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Human CD34-positive hematopoietic stem cells constitute targets for carcinogenic polycyclic aromatic hydrocarbons

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A. Running title
PAH toxicity towards human hematopoietic stem cells

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D. Abbreviations

AhR:	aryl hydrocarbon receptor
αNF:	α-naphthoflavone
ARNT:	AhR nuclear translocator
BFU-E:	burst forming unit-erythroid
BP:	benzo(a)pyrene
BPDE:	benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide
CFU:	colony forming unit
CFU-GM:	colony forming unit-granulocyte-macrophage
CFU-M:	colony forming unit-macrophage
CFU-MK:	colony forming unit-megacaryocyte
CYP:	cytochrome P450
$DiOC_6(3)$:	3-3'-dihexyl-oxocarbocyanine
DMBA:	7-12 dimethylbenzanthracene
3'M4'NF:	3'-methoxy-4'-nitroflavone
NAC:	N-acetylcysteine
PAH:	polycyclic aromatic hydrocarbon
ROS:	reactive oxygen species
TCDD:	2, 3, 7, 8-tetrachlorodibenzo-p-dioxin

E. Recommended section: Toxicology

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are major carcinogenic environmental contaminants known to exert bone marrow toxicity and to induce leukemias, suggesting that these chemicals target hematopoietic stem cells. To investigate this hypothesis, we have studied the effects of PAHs towards cell proliferation and differentiation in human hematopoietic CD34+ cell cultures. Benzo(a)pyrene (BP), a prototypical PAH, was shown to markedly impair CD34+ cell expansion and to inhibit CD34+ cell differentiation into various haematological cell lineages, including erythroid, granulo-macrophagic and megakaryocytic lineages. This was associated with induction of a caspase- and mitochondrion-related apoptosis process. CD34+ progenitor cells were found to exhibit functional expression of the aryl hydrocarbon receptor (AhR) and the use of the pure AhR antagonist 3'-methoxy-4'-nitroflavone partially counteracted the deleterious effects of BP in CD34+ cell cultures, underlining the involvement of AhR in BP toxicity. Additional events such as cytochrome P-4501A1/1B1-dependent PAH metabolism and adduct formation were also required since (i) 2,3,7,8-tetrachlorodibenzo-p-dioxin, a very potent ligand of the AhR, which is poorly metabolized and therefore does not generate reactive metabolites in contrast to PAHs, failed to affect CD34+ cell expansion, (ii) the cytochrome P-4501A1/1B1 inhibitor α-naphthoflavone blocked both BP adduct formation and BP toxicity and (iii) benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide, a highly reactive BP metabolite, exerted a marked toxicity towards CD34+ cell cultures. Overall, these data indicate that human hematopoietic CD34+ cells can bio-activate chemical carcinogens like PAHs and, by this way, constitute targets for such carcinogenic environmental contaminants.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BP) constitute ubiquitous environmental contaminants. They are usually generated through the incomplete combustion of organic materials, and are notably found in automobile exhaust, cigarette smoke, charcoal-broiled-foods and industrial waste by-products (Zedeck, 1980; Phillips, 1999). Some of them, including BP, are well recognized as major chemical carcinogens for rodents and humans (Zedeck, 1980). This has been linked to mutagenic properties of PAH metabolites, such as benzo(a)pyrene-trans-7,8-dihydrodiol-9,10-epoxide (BPDE), which form covalent adducts with DNA (Melendez-Colon et al., 1999). Generation of these toxic metabolites is mediated by cytochromes P450 (CYPs), especially CYP1A1 and 1B1, whose expression can be markedly up-regulated by PAHs (Whitlock, 1999). This induction of xenobiotic metabolizing enzymes is caused by PAH-mediated activation of the aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helix transcription factor. Indeed, PAHs bind to cytoplasmic AhR, thereby triggering its translocation into the nucleus, association with the AhR nuclear translocator (ARNT) and interaction with xenobiotic responsive elements found in the 5'-flanking regions of PAH-regulated genes, including CYPs (Whitlock, 1999).

Besides carcinogenic properties, PAHs exert a wide range of deleterious effects towards tissues and cells. PAHs are thus potent immunotoxic agents which impair functional activation of lymphocytes (Burchiel et al., 1990; Davila et al., 1996) and inhibit macrophagic differentiation (van Grevenynghe et al., 2003). They reduce fertility, which may reflect alterated survival of ovocytes (Matikainen et al., 2001), favour the development of cardiovascular diseases (Ambrose and Barua, 2004) and trigger apoptosis in various cell lines (Salas and Burchiel, 1998; Solhaug et al., 2004).

Bone marrow is also affected by PAHs, at least in rodents. Indeed, acute treatment of mice by 7-12 dimethylbenzanthracene (DMBA) results in a profound hypocellularity in the marrow (Galvan et al., 2003; Page et al., 2004). In addition, B-cell development, which takes place in the bone marrow, is markedly altered by PAHs (Mann et al., 1999). Moreover, PAHs are well-known chemicals used for inducing experimental leukaemias in rodents (Heidel et al., 2000). Taken together, these data suggest

that hematopoietic stem cells may constitute important targets for PAHs. To investigate this hypothesis and to gain insights about the cellular and mechanisms involved, the present study was designed to analyze the effects of PAHs towards normal isolated human CD34+ progenitor cells. Such progenitor cells, characterized by the expression of the immaturity marker CD34, can differentiate into different haematological cell lineages and constitute a suitable *in vitro* model for analyzing the effects of xenobiotics on hematopoiesis, especially in humans (Bartolovic et al., 2004). Our data indicate that BP, used here as a prototypical PAH, markedly impairs proliferation and differentiation of these hematopoietic stem cells and induces their apoptosis. Moreover, such toxic effects are demonstrated to be related, at least in part, to CYP-dependent BP metabolite formation. These data thus provide evidence that human hematopoietic CD34+ cells are capable of bio-activating chemical carcinogens like PAHs and, by this way, constitute targets for these major environmental contaminants.

