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ABSTRACT

Benz[*j*]aceanthrylene (B[*j*]A) is a cyclopenta-fused polycyclic aromatic hydrocarbon with strong mutagenic and carcinogenic effects. We have identified B[*j*]A in air particulate matter in samples collected in Stockholm, Sweden and in Limeira, Brazil using LC-GC/MS analysis. Determined concentrations ranged between 1.57-12.7 and 19.6-30.2 pg/m^3 in Stockholm and Limeira, respectively, which was 11-30 times less than benzo[*a*]pyrene (B[*a*]P) concentrations. Activation of the DNA damage response was evaluated after exposure to B[*j*]A in HepG2 cells in comparison to B[*a*]P. We found that significantly lower concentrations of B[*j*]A was needed for an effect on cell viability compared to B[*a*]P and equimolar exposure resulted in significant more DNA damage with B[*j*]A. Additionally, levels of γH2AX, pChk1, p53, pp53 and p21 proteins were higher in response to B[*j*]A than B[*a*]P. Based on dose response induction of pChk1 and γH2AX, B[*j*]A potency was 12.5 and 33.3 higher than B[*a*]P, respectively. Although B[*j*]A levels in air were low, including B[*j*]A in the estimation of excess lifetime cancer risk increased the risk up to 2-fold depending on which potency factor for B[*j*]A was applied. Together our results show that B[*j*]A could be an important contributor to the cancer risk of air PM.

INTRODUCTION

Particulate matter (PM) from urban air contains a mixture of different chemicals that can interact and cause adverse effects to human health. Outdoor air pollution and its PM component have been classified as carcinogenic to humans by IARC.[1] One group of chemicals that are found in air PM is the polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental contaminants that are formed during incomplete combustion of organic matter. PAHs in the environment are of concern because of their carcinogenic activity and several individual and mixtures of PAHs have been classified as possible or probable carcinogens to humans .[2] To date, benzo[*a*]pyrene (B[*a*]P) is the only individual PAH classified as carcinogenic to humans by IARC.[2]

Health risk assessment of PAH mixtures is often carried out using B[*a*]P for comparison as its toxicological profile has been extensively characterized. One approach to perform risk assessment is based on additivity of toxic equivalency factors (TEFs) or relative potency factors (RPFs) where the carcinogenic potential is expressed relative to B[*a*]P.[3] A second method is to use B[*a*]P as a surrogate marker for all PAHs. This has been applied to air[4] and the European Commission Air Quality Standards has put a target value of PAHs in air expressed as 1 ng B[*a*]P/m^3 over an exposure period of one year (Directive 2004/107/EC). However, as has recently been discussed, these risk assessment approaches have limited application to studying mixture effects and are likely to misestimate the actual risk to human health.[5] Most PAHs have not been assigned TEF/RPF values or their contribution to mixtures has been overlooked due to a lack of sensitivity in detection methods. This is particularly applicable to PAHs which are found at low levels in the environment but have demonstrated high carcinogenic potentials. An example of this is dibenzo[*def,p*]chrysene (DBC, also

63 known as dibenzo[*a,l*]pyrene), the most potent PAH known to date[6-7] yet not included among
64 the US EPA priority PAHs nor routinely used as an indicator of carcinogenic PAHs.[3,8-9]
65 Recent studies have suggested however that the quantification of DBC in urban air has
66 become more common as a result of improved detection methods.[10-14] For the reasons stated
67 above it is important to analyze and determine the levels of PAHs with high carcinogenic
68 potential in environmental samples to include them in the evaluation of mixture exposure
69 risks.
70
71 One poorly studied PAH that has shown high carcinogenic potential is benz[*j*]aceanthrylene
72 (B[*j*]A).[15-16] B[*j*]A is a cyclopenta-fused and bay-region containing PAH. As with other
73 PAHs B[*j*]A requires metabolic activation in order to exert is biological activity and studies
74 have identified two major routes of activation: epoxidation of the cyclopenta-ring, resulting
75 in the B[*j*]A-1,2-epoxide, and diol-epoxidation of the bay-region, resulting in the B[*j*]A-9,10-
76 diol-7,8-epoxide.[15,17-18] The former is the major metabolite found as a 1,2-diol in human and
77 rat liver tissues and cells.[19-20] B[*j*]A is a potent bacterial and mammalian cell mutagen and is
78 more tumorigenic than B[*a*]P in SENCAR mice and in A/J mice lung.[15-16] Trace amounts of
79 B[*j*]A have been found in the emissions from coal-fired residential furnaces and hard-coal
80 combustion[21-22] but not in wood smoke PM.[23] Substituted B[*j*]As, such as 3-methyl- and 1,2-
81 dihydro-3-methyl-B[*j*]A, have been determined in coal tar pitch, cigarette smoke and air
82 PM.[24-27] To date, very few studies have analyzed the levels of B[*j*]A in air PM[28] and to the
83 authors' knowledge no epidemiological studies have investigated the carcinogenicity of
84 B[*j*]A.
85
86 In the present study we have quantified the concentration of B[*j*]A in urban air PM collected
87 in Stockholm, Sweden and Limeira, Brazil. Our analytical setup allowed for detection and

quantification of very low levels of B[*j*]A in air PM samples from the two city locations. We have also investigated the effects of B[*j*]A on cell viability, DNA damage and activation of DNA damage signaling in human-derived hepatocellular carcinoma (HepG2) cells to evaluate its potency relative to B[*a*]P. Taken together our results show that although levels of B[*j*]A are very low in the air PM samples, it is significantly more potent than B[*a*]P and thus is likely to be an important contributor to the cancer risk of air PM.

