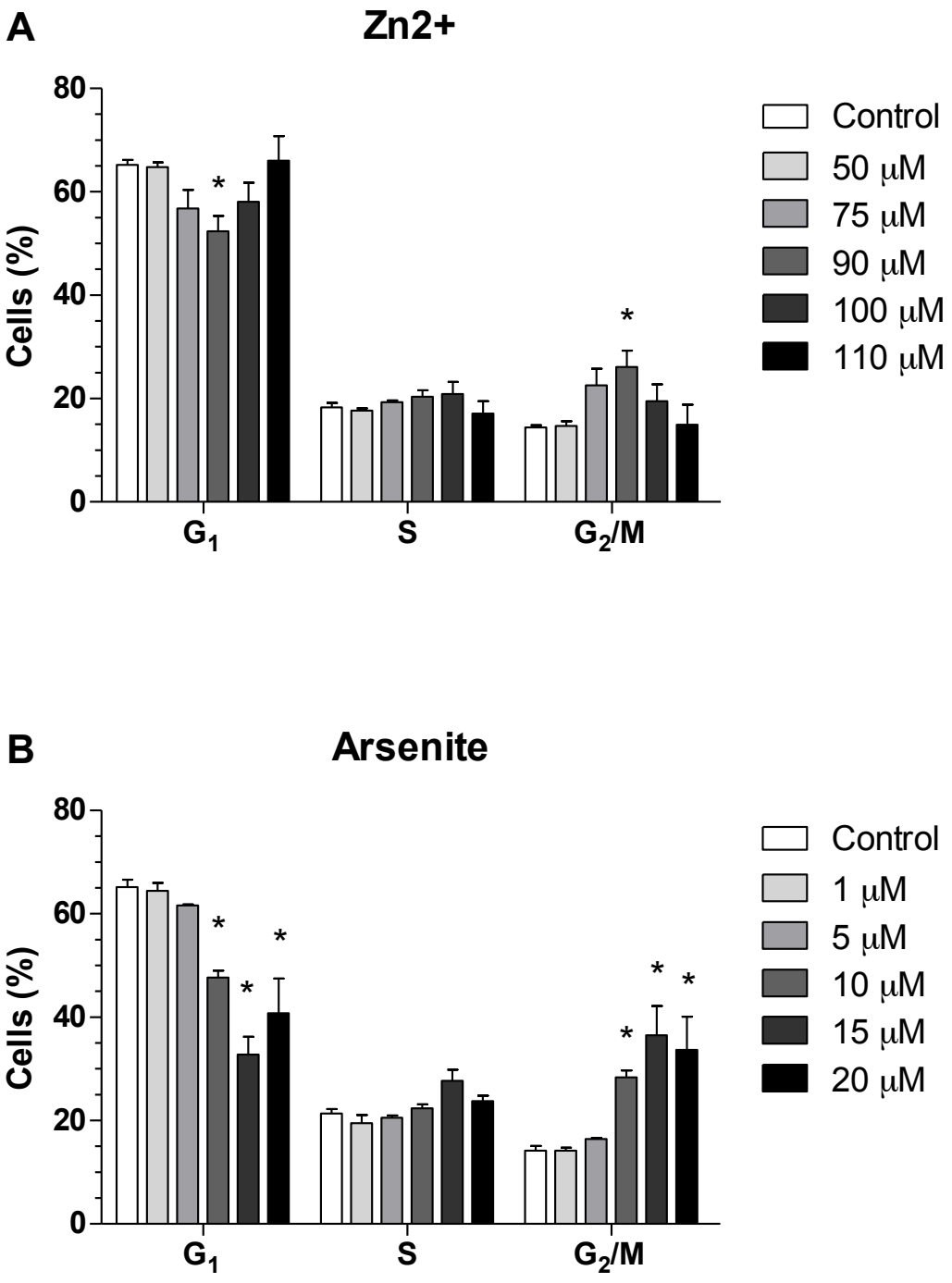

A
Zn2+
Cells (%)
80
60
40
20
0
G1
S
G2/M
Control
50 μM
75 μM
90 μM
100 μM
110 μM
B
Arsenite
Cells (%)
80
60
40
20
0
G1
S
G2/M
Control
1 μM
5 μM
10 μM
15 μM
20 μM

Figure 6

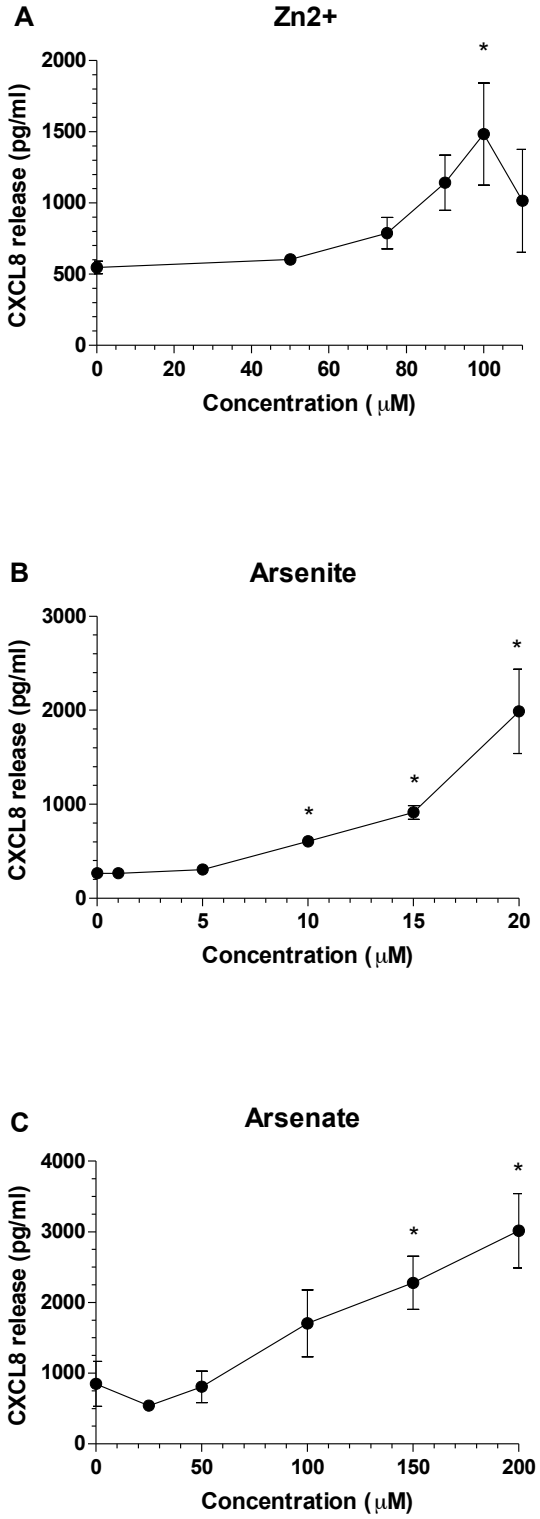
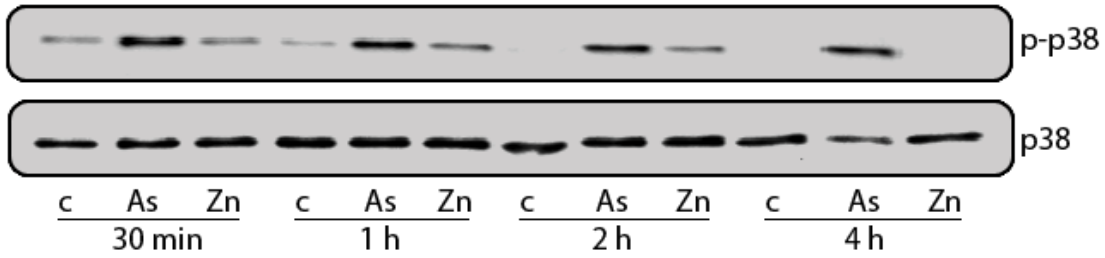
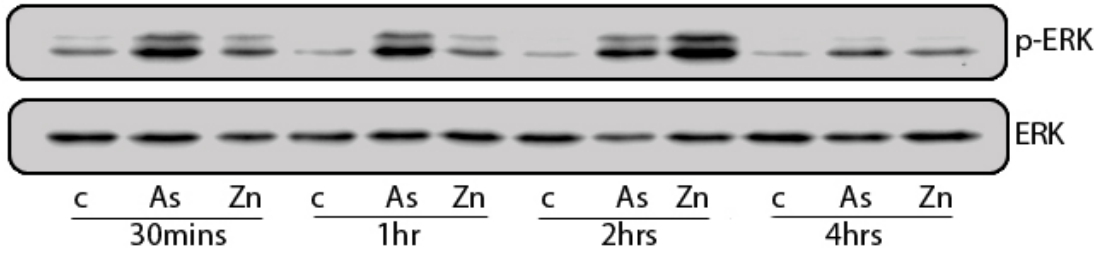