MATERIALS AND METHODS

Chemicals and reagents

BP, benzo(e)pyrene, DMBA, 3-methylcholanthrene, N-acetylcysteine (NAC), pyrene and α-naphthoflavone (αNF) were provided by Sigma (St Louis, MO), whereas 2,3,7,8–tetrachlorodibenzo-p-dioxin (TCDD) was from Cambridge Isotope Laboratories (Cambridge, MA). The pure AhR antagonist 3'-methoxy-4'-nitroflavone (3'M4'NF) was a generous gift from Pr. Thomas A. Gasiewicz (University of Rochester, USA) and BPDE was obtained from Promochem (Molsheim, France). All chemicals were used as stock solutions in dimethyl sulfoxide, except BPDE which was dissolved in anhydrous tetrahydrofuran (Sigma). Final concentrations of solvent in culture medium did not exceed 0.2 % (v/v) and control cultures received the same dose of solvent. Recombinant human stem cell factor, Flt3-ligand and interleukin-3 were provided by Tebu-bio (Le Perray-en-Yvelines, France) and StemCell Technologies (Meylan, France).

Cellular culture

Peripheral human CD34+ cells mobilized by G-CSF were purified from samples of blood cytapheresis products using anti-CD34 Abs-coated magnetic beads (Miltenyi Biotec, Paris, France). Purity of isolated CD34+ cellular fractions was routinely greater than 98%, as determined by immunophenotyping analysis. CD34+ cells were then cultured for 3 to 10 days in conditions triggering their proliferation in the absence or presence of PAHs. Such mitogenic culture conditions consisted of Iscove's modified Dulbecco's medium (In Vitrogen, Cergy-Pontoise, France) containing 10% fetal bovine serum, 2 mM L-glutamine, antibiotics and the following cytokines: 20 ng/ml stem cell factor, 50 ng/ml Flt3-ligand and 50 ng/ml interleukin-3 (Kohler et al., 1999). Initial cellular plating concentration was 5.10^4 CD34+ cells/ml.

Quantitation of clonogenic progenitors in semi-solid assays

Ability of CD34+ cells to differentiate along the erythroid, granulo-macrophagic and megakaryocytic cell lineages was analyzed using methylcellulose- or collagen-based progenitor assays for colony-

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formed units (CFU). Briefly, for erythoid and granulo-macrophagic lineages, 1000 CD34+ cells/dish were cultured in STEMα.IA semi-solid medium (Tebu-bio) containing erythropoietin and granulocyte-macrophage colony stimulating factor for 2 weeks at 37°C in a humidified atmosphere with 5 % CO_2 in the absence or presence of BP. Burst-forming units-erythroid (BFU-E), CFU-granulocyte-macrophage (CFU-GM) and CFU-macrophage (CFU-M) were then scored on an inverted microscope. For megacaryocyte lineage, 5000 CD34+ cells/dish were cultured for 2 weeks in collagen-Megacult^{TM}-C complete medium (StemCell Technologies) containing 50 ng/ml thrombopoietin. After 14 days of culture, CFU-megacaryocytes (CFU-MK) were immunolabeled with mouse mAbs anti-CD41a (Beckton Dickinson) and thereafter visualized using an alkaline phosphatase system (Dako, Trappes, France).

Flow cytometric immunolabelling assays

Cells were labelled with FITC-conjugated mAbs purchased by Immunotech (Marseille, France) and directed against CD34, CD11b or CD15. Isotypic control labeling was performed in parallel. Cells were then analysed analysis using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA).

Cytotoxicity and apoptosis detection

Cellular viability in CD34+ cell cultures was evaluated using the trypan blue dye exclusion test (Eurobio, Les Ullis, France). Assessment of apoptosis was performed using the FITC-conjugated annexinV Kit (Beckman Coulter, Marseille, France) for detection of externalized phosphatidylserine membrane residues of apoptotic cells. Briefly, after washing in phosphate-buffered saline at 4°C, cells were incubated with 2 µL FITC-annexinV and 5 µl propidium iodide in Ca^{2+} binding buffer for 10 min at 4°C and were finally analyzed by flow cytometry.

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Caspase 3 activity measurement

Caspase 3 activity was determined using the caspase 3 fluorogenic substrate Ac-DEVD-AMC as previously described (Gorman et al., 1999). Briefly, protein cellular lysates (10 µg) were incubated with 50 µM Ac-DEVD-AMC in buffer assay (20 mM Pipes, pH 7.2, 100 mM NaCl, 1mM EDTA, 0.1 % CHAPS, 10 % sucrose, and 10 mM dithiothreitol). Caspase-mediated release of AMC was then monitored continuously over a 30 min period at 37°C using a spectrofluorimeter Spectra Max Gemini (Molecular Devices, Sunnyvale, CA); excitation and emission wavelengths were 380 and 460 nm, respectively. Data were expressed as fluorescence arbitrary units (F.A.U)/30 min.

Measurement of mitochondrial membrane potential ($\Delta\psi m$*)*

Mitochondrial energization was evaluated through analysis of mitochondrial retention of the cationic fluorescent dye 3-3'-dihexyl-oxocarbocyanine ($DiOC_6(3)$), which depends on $\Delta\psi m$ (Kalbacova et al., 2003). Briefly, cells were first washed with phosphate-buffered saline before incubation with 50 nM $DiOC_6(3)$ at 37°C for 30 min. Cells were then washed twice with phosphate-buffered saline and $DiOC_6(3)$-related fluorescence was analyzed by flow cytometry using a FACSCalibur cytometer. Positive control of mitochondrial potential depolarisation was performed in parallel using cyanide p-(trifluoromenthoxy) phenyl hydrazone (Kalbacova et al., 2003).

RNA isolation and RT-PCR assay

Total RNA was isolated from cells using the TRIzol method (Life Technologies, Cergy Pontoise, France). Total RNA (1 µg) was reverse transcribed using the Supercript II reverse transcriptase (Life Technologies) and aliquots of cDNA were subsequently amplified with the PCR Master mix from Promega (Madison, WI). The gene-specific primers used were as follows: CYP1B1 sense: 5'-AAAGAGGTACAACATCACCT-3', CYP1B1 antisense: 5'-GTATATTGTTGAAGAGACAG-3', AhR sense: 5'-CTCATACAACACAGCTTCTCC-3', AhR antisense: 5'-TACTGAAGCAGAGCTGTGCA-3', ARNT sense: 5'-ACAGAAAGCCATCTGCTGCC-3', ARNT antisense: 5'-CGGAACA AGATGACAGCCTAC-3' and GAPDH sense: 5'-TTCACCACCATGGAGAAGGC-3', GAPDH antisense: 5'-GGCATGGACTGTGGTCATGA-3'.