MATERIAL AND METHODS

Chemicals and reagents

Hexane, acetone and toluene (HPLC grade) were obtained from Rathburn Ltd. (Walkerburn Scotland) and dodecane (anhydrous, ≥99%) from Sigma-Aldrich (St. Louis, MO, USA). Synthesized B[*j*]A (purity determined to be at least 95% by NMR) was kindly provided by Dr. Avram Gold and Dr. Zhenfa Zhang, University of North Carolina, Chapel Hill, NC, USA. B[*a*]P-D_{12} (98.7%) was supplied by Chiron AS (Trondheim, Norway) and benzo[*b*]fluoranthene (100%) and benzo[*k*]fluoranthene (98.3%) were obtained from Chem Service (West Chester, PA, USA). B[*a*]P (97.6%), benzo[*e*]pyrene (99.7%) and perylene (99.5%) were from Sigma-Aldrich (St. Louis, MO, USA). Benzo[*a*]fluoranthene was obtained from National Institute of Standards and Technology (Gaithersburg, MD, USA), and benzo[*j*]fluoranthene (100%) was from Larodan Fine Chemicals AB (Limhamn, Sweden). All cell culture reagents were supplied by Gibco (Life Technologies, Stockholm, Sweden). Antibodies used for Western blotting were phospho-Chk1 (Ser317), phospho-H2AX (Ser139) and phospho-p53 (Ser15)from Cell Signaling Technology (Beverly, MA, USA), and p53 (DO-1), Cdk2 (M2) and secondary anti-mouse and anti-rabbit antibodies from Santa Cruz

(Santa Cruz, CA, USA). For immunocytochemistry, anti-phospho H2AX (Ser139, clone JBW301) was from EMD Millipore Corp. (Billerica, MA, USA) and secondary Alexa Fluor 594 goat anti-mouse from Molecular Probes (Life Technologies, Stockholm, Sweden).

Air sampling

Two collection sites for air PM were used: Stockholm University, Stockholm, Sweden and the Faculty of Technology, UNICAMP, Limeira, Brazil. The Stockholm sampling has been described previously.[10] Briefly, three air PM samples (STO1, STO2 and STO3) were collected on fluorocarbon coated glass fiber filters (Ø = 235 mm, Fiberfilm Filters, Pallflex, Pall Corporation, Putnam, CT, USA) at roof top level using an in-house pump device for three or seven days at an average flow rate of 70.6 m^3/hr. The filters were desiccated for at least 24 h before and after the sampling. In Limeira, two air PM samples (LMR1 and LMR2) were collected on glass fiber filters (254 × 233 mm, 0.33 mm pore size, Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) at the street level. The sampling was performed with a high-volume sampler (Energética Ind. Com. LTDA, Rio de Janeiro, RJ, Brazil) for 24 h at an average flow rate of 67.8 m^3/hr. Until extraction filters from both Stockholm and Limeira were wrapped in aluminum and stored at -20 ^oC.

Sample preparation and LC-GC/MS analysis

A pressurized liquid extraction system (ASE 200 Accelerated Solvent Extraction System, Dionex Co., Sunnyvale, CA, USA) was used for the filter extraction. Samples were extracted with toluene for 5 consecutive cycles of 30 min. Then, 0.6 mL of the extract (corresponding to 56.1 m^3, 214.6 m^3, 144.5 m^3, 34.5 m^3 and 26.3 m^3 for STO1, STO2, STO3, LMR1 and

LMR2, respectively) was spiked with 60 µL of 442 pg/µL B[*a*]P-D_{12} and applied to an SPE column (silica, 100 mg, IST Isolute, Biotage, Cardiff, UK). A PAH enriched fraction was obtained by elution with 2 mL of hexane. The final hexane eluate was evaporated until 100 µL under a gentle nitrogen stream and transferred to a 300 µL micro vial for LC-GC/MS analysis. All sample preparations were performed in triplicate. The extraction and SPE clean-up procedures[29] and the LC-GC/MS method[30] are described in detail elsewhere. Blank samples were prepared in the same manner using blank filters.

Cell culture and exposure to PAHs

Human-derived hepatocellular carcinoma cells (HepG2) were obtained from American Type Culture Collection (Rockville, MD, USA) at passage 78 and used within 20 passages for all experiments. The rationale for using this cell line in these studies is the capacity to metabolize PAHs[31] and previously demonstrated response to low levels of PAHs.[32-34] Cells were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 units/ml *penicillin* and 0.1 mg/ml *streptomycin*, and maintained at 37°C in 5% CO_2. Cells were exposed to solvent control (0.1% DMSO), B[*j*]A or B[*a*]P for up to 48 h.

MTT assay

Cell viability was assessed using MTT assay as described previously.[35] Briefly, HepG2 cells (0.3×10^5) were plated in 24-well plates (Corning Inc., Corning, NY, USA) and allowed to grow for 24 h. Cells were exposed to solvent control, B[*j*]A or B[*a*]P for 48 h in 1.5 ml medium and thereafter incubated with 0.5 mg/ml MTT reagent in 250 µl HBSS for 4 h.

Subsequently, formazan crystals were dissolved in 500 µl DMSO (15 min, shaking) and plates were spectrophotometrically analyzed at 570 nm with a reference wavelength at 690 nm (Wallac Victor^3 V 1420 multilabel counter, Perkin Elmer, Waltham, MA, USA). Results are presented as percent of solvent control and EC_{50} was established using non-linear regression in GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Comet assay

The alkaline comet assay was performed as described previously.[36] In brief, HepG2 cells (0.3 x 10^5) were plated in 24-well plates (Corning Inc., Corning, NY, USA) and grown for 24 h prior to exposure to solvent control, B[*j*]A or B[*a*]P in 1.5 ml for 48 h. Cells were trypsinized and mixed with 0.75% w/v low melting point agarose and smeared on slides pre-coated with 0.3% agarose. Slides were incubated in cold lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h on ice followed by incubation in cold alkaline solution (0.3 M NaOH, 1 mM EDTA, pH >13) for 40 min on ice. Electrophoresis was run in the alkaline solution at 29 V (1.15 V/cm) for 30 min and thereafter slides were neutralized in 0.4 M Tris-HCl, pH 7.5, dried overnight and fixed in methanol for 5 min. After ethidium bromide staining, at least 100 cells were scored per treatment using a Leica DMLB fluorescent microscope and Comet Assay IV (Perceptive Instruments Ltd., Haverhill, UK).