A
Zn2+
CXCL8 release (pg/ml)
Concentration (μM)
B
Arsenite
CXCL8 release (pg/ml)
Concentration (μM)
C
Arsenate
CXCL8 release (pg/ml)
Concentration (μM)

Figure 7

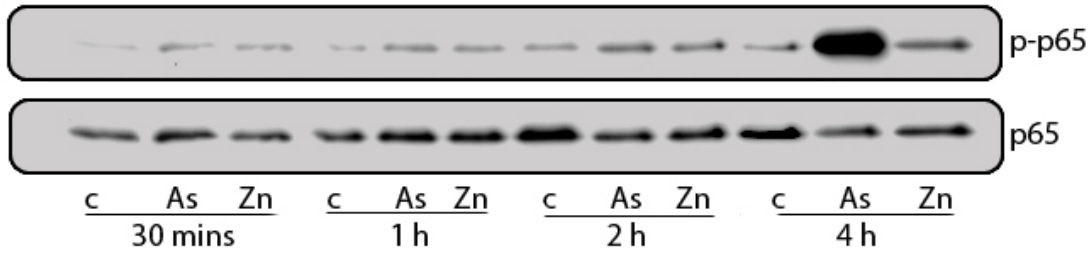
A



B



C



D

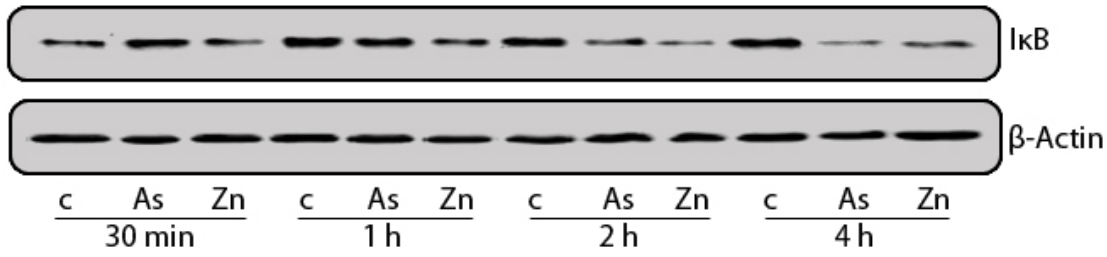


Figure8

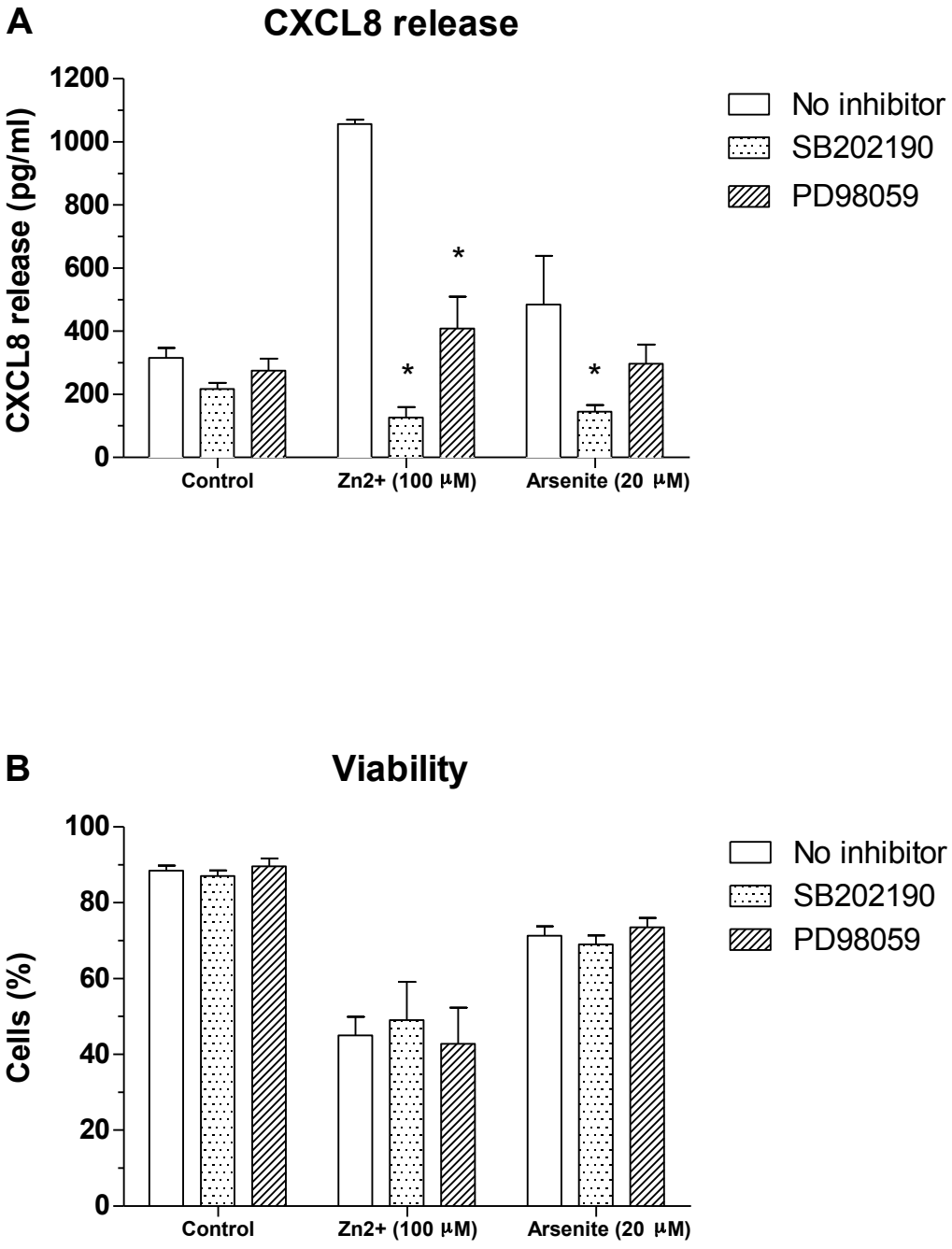
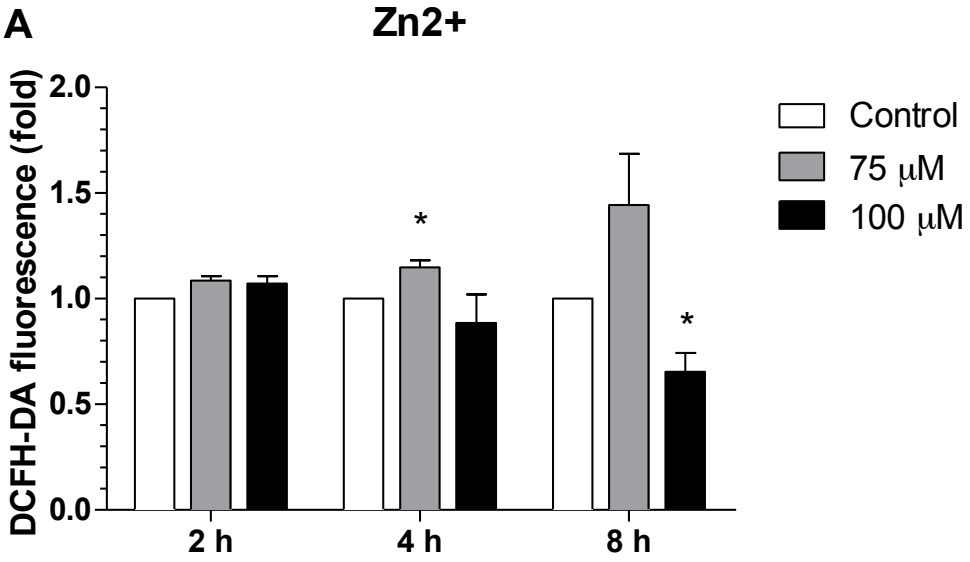

A
CXCL8 release
CXCL8 release (pg/ml)
1200
1000
800
600
400
200
0
*
*
*
Control
Zn2+ (100 µM)
Arsenite (20 µM)
No inhibitor
SB202190
PD98059
B
Viability
Cells (%)
100
80
60
40
20
0
Control
Zn2+ (100 µM)
Arsenite (20 µM)
No inhibitor
SB202190
PD98059

Figure 9


A
Zn2+
DCFH-DA fluorescence (fold)
2.0
1.5
1.0
0.5
0.0
2 h
4 h
8 h
*
*
Control
75 μM
100 μM

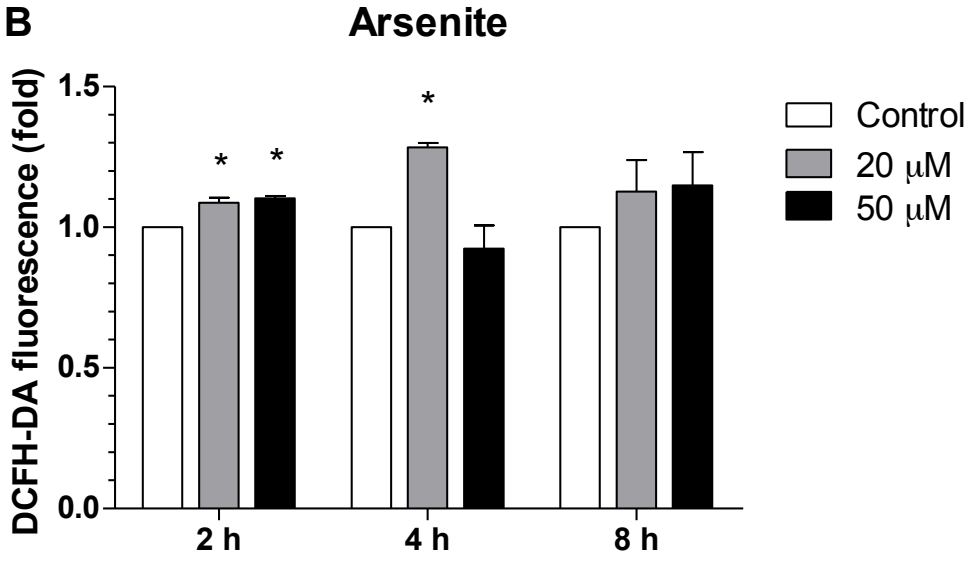

B
Arsenite
DCFH-DA fluorescence (fold)
1.5
1.0
0.5
0.0
2 h
4 h
8 h
*
*
*
Control
20 μM
50 μM