The primers used for CYP1A1 detection were exactly those used by van Grevenynghe et al. (2003). Analysis of GAPDH mRNA levels, not affected by PAHs, was routinely performed as a control. The numbers of PCR cycles were 32 (for AhR, ARNT, CYP1A1 and CYP1B1 detection) and 26 (for GAPDH detection). PCR products were resolved by electrophoresis on 1% agarose gels and visualized by ethidium bromide.

Western Blotting

Total cell lysates were obtained by incubating cells in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 0,1 % Tween 20, 10 % glycerol, 100 µM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 2 µg/ml leupeptin and 1 µg/ml pepstatin. Protein lysates were then subjected to 12 % SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes. After blocking with 5 % bovine serum albumin in 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.1 % Tween 20, membranes were incubated overnight at 4°C with the following primary Abs provided by Santa Cruz Biotechnology (Santa Cruz, CA): rabbit anti-caspase 3 and anti-caspase 9 or mouse anti-HSC70 Abs. Anti-rabbit or anti-mouse peroxidase-conjugated Abs were then used as secondary Abs and the immunocomplexes formed were detected by autoradiography using the chemiluminescence ECL detection kit (Amersham, Orsay, France). Analysis of HSC70 expression, not affected by HAPs, was performed as a control.

Measurement of BP metabolite-adducts

CD34+ cells, previously exposed to 10 nM TCDD for 24 h in order to induce CYP1A1/1B1 expression, were treated by 0.1 µg/ml [^{3}H]BP (sp. act. 50 Ci/ mmol) (Isobio, Fleurus, Belgium) in the absence or presence of 1 µM αNF for 4 h. After, two phosphate-buffered saline washes, total proteins and nucleic acids were extracted using a trichloroacetic acid precipitation method. Amounts of tritiated BP metabolites covalently bound to cellular nucleophile macromolecules were then determined by scintillation counting as previously described (Solhaug et al., 2004). Background levels of BP-related radioactivity found in cell-free incubation experiments were subtracted and data were normalized to

amounts of total proteins quantified by the Bradford's method utilizing the principle of protein-dye binding (Bradford, 1976).

Statistical analysis

Data were analyzed with the non-parametric Wilcoxon's test or with Student's *t* test. The level of significance was $p<0.05$.

RESULTS

PAH exposure alters both proliferation and differentiation of human CD34+ progenitor cells

Isolated human CD34+ cells were first cultured in conditions allowing their expansion. As indicated in Fig. 1A and in agreement with previous studies (Kohler et al., 1999), CD34+ cells actively proliferated in such culture conditions, yielding approximately an expansion factor of 18-fold after 10 days of culture. This cellular proliferation was associated with partial myeloid differentiation, i.e. increased expression of the myeloid markers CD11b and CD15 and concomitant down-regulation of the relative percentage of CD34+ cells (Fig. 1B). In the presence of 10 µM BP, cell expansion in CD34+ cell cultures was altered; such a BP-mediated effect, although observed for a 3-day treatment, was more pronounced after a 6- or a 10-day treatment (Fig. 2A). This inhibitory role of BP towards CD34+ cell expansion was similarly observed for the doses of 1, 5 and 10 µM whereas a lower BP dose (0.1 µM) had no effect (Fig. 2B). Besides BP, other PAHs such as DMBA and 3-methylcholanthrene were found to decrease the number of viable cells in CD34+ cell cultures whereas, by contrast, benzo(e)pyrene and pyrene had no significant effect (Fig. 2C). We then analyzed whether a continuous exposure to BP was required for altering expansion in CD34+ cell culture. For this, isolated CD34+ cells were treated with 10 µM BP for 1, 2 or 3 days, washed and cultured in BP-free medium until day 10; number of viable cells was then compared to those found in untreated cultures and in cultures permanently exposed to BP for 10 days. As shown in Fig. 2D, amounts of viable cells were similarly down-regulated whatever the time of BP treatment, i.e. 1, 2 or 3 days, when compared to untreated cultures; they were similar to those found in CD34+ cell cultures continuously exposed to BP for 10 days, suggesting that an initial short 1-, 2- or 3-day exposure to BP was sufficient to markedly impair CD34+ cell expansion.

We then determined whether BP treatment can alter differentiation of CD34+ cells into different hematological cell lineages upon the action of appropriate cytokines in semi-solid medium assays. As shown in Fig. 3, exposure to 10 µM BP resulted in a marked reduction of colonies formed by CFU-GM, CFU-M, CFU-MK and BFU-E, when compared to untreated cultures.

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PAH treatment leads to caspase- and mitochondrion-dependent apoptosis of human cultured CD34+ cells

Cultured CD34+ cells exposed to 1 or 10 µM BP for 6 days were found to undergo apoptosis as assessed by determination of annexinV-positive cells by flow cytometry (Fig. 4A), whereas BP failed to increase the % of non-apoptotic necrotic cells as demonstrated by propidium iodide staining (data not shown). Moreover, BP-treated CD34+ cells displayed increased caspase 3 activity and enhanced expression of the cleaved form of caspase 3 when compared to their untreated counterpart (Fig. 4B and 4C). In addition, BP-exposed cells were found to exhibit a reduced $\Delta\psi m$ as shown by decreased $DiOC_6(3)$ labeling (Fig. 4D) and an increased expression of cleaved forms of caspase 9 (Fig. 4C); such events have been reported to represent important steps in numerous mitochondrion-related apoptotic processes (Delhalle et al., 2003).