Immunocytochemistry

HepG2 cells (0.2 x 10^4) were plated in 12-well plates containing 13 mm glass cover slips and grown for 24 h before exposure to solvent control, B[*j*]A or B[*a*]P in 2.0 ml for 48 h. Cells were washed and fixed in 4% paraformaldehyde for 20 min at room temperature and then

permeabilized with 0.2% Triton X-100 for 10 min followed by blocking in 2% bovine serum albumin in TBS-Tween 0.1% with 5% normal goat serum. Incubation with primary antibody was overnight at 4°C followed by washing and incubation with secondary antibody for 1 h in the dark. Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) prior to mounting with Vectashield H-1000 mounting medium (Vector Labs, Burlingame, CA, USA). Images were captured using a 63x oil immersion objective on an LSM 510 Meta confocal laser scanning microscope (Zeiss, Göttingen, Germany). Cell foci were counted using CellProfiler cell image analysis software version 2.1.0 (MIT and Broad Institute, Cambridge, MA, USA).[37]

For determining statistical effects in the immunocytochemistry experiments, we estimated the expected mean count in each exposure group with a linear regression model for each experiment separately. The experimental groups were introduced by means of indicator variables and count data analyzed marginally with respect to the experiment's day. We used the sandwich robust estimator for the standard errors[38] which is robust to misspecification of the modeling assumptions (e.g. homoscedasticity). The analyses were performed in Stata version 13 (StatCorp, Collage Station, TX, USA).

Western Blotting

Western blotting was performed as described previously[32] with minor modifications. HepG2 cells (4.0×10^5) were plated in 6-well plates (Corning Inc., Corning, NY, USA) and cultured for 72 h. Cells were exposed to solvent control, B[*j*]A or B[*a*]P for 48 h, washed with ice-cold PBS and scraped into IPB-7 buffer containing protease inhibitors. Protein concentration was measured and samples subjected to standard SDS-PAGE. Subsequently, proteins were

transferred to PVDF membrane (Bio-Rad, Hercules, CA) and protein levels were detected using specific antibodies and visualized using enhanced chemiluminescence (Amersham GE Healthcare, Bio-Sciences AB, Uppsala, Sweden). Densitometric analysis was performed using ImageJ software version 1.48f (National Institute of Health, USA).

Cancer risk assessment

In order to estimate the excess lifetime cancer risk for inhalation exposure to PAHs in air PM, B[*a*]P equivalency concentrations (B[*a*]P_{eq}) were determined using current RPF scales (Table S1).[39] The contribution to the cancer risk from B[*j*]A was estimated using published RPFs[15-16,40-41] and potency factors based on H2AX and Chk1 activation. The cancer risk was determined by multiplying PAH concentration with a unit risk (per 100 000 people) for B[*a*]P set by WHO[4] to 8.7×10^{-5} ng/m^3 (based on epidemiological study of coke oven workers) using the equation below.

$$\text{Cancer risk} = \sum([\text{PAH}] \times \text{RPF}_{\text{PAH}}) \times \text{unit risk}$$

Statistics

All data presented are means ± standard error (SE). With the exception of the immunocytochemistry analysis (described above), One-Way ANOVA with Bonferroni's t-test correction was used to determine statistical significance ($p<0.05$).

RESULTS

238 **Detection limit and linearity**

239

240 A series of six B[*j*]A standard solutions were injected on the LC-GC/MS system in triplicate
241 and a calibration curve of the area ratio (B[*j*]A/B[*a*]P-D_{12}) against the amount of B[*j*]A was
242 plotted in the range of 2−493 pg B[*j*]A spiked with 10 µL of 442 pg/µL B[*a*]P-D_{12}. A GC/MS
243 chromatogram of a calibration standard is shown in Fig. S1. The coefficient of determination
244 (R^2) was 0.9975 and the limit of detection (at a signal to noise ratio (S/N) of 4.2) and limit of
245 quantification (at a S/N of 14.9) were determined to be 2.5 and 12 pg, respectively.

246

247 **Determination of B[*j*]A in air PM**

248

249 Representative GC/MS chromatograms of air PM samples from Stockholm and Limeira are
250 shown in Fig. 1A and 1B, respectively. The B[*j*]A peak was well-separated from the other
251 seven molecular weight 252 Da isomers detected, whilst partially co-eluting with an
252 unknown peak displaying a characteristic PAH mass spectrum (mother ion *m/z* 268).
253 Together with mass spectra, the peak identification was confirmed by comparing the
254 retention time of B[*j*]A from the original and spiked samples in the GC/MS chromatograms
255 as shown in Fig. 1C and 1D. No detectable amount of B[*j*]A was found in blank samples
256 generated by extracting the different filter types used for sampling air PM in Stockholm and
257 Limeira (Fig. S2). Sample identities and collection details including levels of B[*a*]P and
258 B[*j*]A in the air PM from Stockholm and Limeira are summarized in Table 1. Determined
259 B[*j*]A concentrations were between 11 to 30 times less than those of B[*a*]P. The
260 concentrations of all PAHs determined in the air PM samples are shown in Table S2. The
261 levels of PAHs in STO1 and LMR2 have previously been published.[42]

262

B[*j*]A exerts stronger effects on cell viability than B[*a*]P consistent with increased DNA damage

In vitro studies have shown that B[*j*]A is a genotoxic agent and a more potent inducer of apoptosis than B[*a*]P.[17,43] In agreement, we found that B[*j*]A was more toxic to the cells than B[*a*]P in the MTT assay (Fig. 2A). The estimated EC_{50} value for cell viability was 0.39±0.20 and 1.45±0.09 μM for B[*j*]A and B[*a*]P, respectively. A statistically significant decrease in cell viability compared to control levels was observed after exposure to 2.0 μM B[*a*]P or 0.3 μM B[*j*]A ($p<0.001$). There was also a significant difference between cells exposed to equimolar concentrations of B[*j*]A and B[*a*]P at 0.3 and 1.0 μM ($p<0.001$).