Figure 10

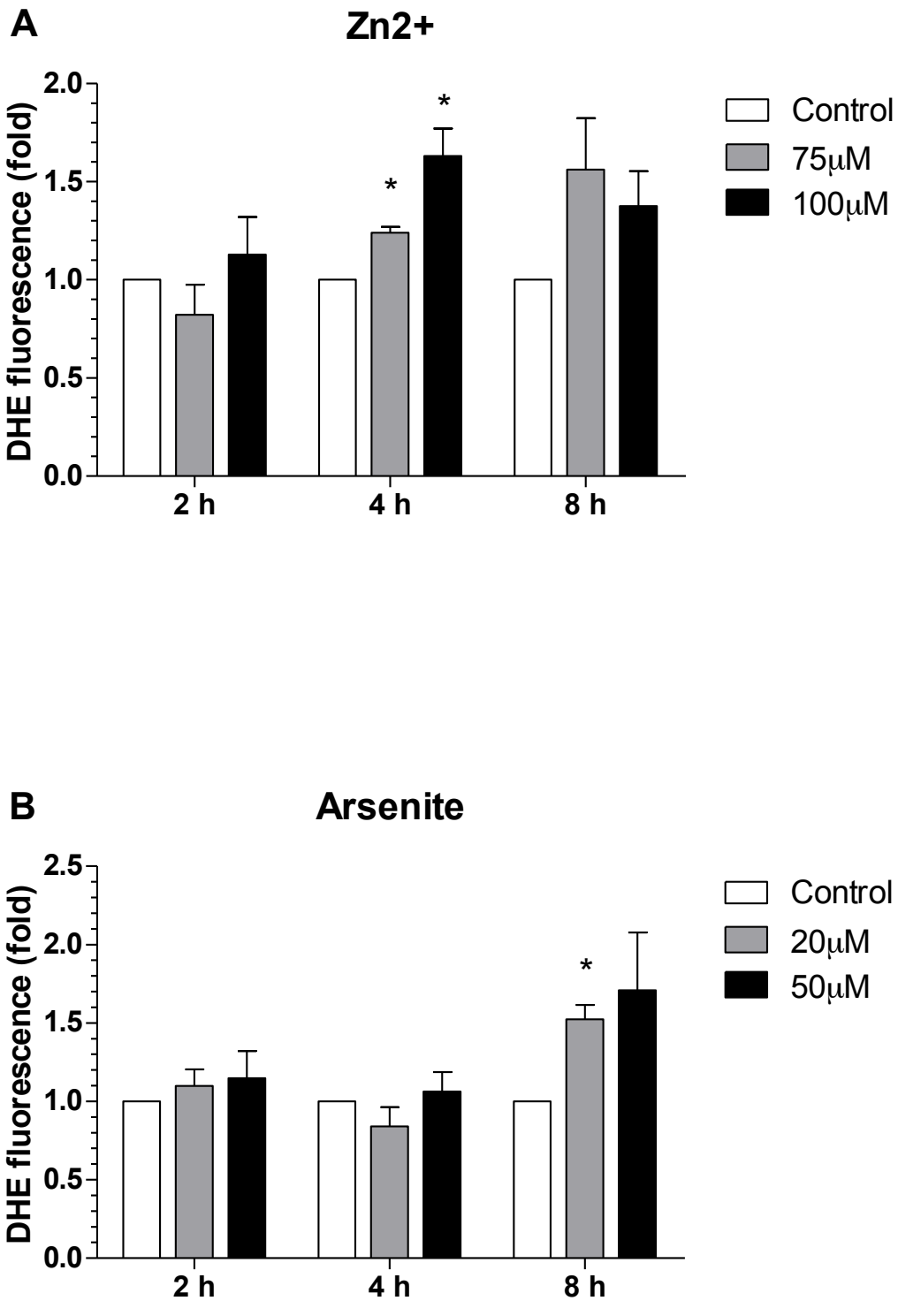

A
Zn2+
DHE fluorescence (fold)
2.0
1.5
1.0
0.5
0.0
2 h
4 h
8 h
*
*
Control
75μM
100μM
B
Arsenite
DHE fluorescence (fold)
2.5
2.0
1.5
1.0
0.5
0.0
2 h
4 h
8 h
*
Control
20μM
50μM