Activation of AhR is required but not sufficient for accounting for PAH deleterious effects towards CD34+ cells

The AhR, which mediates many deleterious effects of PAHs (Matikainen et al., 2001; Landers et al., 1991), and its co-factor ARNT were found to be expressed in CD34+ cells as assessed by RT-PCR assays (Fig. 5A). Their levels of mRNA expression were however lower than those detected in human hepatocytes used here as positive controls. BP and TCDD, a very potent agonist of the AhR (Landers and Bunce, 1991), were found to induce expression of protypical AhR-regulated genes such as CYP1A1 and CYP1B1 in cultured CD34+ cells (Fig. 5B), indicating that AhR was fully functional in these cells. CYP1B1 mRNAs were however constitutively present at low levels in untreated CD34+ cell cultures (Fig. 5B). To determine whether AhR may play a role in PAH toxicity towards CD34+ cells, we then analyzed the effects of co-treatment by 3'M4'NF, used at a 0.1 µM dose for which this compound acts as a pure AhR antagonist (Lu et al., 1995). As indicated in Fig. 5C, 3'M4'NF was able to partially suppress BP toxicity towards CD34+ cells as assessed by counting viable cells in CD34+ cell cultures, indicating an involvement of AhR. We then determined whether AhR activation was sufficient to trigger alteration of CD34+ cell proliferation. For this, cells were treated by 10 nM TCDD, a potent AhR ligand fully active in CD34+ cell cultures as revealed by its inducing effects on

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CYP1A1/1B1 expression (Fig. 5B). As shown in Fig. 5C, TCDD failed to alter expansion of CD34+ cells, suggesting that activation of AhR is not sufficient by itself for exerting toxicity in CD34+ cell cultures.

Involvement of CYP P450-dependent BP metabolites and adducts in BP toxicity towards CD34+ cell cultures

A major difference between TCDD and PAHs lies in the fact that the former is poorly metabolized, if any, whereas the latter can be converted in toxic reactive metabolites through the action of CYP, especially CYP1A1 and CYP1B1, and, by this way, form covalent adducts to cellular macromolecules (Zedeck, 1980). Interestingly, BP-exposed CD34+ cells exhibited up-regulation of CYP1A1 and CYP1B1 expression (Fig. 5B), suggesting that PAHs can be metabolized and can form adducts in these cells. To examine this point, formation of reactive BP adducts was analyzed through measuring covalent binding of radiolabelled metabolites of BP to macromolecules in the presence or absence of the CYP1A1/1B1 inhibitor αNF (Chang et al., 1994). As shown in Fig. 6A, BP adducts were detected in CD34+ cells not co-exposed to αNF. Such formation of BP adducts was however greatly reduced in the presence of αNF (Fig. 6A), most likely indicating that PAHs such as BP can generate reactive metabolites and adducts in CD34+ cell cultures through their CYP-dependent metabolism.

To further investigate the potential role of CYP-related metabolism of PAHs in the toxicity of these environmental contaminants in CD34+ cell cultures, we analyzed the effects of αNF on BP-mediated alteration of CD34+ cell culture expansion. αNF was able to fully abolish the toxic effects of BP towards cell expansion in 6-day old CD34+ cell cultures (Fig. 6B). To confirm the involvement of BP metabolites, we then directly added BPDE, one of the major toxic BP metabolites (Zedeck, 1980; Chen et al., 2003), to CD34+ cell cultures for 6 days and studied the effects of this reactive compound on cell proliferation. BPDE was found to markedly inhibit cell growth; this effect was dose-dependent and even very low doses (10 to 50 nM) exert significant toxicity (Fig. 6C). In addition, BPDE induced apoptotic process as assessed by annexinV labeling (data not shown). Moreover, the antioxidant NAC, already described as able to prevent the toxicity of BP metabolites, i.e. the formation of BPDE adducts

to liver and lung DNA (De Flora et al., 1991; Izzotti et al., 1995), was demonstrated to counteract significantly BP- and BPDE-mediated alteration of cell proliferation in CD34+ cell cultures (Fig. 6D).

DISCUSSION

The results reported in the present study indicated for the first time to the best of our knowledge that human CD34+ hematopoietic cells constitute cellular targets for PAHs. Indeed, PAHs were able to markedly decrease CD34+ cell expansion as demonstrated by counting viable cells in proliferating CD34+ cell cultures. BP concomitantly altered CD34+ cell differentiation towards erythroid, granulo-macrophagic, macrophagic and megakaryocytic cell lineages as assessed by semi-solid CFU assays, which may be due to its inhibitory effect towards cell proliferation since cell growth and cell differentiation are well-established as coordinated and concomitant processes during hematopoiesis (Ogawa, 1993). Moreover, BP triggered apoptosis in CD34+ cell cultures as shown by annexinV labeling, measurement of caspase 3 activity, detection of cleaved forms of caspase 3 and 9 and loss of mitochondrial potential. Interestingly, a short exposure to BP (24 h or 48 h), starting at the beginning of CD34+ cell cultures, was sufficient to markedly impair proliferation after 10 days of culture, although BP was withdrawal after initial incubation. Such results therefore provide evidence that CD34+ cells, which represent more than 90 % of cultured cells during the first 2-days of culture (data not shown), were targets by themselves for PAHs. An additional toxicity of PAHs towards more differentiated myeloid CD34- cells, which appeared during cell expansion with time in culture, can however not be excluded. Indeed, several studies have indicated that differentiated myeloid cells such as monocytes/macrophages (van Grevenynghe et al., 2004) or dendritic cells (Laupeze et al., 2002) constitute targets for PAHs.

AhR appeared to be expressed and fully functional in progenitor cells as notably demonstrated by the up-regulation of CYP1A1/1B1 expression in BP-treated CD34+ cells. Its activation was required for the deleterious effects of PAHs since (i) the pure AhR antagonist 3'M4'NF partially counteracted BP toxicity and (ii) PAHs such as benzo(e)pyrene or pyrene, known to not or only poorly interact with the AhR (Zedeck, 1980), failed to exert toxicity towards CD34+ cell cultures. The lack of deleterious effects of TCDD, a very potent ligand of the AhR, however clearly demonstrated that activation of the AhR is not sufficient by itself and, therefore, that additional events are required. In this context, we have focused on PAH metabolites since (i) their formation requires prior AhR-