Increased potency between different PAHs has previously been shown to be intrinsically linked to their ability to form DNA damage and the persistence of this damage/resistance to repair.[44-46] We therefore hypothesized that in a comparable manner, the increased toxicity of B[*j*]A over B[*a*]P correlates with differences in DNA damage levels. A single exposure time of 48 h was chosen for these analyses as we have previously used this time point to study the effects of individual and mixtures of PAHs on DNA damage.[32-33] Significantly increased levels of DNA damage were observed in the Comet assay after exposure to 1.0 μM B[*a*]P ($p<0.001$) or 0.3 μM B[*j*]A ($p<0.001$) when compared to control levels (Fig. 2B). No significant changes in DNA damage levels were observed after exposure to 0.3 μM B[*a*]P (Fig. 2B) or 0.1 μM B[*j*]A (data not shown). Comparing equimolar concentrations showed that B[*j*]A induced significantly more DNA damage than B[*a*]P ($p<0.001$). These results were further confirmed by immunostaining for formation of phosphorylated H2AX (Ser139) foci (γH2AX). Visual comparison between treatments showed more foci formation in response to B[*j*]A>B[*a*]P>DMSO control (Fig. 2C). Both the B[*a*]P group and the B[*j*]A group had

higher foci rate compared to control group, 5.7 and 17.2 times more, respectively ($p<0.001$) (Fig. 2D). The number of foci in B[*j*]A exposed cells were also significantly higher from that in the equimolar B[*a*]P group ($p<0.001$). Taken together these data show that B[*j*]A is significantly more potent than B[*a*]P in reducing cell viability and that this most likely results from increased damage to DNA.

B[*j*]A induces a stronger activation of DNA damage signaling than B[*a*]P

In agreement with the Comet assay and γH2AX foci results, our data revealed B[*j*]A to be a more potent inducer of DNA damage signaling than B[*a*]P. At 1 μM, B[*j*]A induced phosphorylation of Chk1 Ser317 (pChk1), γH2AX and p53 Ser15 (pp53) to a higher extent than B[*a*]P (Fig. 3A). Total p53 level was also more elevated in response to B[*j*]A compared to B[*a*]P as well as the protein level of the cell cycle regulator p21. To further study the difference in potencies between B[*j*]A and B[*a*]P we applied a dose response analysis for the induction of pChk1, pp53 and γH2AX. As can be seen, B[*j*]A induced phosphorylation of all proteins at lower concentration compared to B[*a*]P (Fig. 3B and 3C). To allow for a quantitative comparison of potency, densitometric analysis of pChk1 and γH2AX levels (Fig. 3C) was used to estimate the concentrations of B[*j*]A and B[*a*]P required to induce a particular fold induction of the proteins (Table S3). Due to no visible bands of pp53 in control and at lower concentrations of B[*j*]A and B[*a*]P proper densitometry analysis and comparison of potencies could not be accurately performed. For comparison, we included previous data from DBC exposures.[32] DBC induces pChk1 at lower concentrations compared to B[*j*]A whereas γH2AX was induced similarly by DBC and B[*j*]A (Fig. 3C). The results showed that levels of pChk1 increased at lower levels for B[*j*]A compared to γH2AX, in

agreement with our previous data.[32] On average B[*j*]A was 12.5- and 33.3-fold more potent inducer of pChk1 and γH2AX levels, respectively, compared to B[*a*]P.

Contribution of B[*j*]A to air PM cancer risk

If only the B[*a*]P concentration in air PM was considered the estimated excess lifetime cancer cases per 100 000 people was 0.15-2.5 (ex. STO1: 0.289 ng B[*a*]P/m^3 × 8.7×10^{-5} = 2.5×10^{-5}) and 4.9-7.8 for Stockholm and Limeira, respectively (Table 2). Next we calculated the excess lifetime cancer cases based on all the determined PAHs in the air PM samples (except B[*j*]A) with an assigned RPF value. Compared to the excess lifetime cancer cases based on levels of B[*a*]P alone, the cases increased to 0.77-11.3 and 16.2-22.7 per 100 000 people for Stockholm and Limeira, respectively (Table 2). The additional contribution to the cancer risk by including B[*j*]A in the $B[a]P_{eq}$ levels was estimated using two different published RPFs for B[*j*]A of 10 and 60[15-16,40-41] and our potency factor of 30 based on H2AX activation (Table 2). The published RPF of 10 was similar to our potency factor based on activation of Chk1. The results showed that inclusion of B[*j*]A increased the estimated cancer risk of the air PM. Depending on which potency factor that was applied, inclusion of B[*j*]A resulted in an up to doubling of expected excess lifetime cancer cases (Table 2). This provides convincing evidence that B[*j*]A contributes to the carcinogenic potency of urban air PM and warrants further investigation.