Figure 11

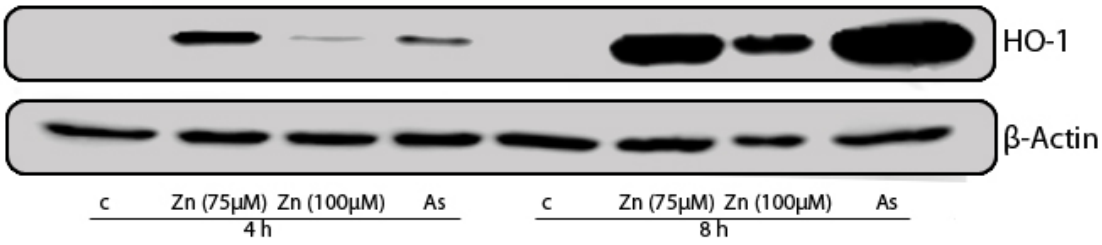


Figure 12

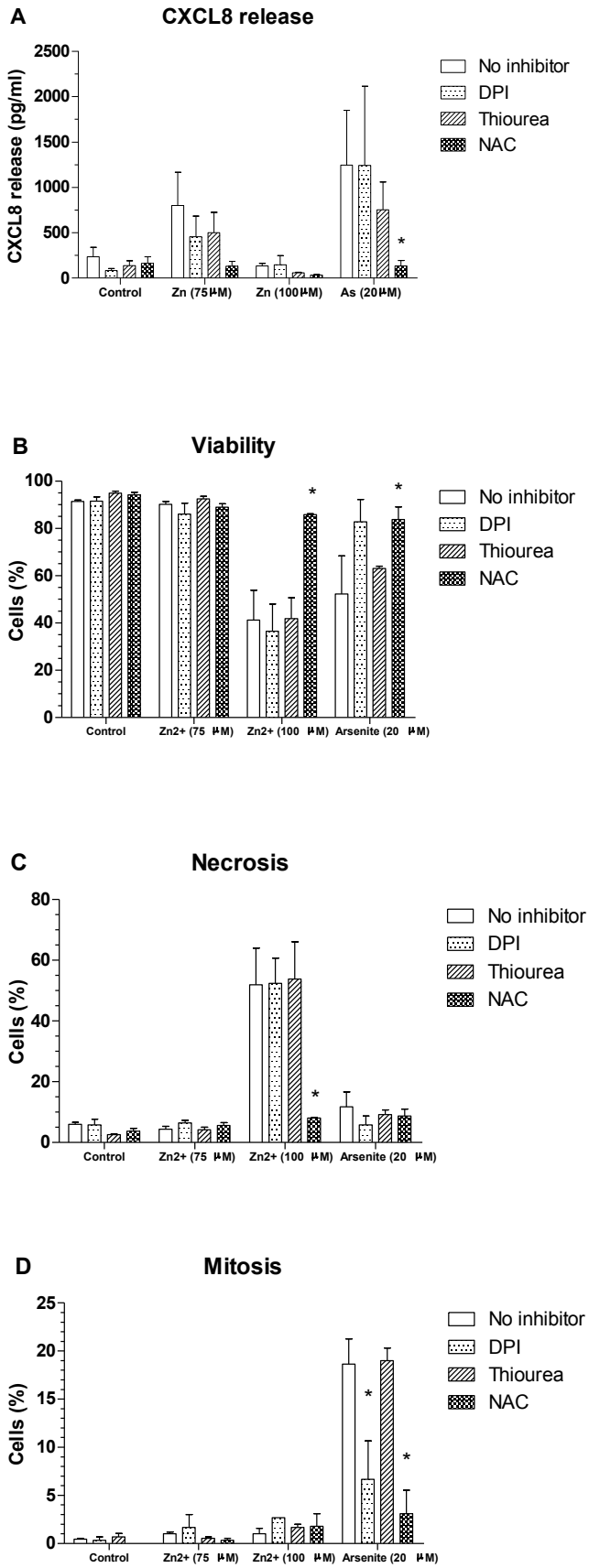

A
CXCL8 release
CXCL8 release (pg/ml)
2500
2000
1500
1000
500
0
Control
Zn (75µM)
Zn (100µM)
As (20µM)
No inhibitor
DPI