dependent up-regulation of CYP1A1/1B1 and BP-exposed CD34+ cells effectively expressed CYP1A1/1B1, (ii) they are well-known to play a major role in many PAH deleterious effects, especially via formation of adducts to macromolecules (Melendez-Colon et al., 1999), and (iii) careful analysis of the effects of the different PAHs used in the present study reveals that only PAHs known to generate toxic reactive intermediates, i.e. BP, DMBA and 3-methylcholanthrene (Zedeck, 1980), affected CD34+ cell expansion. Our results fully supported an involvement of PAH metabolites able to form cellular DNA and protein adducts. Indeed, BP-related adducts were observed in CD34+ cell cultures and their formation required CYP-dependent metabolism of BP as illustrated by the inhibitory effects of αNF. Importantly, αNF was capable of significantly preventing toxic effects of BP toward CD34+ cell expansion, linking formation of adducts and BP toxicity. The fact that (i) BPDE, a major toxic metabolite of BP, was found to directly exert toxicity in CD34+ cell cultures and (ii) that NAC, known to counteract PAH metabolite effects through preventing DNA adduct formation (Izzotti et al., 1995), partially inhibited toxicity of both BP and BPDE towards CD34+ cell expansion, also fully favors the idea of a critical role of PAH metabolites and adducts. It is noteworthy that, besides CD34+ cells, lymphocytes (Salas and Burchiel, 1998; Mann et al., 1999) and macrophages (van Grevenynghe et al., 2004) have been shown to be targeted by PAH metabolites. This emphasizes the fact that PAH metabolism and adduct formation with prior AhR-dependent CYP1A1/1B1 up-regulation probably represent key-steps in PAH toxicity towards these different hematological cells. Interestingly, CD34+ hematopoietic progenitor cells have been previously shown to exhibit slow DNA repair and high sensitivity to DNA damaging drugs, notably when compared to hematological CD34- cells (Buschfort-Papewalis et al., 2002). Our results fully support this conclusion since CD34+ cells were found to be very sensitive to low doses of the mutagenic agent BPDE.

Apoptosis due to BP exposure in CD34+ cell culture involved executioner caspase 3. It also incriminated mitochondrial events: indeed, $\Delta\psi m$ was strongly reduced in response to BP and caspase 9 was activated. Such a participation of both caspases and mitochondria to PAH-related apoptotic processes has already been described in several cell types undergoing apoptosis in response to PAHs such as lymphoma, hepatoma and macrophagic cells (Salas and Burchiel, 1998; van Grevenynghe et al., 2004; Solhaug et al., 2004). In such cells, PAH-induced apoptosis has been linked to altered

expression of both pro- and anti-apoptotic factors such as bax, p53, c-FLIP and Bcl-XL (Salas and Burchiel, 1998; Matikainen et al., 2001; Solhaug et al., 2004). Whether these proteins may also be affected by PAH exposure in CD34+ cell cultures will deserve further studies. Another factor linked to apoptosis is the overproduction of reactive oxygen species (ROS) (Delhalle et al., 2003). Using the ROS-sensitive probes 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium, we however failed to demonstrate an increased formation of ROS in BP-treated cultures of CD34+ cells (data not shown). This likely indicates that the antioxidant NAC protected CD34+ cells from BP toxicity not through counteracting ROS overproduction, but rather through down-modulating BP-related adduct formation (Izzotti et al., 1995) as already described above.

The potential contribution of PAH-related deleterious effects towards hematopoietic CD34+ cells to the global toxicity of these ubiquitous environmental contaminants remains to be determined, notably after *in vivo* exposure. It is however noteworthy that previous experimental data in rodents have well established the bone marrow toxicity of PAHs. Indeed, DMBA treatment of mice resulted in a profound hypocellularity of the bone marrow, with a depletion of various cell lineages, i.e. granulocytes, erythroid precursors and lymphocytes (Galvan et al., 2003; Page et al., 2004). In rats, BP exposure also triggered bone marrow cytotoxicity, micronuclei formation in erythroid precursors (Shimada et al., 1990) and the development of aplastic anemia (Nebert et al., 1977). These data suggest that hematopoeisis is markedly altered in response to PAHs. Our data, indicating that CD34+ progenitor cells constitute targets for PAHs, fully support this hypothesis and provide informations about the cellular bases of PAH hematotoxicity, i.e. a direct deleterious action on human hematopoietic CD34+ cells. In addition, PAHs are well-recognized as immunotoxic agents, which has been linked to alteration of the functions of lymphocytes (Davila et al., 1996) and to perturbation of lymphopoiesis (Mann et al., 1999); moreover, differentiation of antigen-presentating cells such as dendritic cells (Laupeze et al., 2002) and macrophages (van Grevenynghe et al., 2003) may be compromised. Toxicity of PAHs towards CD34+ hematopoietic cells may also directly contribute to their immunosuppressive properties by down-regulating the production of cell lineages playing a major role in the immune response such as lymphocytes, macrophages and neutrophils.

Our study, that used human cells, suggests that PAH hematoxicity, already known for rodents, may concern humans. The fact that human lymphocytes (Davila et al., 1996), dendritic cells (Laupeze et al., 2002) and macrophages (van Grevenynghe et al., 2003) constitute targets for PAHs favors this hypothesis. Interestingly, BP was still active on human CD34+ cells when used at 0.5 µM (125 ng/g), a relatively low concentration close to PAH amounts found in some foods (up to 50 ng/g) (Lijinsky, 1991; Phillips, 1999) or in tobacco smoke (20-40 ng/cigarette) (Izzotti et al., 1991). This suggests that humans may be exposed to PAH concentrations putatively affecting hematopoietic cells.

A major effect of PAHs on health is to favor the development of cancers, including malignant hemopathies (Mills et al., 1990). Indeed, DMBA elicits leukemia and lymphomas in rodents (Ball, 1970; Heidel et al., 2002) whereas oral exposure of mice to BP results in leukemias (Zhu et al., 1995). In this context, the fact that normal hematopoietic stem cells express AhR–related inducible metabolizing enzymes such as CYP1A1/1B1 and therefore can generate reactive PAH metabolites when exposed to these environmental chemicals is probably important to consider. Indeed, mutagenic damage to CD34+ bone marrow cells is thought to constitute an important initial event in leukemogenesis (Irons and Stillman, 1996) and that PAHs can cause adducts in CD34+ cells, after their CYP-dependent metabolism, may thus provide a molecular basis for the potential leukemogenic effects of these chemicals. It is noteworthy that CYP1B1, constitutively present in bone marrow cells, may play a major role in PAH bioactivation as recently suggested (Galvan et al., 2003). The fact that low doses of αNF (0.1 and 0.2 µM), thought to block CYP1B1 activity unlike that of CYP1A1 (Shimada et al., 1998), remain able to decrease BP toxicity towards CD34+ cell proliferation (data not shown) fully supports this conclusion. In addition to CYP1A1/1B1, other AhR-related genes may also be incriminated in PAH-induced leukemogenesis owing to the fact that some genes controlling cell division and apoptosis are known to be AhR-responsive (Nebert et al., 2000).