DISCUSSION

Concentrations of B[*j*]A in urban air PM

337 The present study determined the atmospheric B[*j*]A concentration in air PM collected at two
338 different locations, Stockholm, Sweden and Limeira, Brazil. The air PM from Limeira
339 contained higher levels of PAHs compared to the samples from Stockholm. Besides the
340 probable difference in PAH levels due to emission profiles in the respective areas, the
341 samples from Limeira were collected at street level while in Stockholm sampling was
342 performed on a roof top. PAHs at the Limeira site can be mostly attributed to heavy traffic
343 and biomass burning whereas traffic is a main source at the Stockholm site.[47] The PAH levels
344 in Stockholm air PM displayed large seasonal changes during the different sampling periods:
345 the B[*a*]P concentration was 17.3 pg/m^3 in September and increased to 49.5 and 289 pg/m^3 in
346 December and January, respectively. The B[*j*]A concentration followed the same trend: 1.57,
347 3.14 and 12.7 pg/m^3, as well as the other measured PAHs and this is in line with recently
348 reported PAH levels from the Stockholm atmosphere showing a high annual variability with
349 higher concentrations during the colder parts of the year.[48] Higher PAH levels during the
350 winter season in cities around the world has been attributed to meteorological conditions such
351 as inversion and lower mixing layer, less efficient atmospheric reactions and increase in
352 emissions from domestic heating.[49] The two air PM samples from Limeira were both
353 collected in July and had higher concentration of B[*a*]P, B[*j*]A and of most of the other
354 investigated PAHs with a few exceptions compared to the Stockholm samples. A previous
355 study of air PM PAHs in Saitama City, Japan, reported measurements of 37 PAHs including
356 B[*a*]P, B[*j*]A and DBC and to our knowledge it is the only other study reporting measurement
357 of B[*j*]A in urban air.[28] The concentrations of these PAHs in total air PM was 270, 130 and
358 70 pg/m^3 for B[*a*]P, B[*j*]A and DBC, respectively. The levels of B[*a*]P in Saitama (sampling
359 period November-December 2013) was similar to STO1, exceeded STO2 and STO3 and was
360 less than LMR1 and LMR2. For B[*j*]A and DBC the levels were much higher in Saitama
361 compared to all our investigated samples. Further studies to investigate the generation and

362 deposition of PAHs in the different locales might explain the observed differences in B[*j*]A
363 and DBC levels.
364
365 **Stronger activation of DNA damage and DNA damage signaling after exposure to B[*j*]A**
366
367 In HepG2 cells, B[*j*]A was significantly more toxic than B[*a*]P. This is in agreement with a
368 previous study performed in mouse Hepa1c1c7 hepatoma cells showing that B[*j*]A is a
369 stronger inducer of apoptosis compared to B[*a*]P.[43] As mentioned before, previous studies
370 have correlated increased potency of PAHs with the ability to form DNA adducts and
371 avoidance of repair. The Comet assay revealed that B[*j*]A was significantly more genotoxic
372 than B[*a*]P at equimolar concentrations and this is in line with earlier studies showing that
373 B[*j*]A induces more DNA adducts in rat and human liver microsomes compared to B[*a*]P.[19-20]
374 Comparing the cytotoxic and genotoxic potencies of the two compounds showed that the
375 cytotoxic doses of B[*j*]A were also genotoxic, this was not true for 1 μM B[a]P which was
376 genotoxic but not cytotoxic. The increase in genotoxicity was further confirmed by
377 immunostaining for phosphorylated H2AX (γH2AX). H2AX is rapidly phosphorylated (at
378 Ser139) at sites of DNA damage, both in response to strand breaks and stable DNA adducts,
379 forming γH2AX foci that can be detected by immunocytochemistry.[50-51] Furthermore,
380 phosphorylation of H2AX in the early stages of damage detection is attributed to the
381 propagation of DNA damage signaling and hence we also analyzed levels of γH2AX by
382 Western blotting alongside other major DNA damage signaling proteins including Chk1, p21
383 and p53. We found B[*j*]A to be a stronger inducer of all investigated signaling proteins.
384
385 From the dose response curves of pChk1 and γH2AX we calculated the PAH concentrations
386 needed to induce a fold change in activated protein levels as described earlier.[32] These

proteins have previously been shown to be sensitive markers for studying genotoxic potencies of both individual and mixtures of PAHs.[32-33,52] We calculated the difference in potency of B[*j*]A compared to B[*a*]P to be 12.5- and 33.3-fold for pChk1 and γH2AX, respectively. Previous studies have reported RPFs of 10 and 60 for B[*j*]A. The RPF of 10 is based on Ames test where an approximate 10-fold lower concentration of B[*j*]A compared to B[*a*]P induce optimal activity in S9 dependent test.[40-41] Similarly in SENCAR mice treated topically with B[*j*]A tumor initiation was 12 times higher compared to B[*a*]P.[16] The RPF of 60 is based on B[*j*]A inducing 16-60 times more lung tumors in A/J mice subjected to a single intraperitoneal injection relative to B[*a*]P.[15] These tumor data are in the same range as the fold induction data generated in the present study from the phosphorylation of Chk1 and H2AX. It has previously been shown for DBC that fold induction data generated from DNA damage signaling *in vitro* is in agreement with animal experiments regarding carcinogenic potency suggesting that activation of the DNA damage response could serve as a marker for *in vitro* testing of PAH potency.[5,32] The higher sensitivity of pChk1 and γH2AX compared to Comet assay data and the close agreement with published RPFs motivated us to include our potency factors based on activation of DNA damage signaling in the cancer risk estimation as further discussed below.