Thiourea
NAC
B
Viability
Cells (%)
100
80
60
40
20
0
Control
Zn2+ (75 µM)
Zn2+ (100 µM)
Arsenite (20 µM)
C
Necrosis
D
Mitosis
25
20
15
10
5

Figure 13

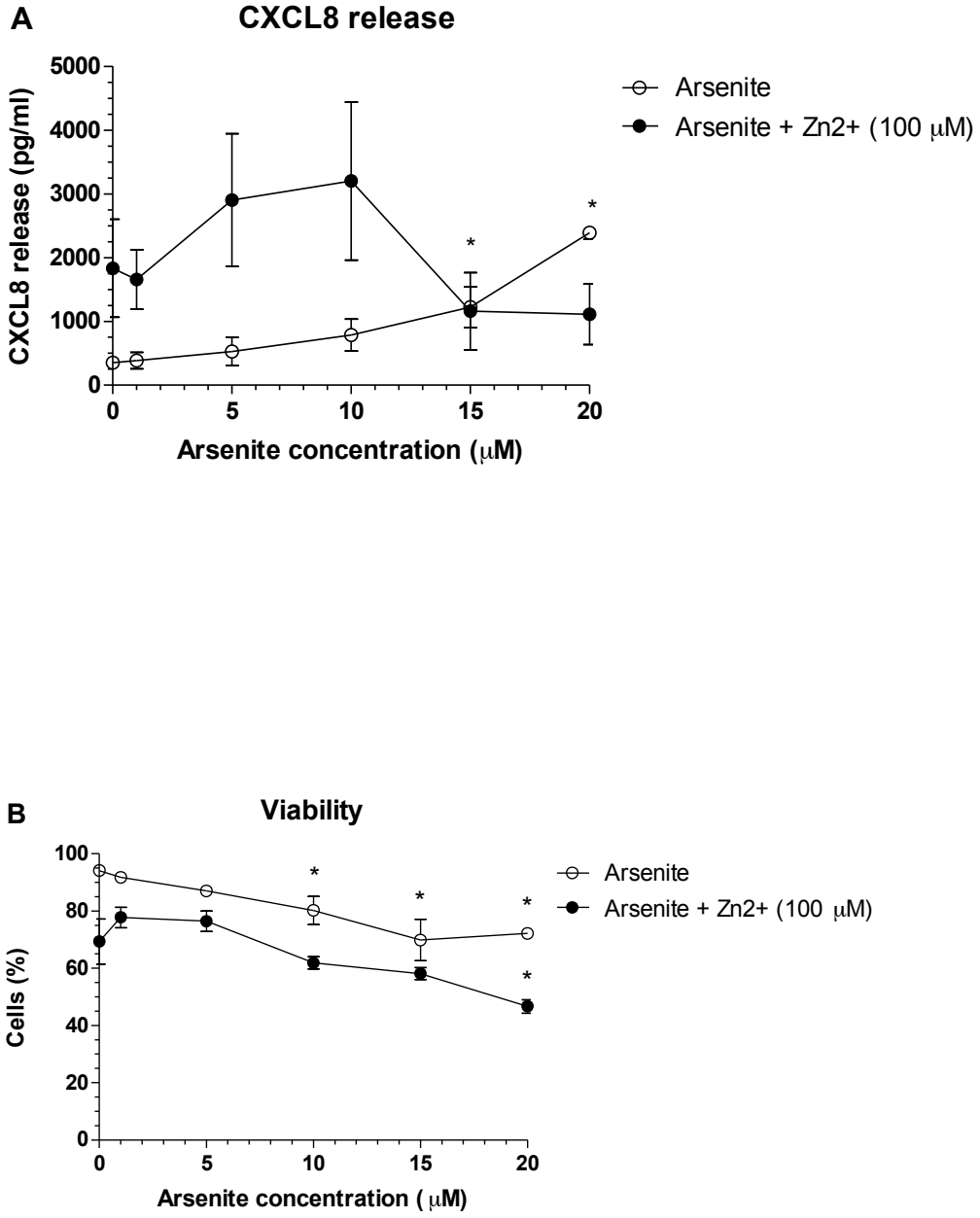
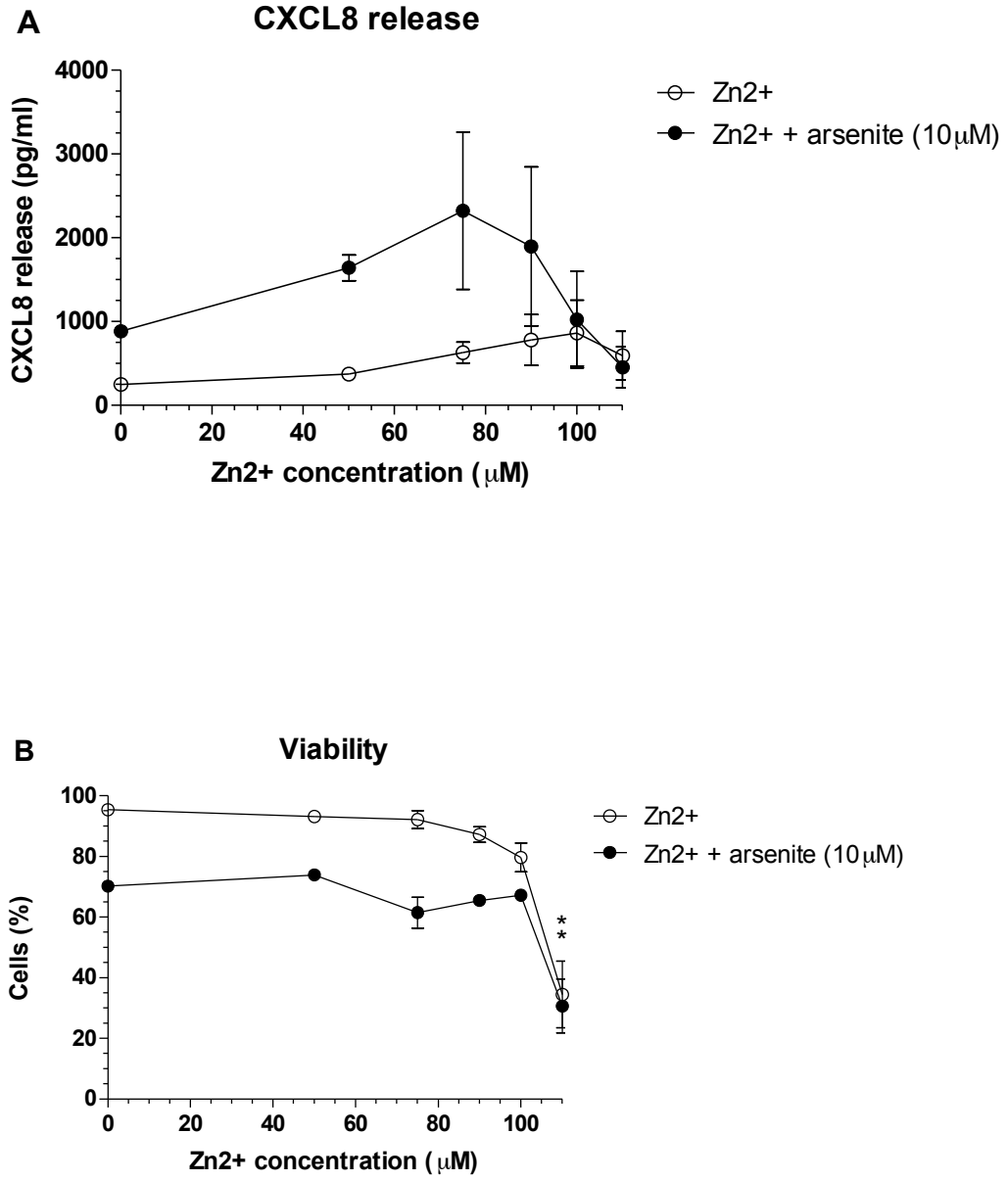