In conclusion, we have demonstrated that PAHs, potent carcinogenic and immunotoxic agents, can markedly alter cell expansion and differentiation in human progenitor CD34+ cell cultures. This is associated with induction of apoptosis and most likely depends on AhR-dependent PAH metabolite formation. These data therefore provide evidence that human hematopoetic CD34+ cells are capable of

bio-activating chemical carcinogens like PAHs and, by this way, constitute potent targets for such major environmental contaminants.

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Footnotes

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LEGENDS TO FIGURES

Fig. 1. *Characterization of human CD34+ cell cultures.*

CD34+ cells were cultured in liquid medium for various lengths of time (up to 10 days) as described in Materials and Methods. (A) Cell number was determined by trypan blue exclusion; results shown are the means ± SD of at least five independent experiments. *, $p<0.05$ when compared to untreated cells. (B) Cells were stained with mAbs directed against CD34, CD11b or CD15 (filled histogrammes) or with isotypic controls (open histogrammes) and were then analyzed by flow cytometry. For each flow cytometric graph, the percentage of positive cells is indicated. The data shown are representative of four independent experiments.

Fig. 2. *PAH treatment alters cell proliferation in human CD34+ cell cultures.*

CD34+ cell cultures were exposed to (A) 10 µM BP for 3, 6 and 10 days, (B) various concentrations of BP (from 0.1 µM to 10 µM) for 6 days, (C) different PAHs (BP, DMBA, 3-methylcholanthrene (MC), benzo(e)pyrene (BeP) and pyrene (PYR)) used at 10 µM for 6 days and (D) to a transient 1-, 2- or 3-day treatment to 10 µM BP followed by an additional culture in BP-free medium until 10 day of culture or to a continuous treatment by 10 µM BP for 10 days. Concentrations of viable cells were then determined by trypan blue exclusion. Results shown are the means ± SD of at least five independent experiments. *, $p<0.05$ when compared to untreated cells.

Fig. 3. *BP inhibits differentiation of human CD34+ cells into various hematological cell lineages.*

Differentiation of human CD34+ cells into various hematological lineages, i.e. granulo-macrophagic, macrophagic, megakaryocytic and erythroid, were analyzed in the absence or presence of 10 µM BP using progenitor semi-solid medium assays as described in Materials and Methods. Results are expressed as the number of colony formed by CFU-granulocyte-macrophage (CFU-GM), CFU-macrophage (CFU-M), CFU-megacaryocyte (CFU-MK) and BFU-erythroid (BFU-E); they are the mean ± SD of five independent experiments. *, $p<0.05$ when compared to untreated cells.

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Fig. 4. *BP triggers apoptotic processes in human CD34+ cell cultures.*

CD34+ cells were cultured for 6 days in the presence or absence of 1 or 10 µM BP. (A) The % of apoptotic annexinV-positive cells was then evaluated as described in *Materials and methods.* (B) Intracellular activity of caspase 3 was determined using its fluorigenic substrate Ac-DEVD-AMC and expressed as fluorescent arbitrary unit (F.A.U)/30 min. (C) 21 kDa-cleaved form of caspase 3, and 40 kDa- and 38 kDa-cleaved forms of caspase 9 and total HSC70 proteins were analyzed by Western blotting. (D) Loss of mitochondrial potential was measured by flow cytometry using the dye $DiOC_6(3)$; positive control of mitochondrial potential depolarization was performed in parallel using 50 µM cyanide p-(trifluoromenthoxy) phenyl hydrazone (FCCP). Data are respectively the means ± SD of five or three independent experiments (A and B) or are representative of three or five experiments (C and D). *, $p<0.05$ when compared to untreated cells.

Fig. 5. *Activation of AhR is required, but not sufficient, to account for BP toxicity in CD34+ cell cultures.*

(A) ARNT, AhR and GAPDH mRNA levels in freshly purified human CD34+ cells and hepatocytes, used here as positive controls, were analyzed by RT-PCR assays. (B) CD34+ progenitors were either untreated or exposed to 10 nM TCDD, 1 µM or 10 µM BP for 8 hours; CYP1A1, CYP1B1 and GAPDH expression were then determined by RT-PCR. (C) CD34+ cell cultures were either untreated or exposed for 6 days to 10 nM TCDD, 1µM BP, 0.1 µM 3'M4'NF or co-treated with 3'M4'NF and BP for 6 days. Concentrations of viable cells were then determined by blue trypan exclusion. Data are representative of three independent experiments (A, B) or are expressed as means ± SD of at least five independent (C). *, $p<0.05$. NS, not statistically significant.

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Fig. 6. *Involvement of CYP1A1/1B activity-dependent metabolites in BP toxicity in CD34+ cell cultures.*

(A) Adducts to cellular macromolecules formed by reactive BP metabolites were determined in CD34+ cells in the absence or presence of the CYP1A1/1B1 inhibitor αNF using a radiometric method as described in *Materials and Methods*. Results are expressed as cpm/ µg of total proteins and are the means ± SD of three independent experiments. *, $p<0.05$ when compared with αNF-untreated cells. (B-D) CD34+ cell cultures were either untreated or exposed for 6 days to (B) 1 µM BP, 1 µM αNF or co-treated with αNF and BP, (C) various doses of the BP-metabolite BPDE and (D) 10 µM BP or 50 nM BPDE in the absence or presence of 2 mM NAC. Concentrations of viable cells were then determined by blue trypan exclusion. Results shown are means ± SD of five independent experiments.*, $p<0.05$ when compared to untreated cells (B and C) or to NAC-untreated counterparts (D).