Risk assessment of air PM and contribution of B[*j*]A to lifetime cancer risk

PAHs have been suggested to be the group of airborne contaminants that contribute most to human health risk. In urban sites 83-94% of the health risk was related to particle associated compounds and of these PAHs was responsible for 99% of the risk.[53] An investigation of excess lifetime cancer risk in different age categories in Cordoba, Argentina, showed that already in the age category 1-6 years one child out of one million would get cancer as a result

of airborne PAH exposure.[54] In the present study the excess lifetime cancer cases was estimated based on B[*a*]P_{eq} levels and the WHO unit risk value 8.7×10^{-5} ng B[*a*]P /m^3.[4] We showed that the air samples from Limeira were more polluted by PAHs than the Stockholm samples which was also reflected in a higher number of expected excess lifetime cancer cases, 17.9-38.4 and 0.91-18.0 cases per 100 000 people, respectively. Furthermore, our results showed that B[*j*]A greatly contributes to the cancer risk of air PM despite the low concentrations that we detected. Although B[*j*]A levels in air were low, including B[*j*]A in the estimation of excess lifetime cancer risk increased the risk up to 2-fold depending on which potency factor for B[*j*]A was applied. The importance of including highly potent PAHs, even though found in low levels, in the cancer risk assessment of air PM is further confirmed by recent studies. Layshock *et al.,* showed that excluding the dibenzopyrenes when assessing air quality most likely results in a substantial underestimation of the health risk.[12] A calculated increase in lifetime risk of developing lung cancer for residents in Beijing, China, due to PAHs in air PM, may be 1 out of 10 000 to over 6 out of 100 and half of that risk was estimated to be related to levels of the dibenzopyrenes.[12] The importance of including high-molecular weight PAHs, such as the dibenzopyrenes, has also been emphasized in other studies showing a significant contribution to the estimated excess lifetime cancer risk of air PM.[11,13,55] It should be noted that B[*j*]A was not included in any of the above mentioned studies which probably further resulted in an underestimation of the cancer risk.

In this study we focused on excess lifetime cancer risk from exposure to inhaled air PM PAHs. We are aware of the limitations in the estimation such as the shortcomings of using RPFs which in the case of B[*j*]A are based on data from Ames test and *in vivo* dermal and intraperitoneal exposure when assessing the cancer risk for inhaled air PM. Further to give a

more comprehensive view of excess lifetime cancer risk, the PAH levels should be monitored over the whole year to include the seasonal variations in PAH constituents. It is very apparent in the three Stockholm samples that the time of sampling greatly affects the PAH levels and in turn the cancer risk estimate.

SUPPORTING INFORMATION

Supporting information available: Tables S1-S3 (air PM B[*a*]P_{eq} levels, chemical analysis of air PM samples and fold induction of DNA damage signaling) and Figures S1-S2 (GC/MS chromatograms). This material is available free of charge via the internet at http://pubs.acs.org.

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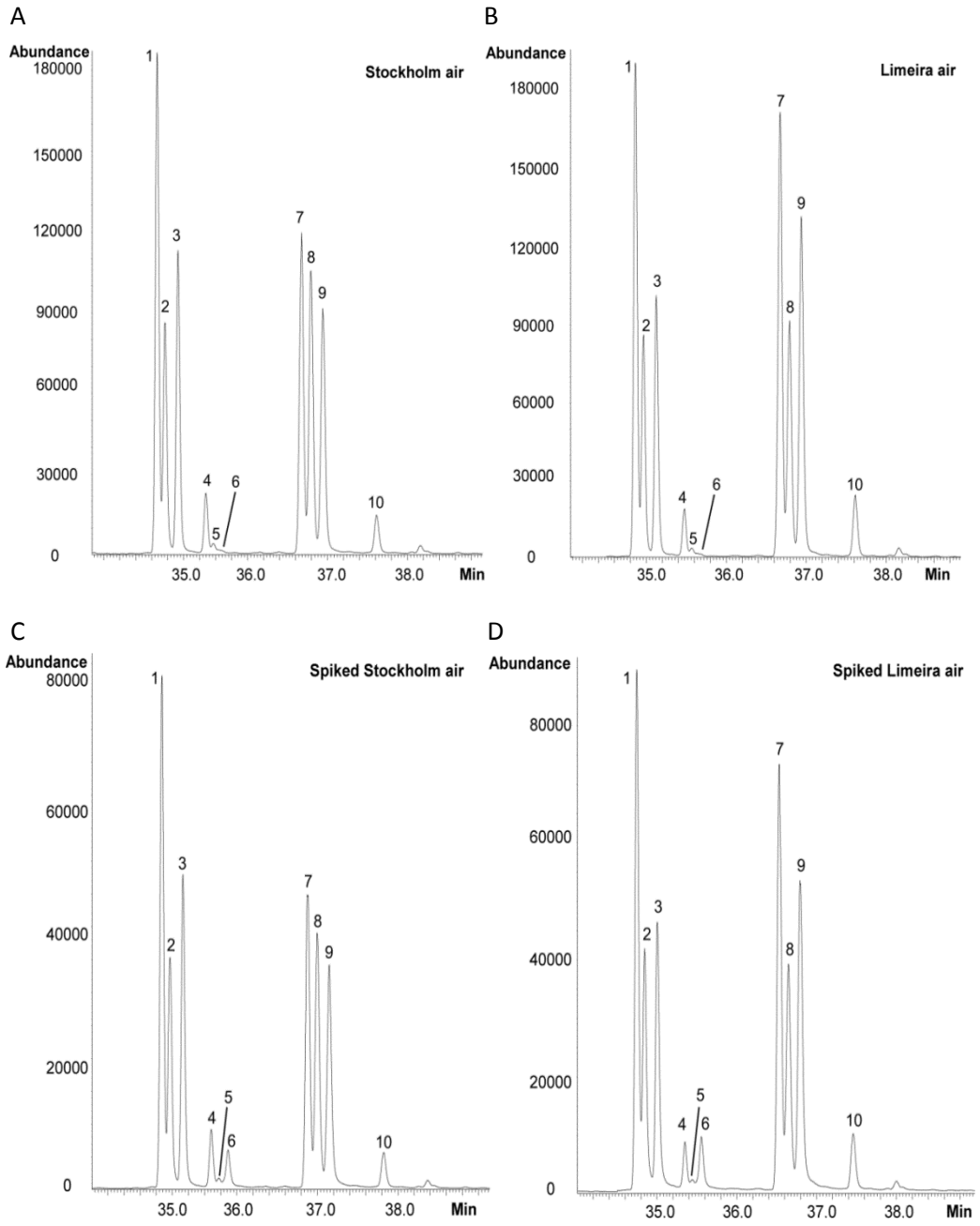