A
CXCL8 release
CXCL8 release (pg/ml)
5000
4000
3000
2000
1000
0
0
5
10
15
20
Arsenite concentration (μM)
Arsenite
Arsenite + Zn2+ (100 μM)
*
*
B
Viability
Cells (%)
100
80
60
40
20
0
0
5
10
15
20
Arsenite concentration (μM)
Arsenite
Arsenite + Zn2+ (100 μM)
*
*
*
*

Figure 14


A
CXCL8 release
CXCL8 release (pg/ml)
4000
3000
2000
1000
0
0
20
40
60
80
100
Zn2+ concentration (μM)
Zn2+
Zn2+ + arsenite (10μM)
B
Viability
Cells (%)
100
80
60
40
20
0
0
20
40
60
80
100
Zn2+ concentration (μM)
**
Zn2+
Zn2+ + arsenite (10μM)

APPENDIX

Experiments not relevant for the aims, or experiments not generating any results were put in the appendix. They were not included in the discussion.

FIGURE LEGENDS

Figure 15. Effects of arsenite on cytotoxicity (A) and CXCL8-release (B).
BEAS-2B cells were exposed to 0-50 μM arsenite for 24 h trypsinated and stained with PI and Hoechst 33342. Analysis of viability, necrosis, apoptosis, mitosis and apoptosis/mitosis was performed by fluorescence microscopy. For each exposure, 300 cells were counted. The CXCL8-levels in the culture media were measured by ELISA. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 16. CXCL8-release (A) and cell viability (B) after combined exposure to arsenite and 1-NP.
BEAS-2B cells were exposed to 0-20 μM arsenite with or without 10 μM 1-NP or 30 μM 1-NP for 24 h. The CXCL8-levels in the culture media were measured by ELISA. Cell viability was determined after cells were trypsinated and stained with PI and Hoechst 33342 by fluorescence microscopy. For each exposure, 300 cells were counted. The graphs show mean ±SEM of a minimum of three independent experiments. * = Significantly different from control ($P<0.05$). Magnification: 1000x.

Figure 17. Effects of Zn^{2+} and arsenite on phospho-histone H3 fluorescence.
BEAS-2B cells were exposed to 0-110 μM Zn^{2+} or 0-20 μM arsenite for 24 h, trypsinated, fixed by PFA and stained with Hoechst 33258 and anti phospho-histone H3 (pS 28) before measuring Hoechst 33258 and phospho-histone H3 fluorescence by flow cytometry. Selecting for phospho-histone H3 fluorescence was done manually in the FACS-Diva software. Only one experiment was conducted. A minimum of 5000

cells were counted. (A) Representative pictures of control, cells exposed to 100 μM Zn^{2+} or 20 μM arsenite are shown to illustrate differences in phosphorylated histone H3-fluorescence. (B) The graph is based on total cells with phospho-histone H3 fluorescence manually selected for in FACS-Diva software.

Figure 18. Effects of $Zn^{2+}$and arsenite on cyclooxygenase.

BEAS-2B cells were exposed to 100 μM Zn^{2+} (Zn) or 20 μM arsenite (As) for 30 min-4 h, trypsinated, lysed and analyzed for cyclooxygenase (COX) expression by Western blotting. β-actin expression was used as loading control. Representative data is shown for one experimental set-up from minimum three.

Figure 19. Involvement of ROS on cell morphology induced by Zn^{2+} and arsenite.

BEAS-2B cells were pretreated with thiourea, DPI or NAC for 1 h before being exposed to 75 μM Zn^{2+}, 100 μM Zn^{2+} or 20 μM arsenite for 24 h. Representative pictures by light microscopy are shown to illustrate differences in the involvement of ROS.