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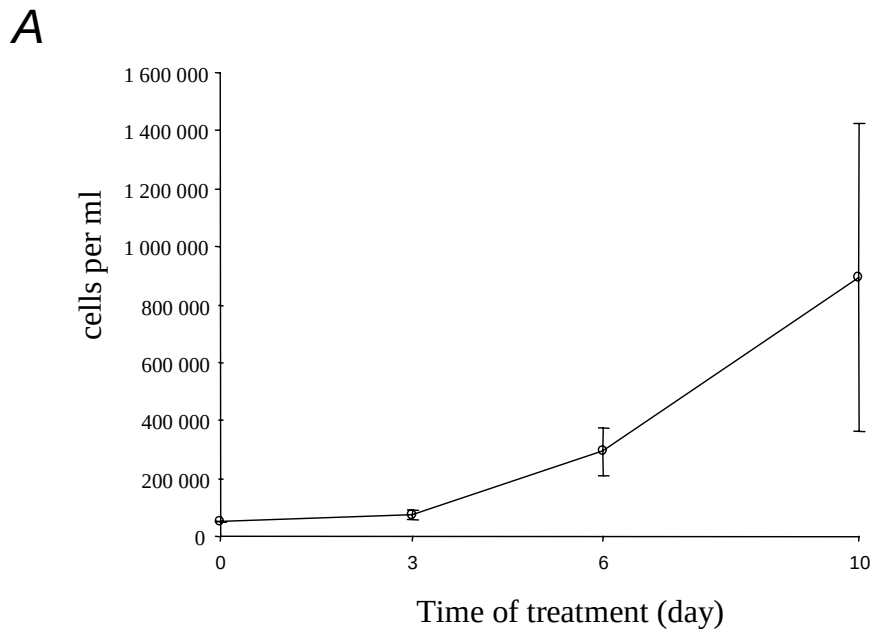

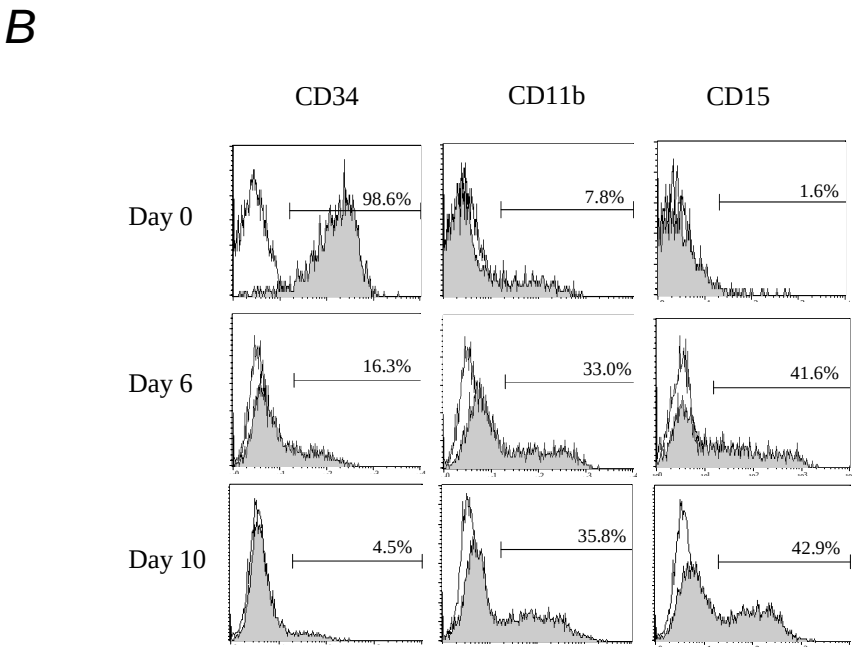

Figure 1

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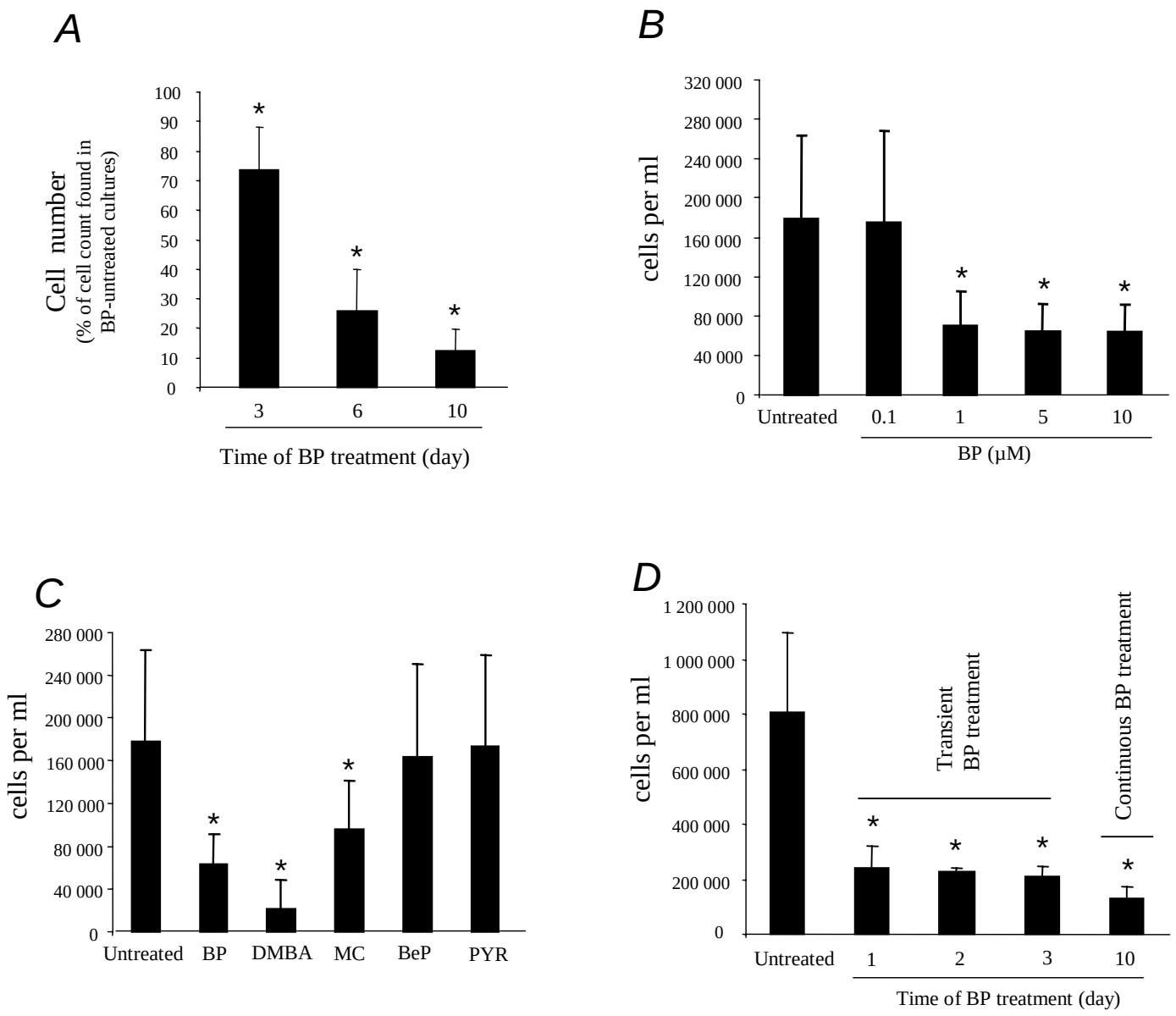