Fig. 1. Detection of B[*j*]A in air PM samples. GC/MS chromatograms obtained in SIM mode of air PM samples from Stockholm (A), Limeira (B), Stockholm spiked with B[*j*]A (C) and Limeira spiked with B[*j*]A (D). 1: benzo[*b*]fluoranthene, 2: benzo[*k*]fluoranthene, 3: benzo[*j*]fluoranthene, 4: benzo[*a*]fluoranthene, 5: unknown, 6: benz[*j*]aceanthrylene, 7: benzo[*e*]pyrene, 8: benzo[*a*]pyrene-D_{12}, 9: benzo[*a*]pyrene, 10: perylene.

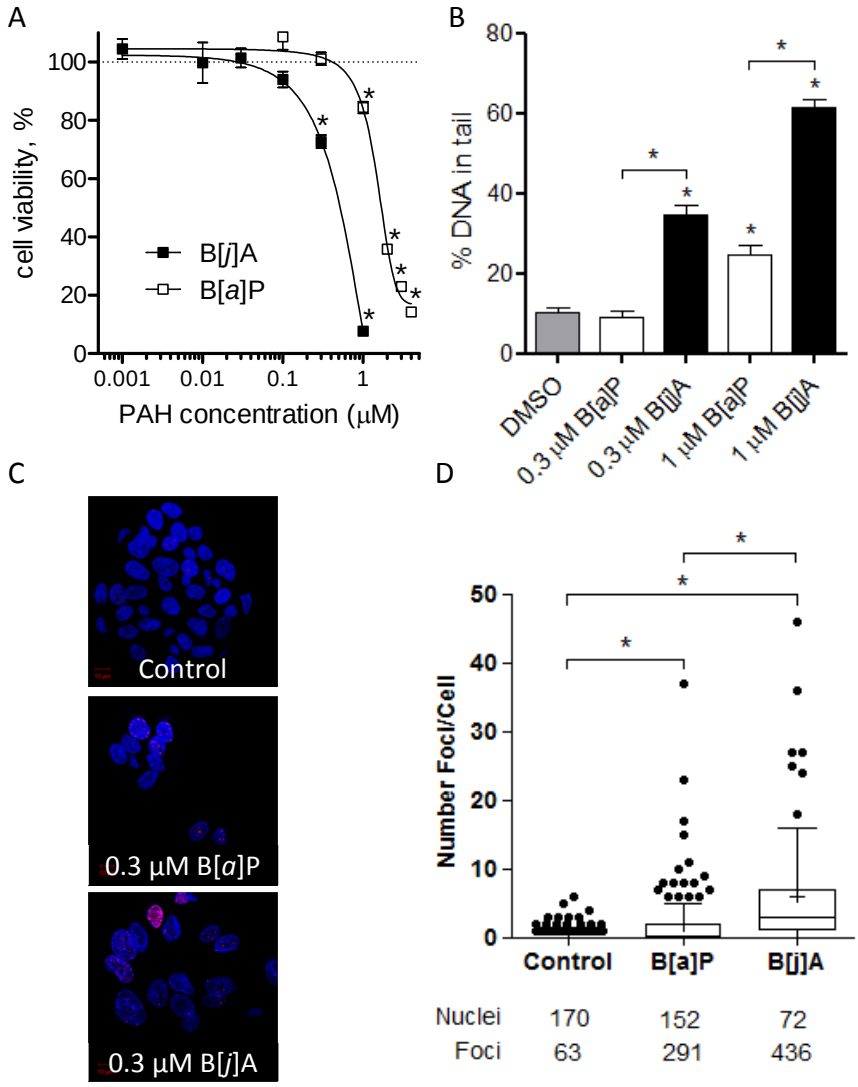

Fig. 2. B[*j*]A reduce cell viability and induce DNA damge stronger than B[*a*]P. HepG2 cells were exposed to B[*j*]A, B[*a*]P or control solvent (DMSO) for 48 h. MTT assay (A) of cells exposed to 0.001-1 μM B[*j*]A or 0.01-4 μM B[*a*]P where cell viability is presented as percent of DMSO treated cells. Comet assay (B) was performed on cells exposed to 0.3 and 1 μM B[*j*]A or B[*a*]P, or DMSO and 100 cells were scored (n=2). Sigmoidal dose response curve fit (n=6) was applied in A. One-way ANOVAs with Bonferroni's post test was applied in A and B, * $p<0.05$ compared DMSO. Immunostaining (C) of γH2AX (red) and DAPI (blue) in cells exposed to 0.3 μM B[*a*]P or B[*j*]A, or DMSO. Scatter plot (D) of counted cells and foci from immunostaining with statistical significance obtained using a linear regression model.