FIGURES

Figure 15

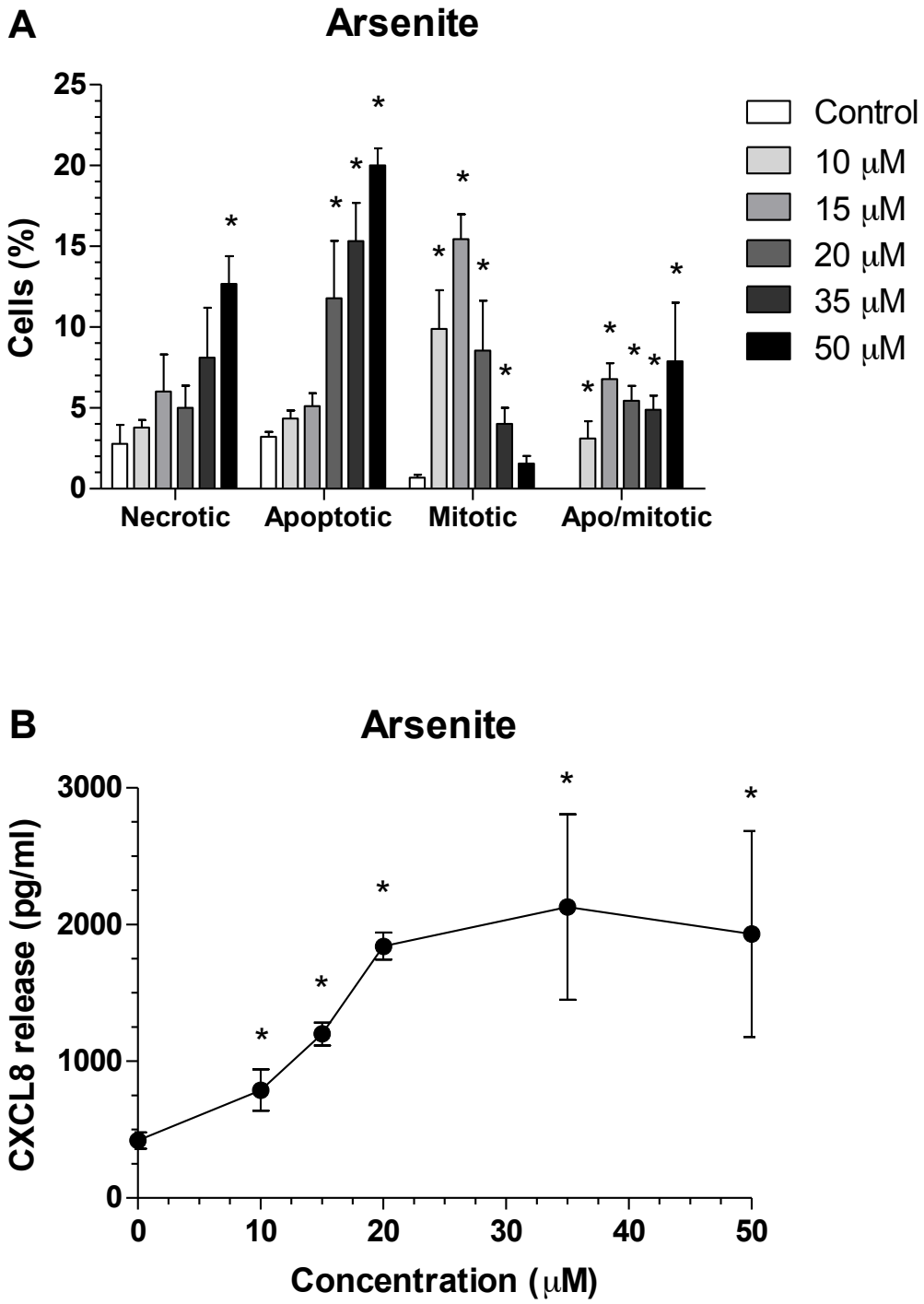


Figure 16

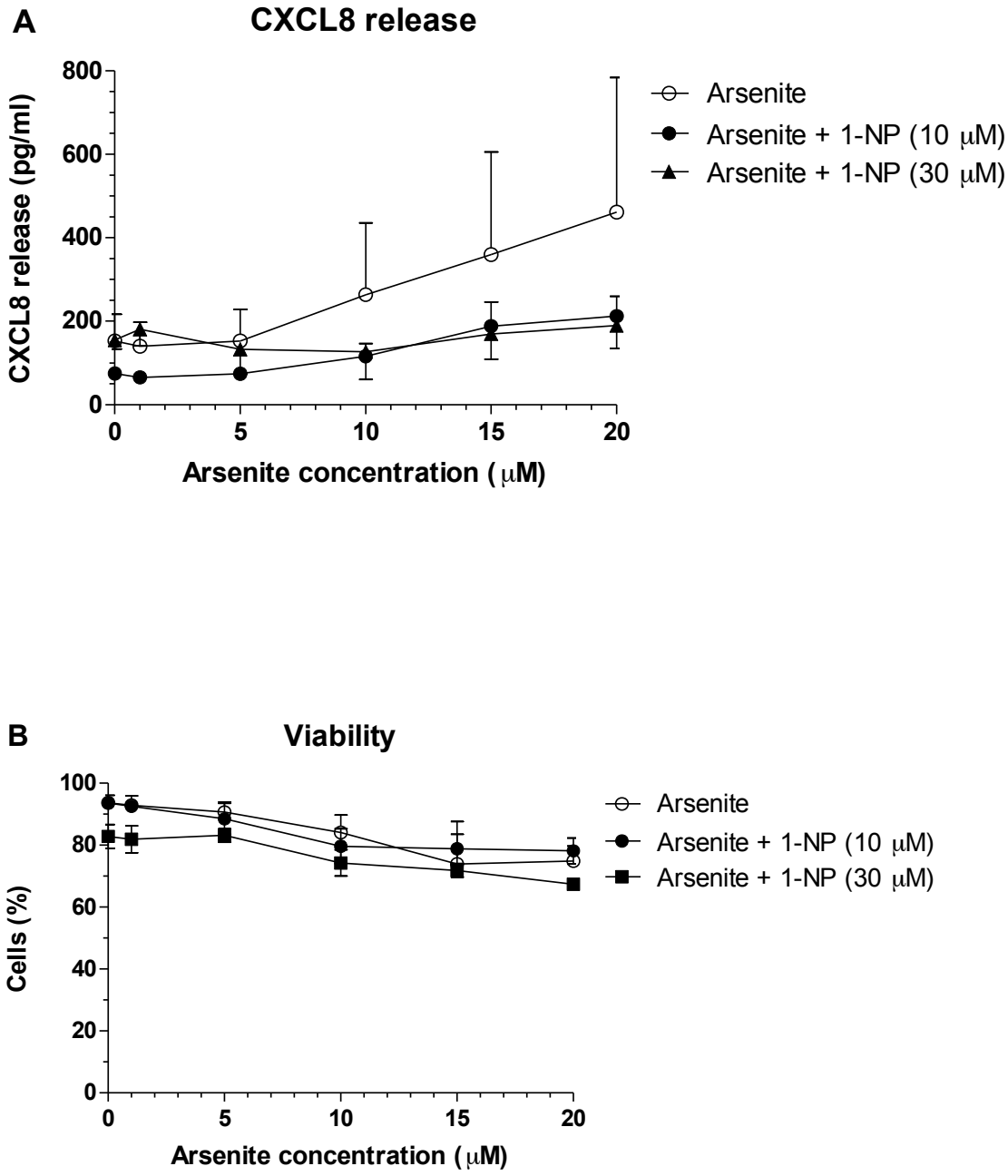
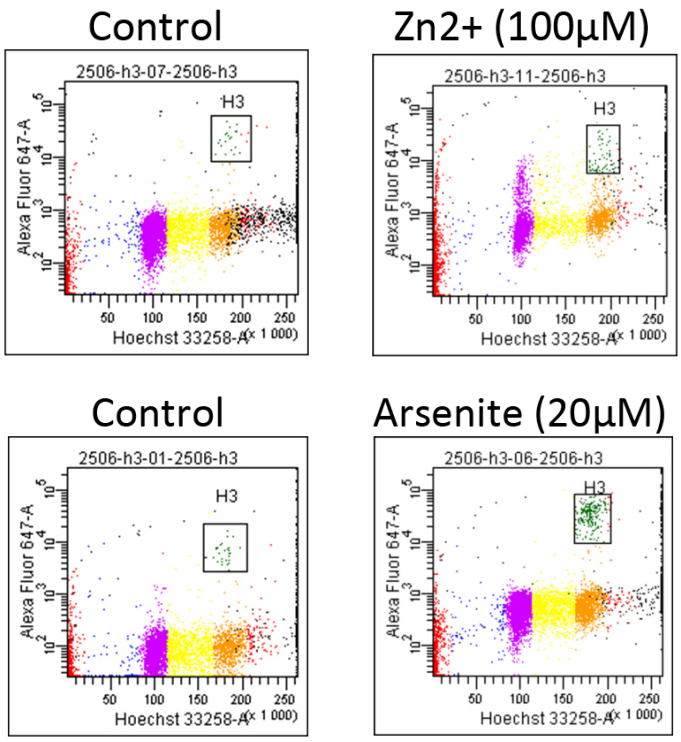
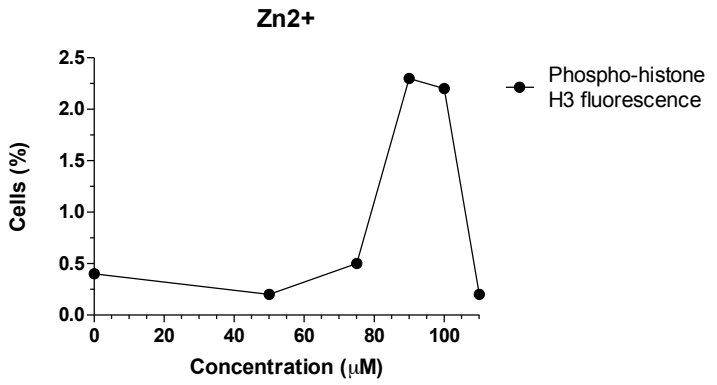

A
CXCL8 release
CXCL8 release (pg/ml)
800
600
400
200
0
0
5
10
15
20
Arsenite concentration (μM)
Arsenite
Arsenite + 1-NP (10 μM)
Arsenite + 1-NP (30 μM)
B
Viability
Cells (%)
100
80
60
40
20
0
0
5
10
15
20
Arsenite concentration (μM)
Arsenite
Arsenite + 1-NP (10 μM)
Arsenite + 1-NP (30 μM)

Figure 17

A


Control
2506-h3-07-2506-h3