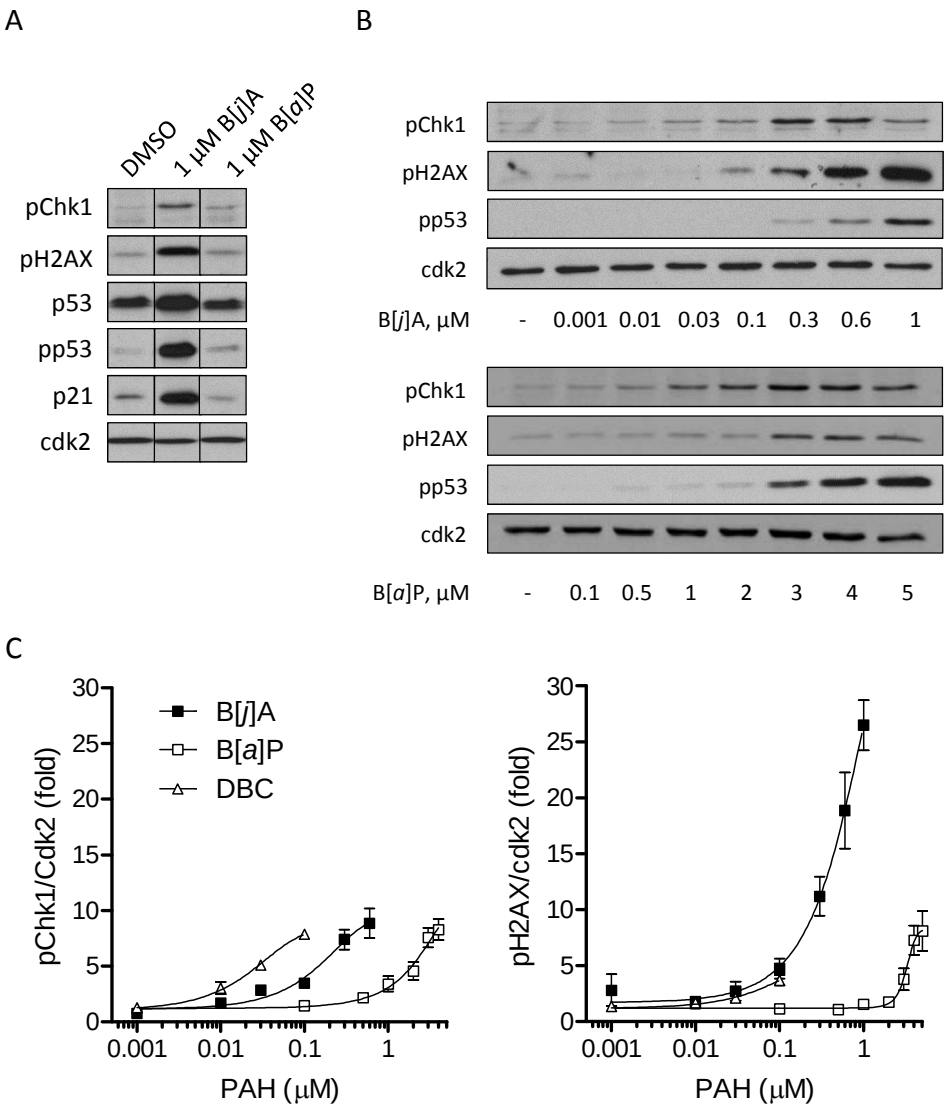

Fig. 3. Stronger activation of DNA damage signaling proteins in cells exposed to B[*j*]A compared to B[*a*]P. HepG2 cells were exposed to PAHs for 48 h and protein levels were measured with Western blot. In (A) the effect of equimolar concentration of B[*j*]A and B[*a*]P (1 µM) on pChk1, γH2AX, p53, pp53 and p21. Cdk2 was used as loading control. In (B) dose response of 0.001-1 µM B[*j*]A and 0.1-5 µM B[*a*]P on pChk1 and γH2AX. In (C) densitometric analysis of pChk1 and γH2AX. Densitometric analysis of DBC was previously published in Jarvis et al., 2013. Non-linear curve fit, sigmoidal dose response, n=4.

709 **Table 1**. Information of the air PM samples collected in Stockholm and Limeira.

Name	**Abbreviation**	**Sampling period**	**Duration** (hours)	**Sampled air** (m^3)	**Total PAH** (pg/m^3)	**B[*a*]P** (pg/m^3, n=3)	**B[*j*]A** (pg/m^3, n=3)	**Total PM** (mg)
Stockholm 1	STO1	Jan 18-21, 2013	71	5141	4440 ± 138	289 ± 4	12.7 ± 0.2	36.8
Stockholm 2	STO2	Sep 10-17, 2013	168	12265	351 ± 4.1	17.3 (SD<0.02)	1.57 ± 0.07	91.3
Stockholm 3	STO3	Dec 10-17, 2013	167	11034	793 ± 7.1	49.5 ± 0.5	3.14 ± 0.15	60.3
Limeira 1	LMR1	Jul 12, 2010	24	2352	7958 ± 158	560 ± 10	19.6 ± 0.8	263
Limeira 2	LMR2	Jul 19, 2010	24	2441	10693 ± 297	899 ± 17	30.2 ± 0.7	234

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Table 2. Contribution of B[*j*]A levels to the excess lifetime cancer cases from exposure to air PM. Based on B[*a*]P_{eq} levels and the WHO unit risk value of 8.7×10^{-5} ng/m^3 B[*a*]P.[4]

Air PM	Excess lifetime cancer cases / 100 000 people				
	B[*a*]P	B[*a*]P_{eq} excl. B[*j*]A	B[*a*]P_{eq} incl. B[*j*]A[a]		
			RPF of B[*j*]A = 10[b]	RPF of B[*j*]A = 30[c]	RPF of B[*j*]A = 60[d]
STO1	2.5	11.3	+ 1.1	+ 3.4	+ 6.7
STO2	0.15	0.77	+ 0.14	+ 0.41	+ 0.82
STO3	0.43	2.3	+ 0.3	+ 0.9	+ 1.7
LMR1	4.9	16.2	+ 1.7	+ 5.1	+ 10.2
LMR2	7.8	22.7	+ 2.6	+ 7.9	+ 15.7

[a] increase in cancer cases compared to B[*a*]P_{eq} excl. B[*j*]A; [b] from[16,40-41], [c] our fold induction data for γH2AX (Table S2), [d] from[15]

717 **Abstract Art**

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