H3
Alexa Fluor 647-A
Hoechst 33258-A (x 1 000)
Zn2+ (100µM)
2506-h3-11-2506-h3
H3
Alexa Fluor 647-A
Hoechst 33258-A (x 1 000)
Control
2506-h3-01-2506-h3
H3
Alexa Fluor 647-A
Hoechst 33258-A (x 1 000)
Arsenite (20µM)
2506-h3-06-2506-h3
H3
Alexa Fluor 647-A
Hoechst 33258-A (x 1 000)

B


Zn2+
Cells (%)
Concentration (µM)
Phospho-histone H3 fluorescence

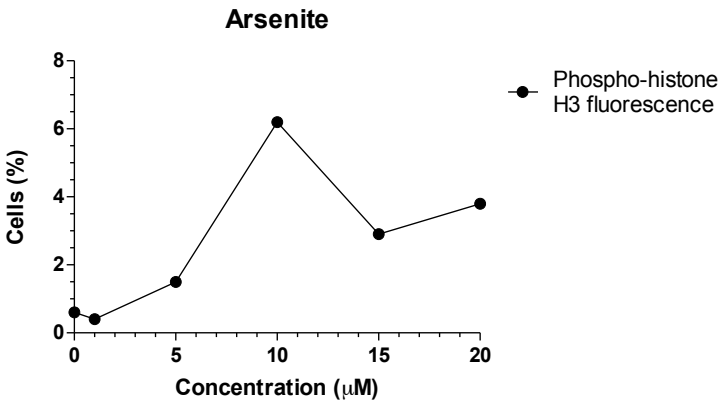

Arsenite
Cells (%)
Concentration (µM)
Phospho-histone H3 fluorescence

Figure 18

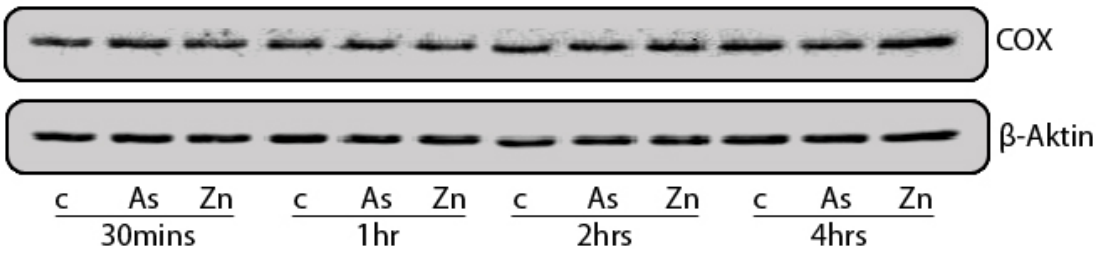


Figure 19