Figure 2

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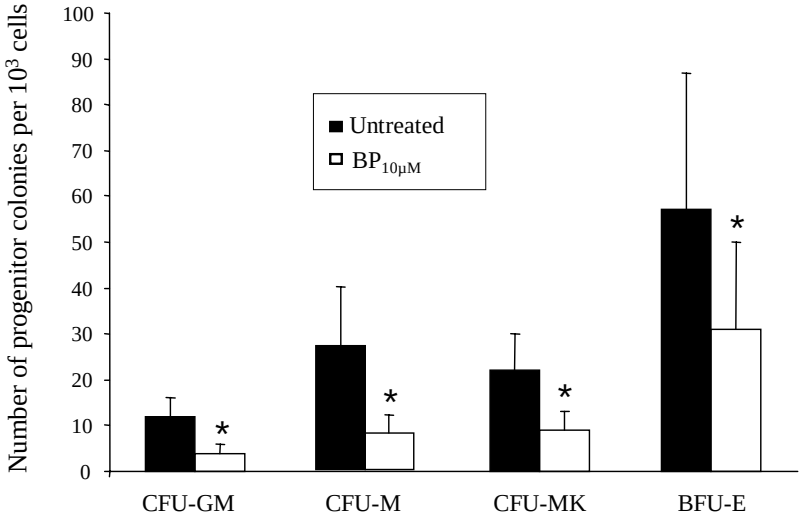

Figure 3

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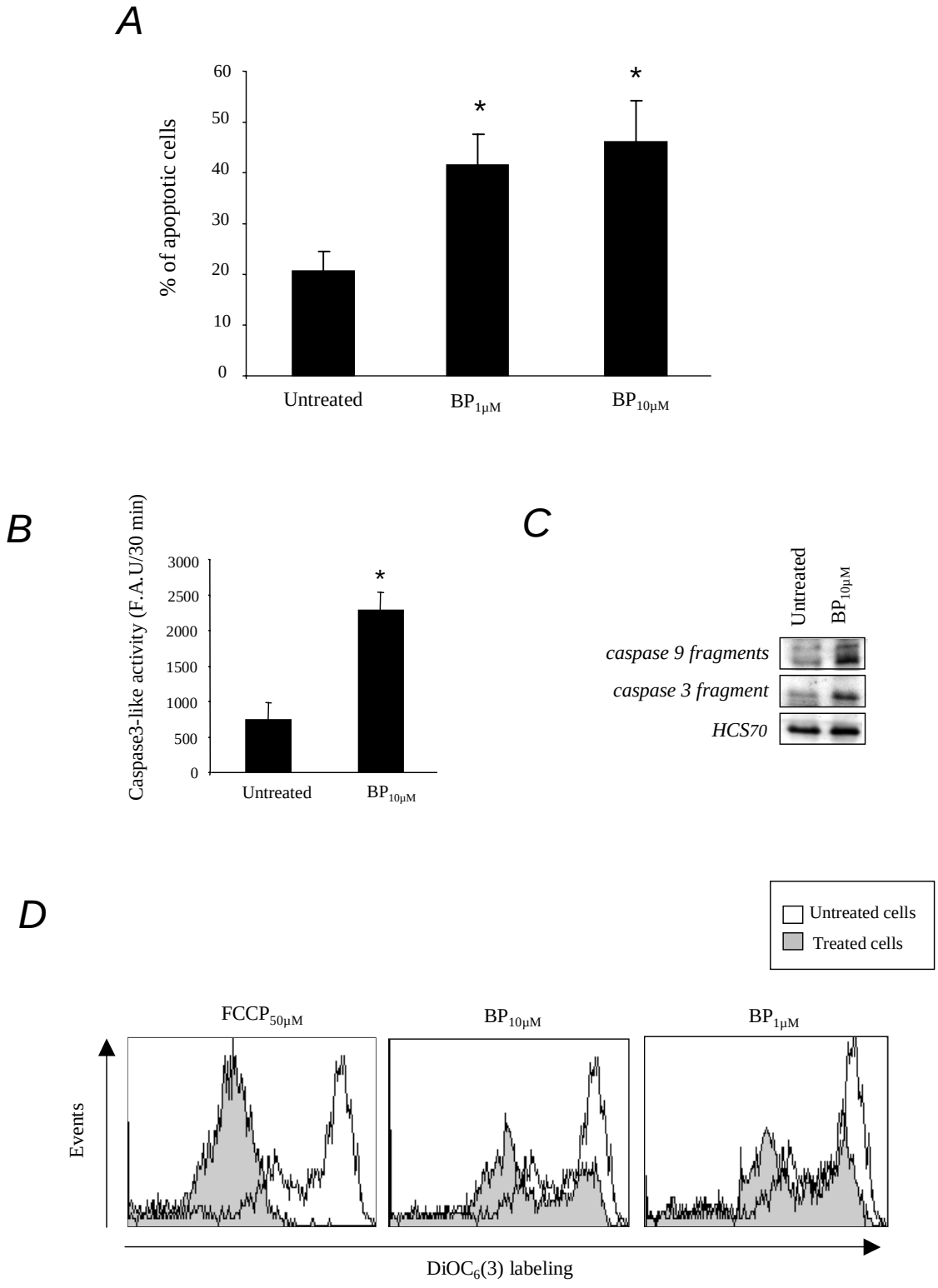

Figure 4

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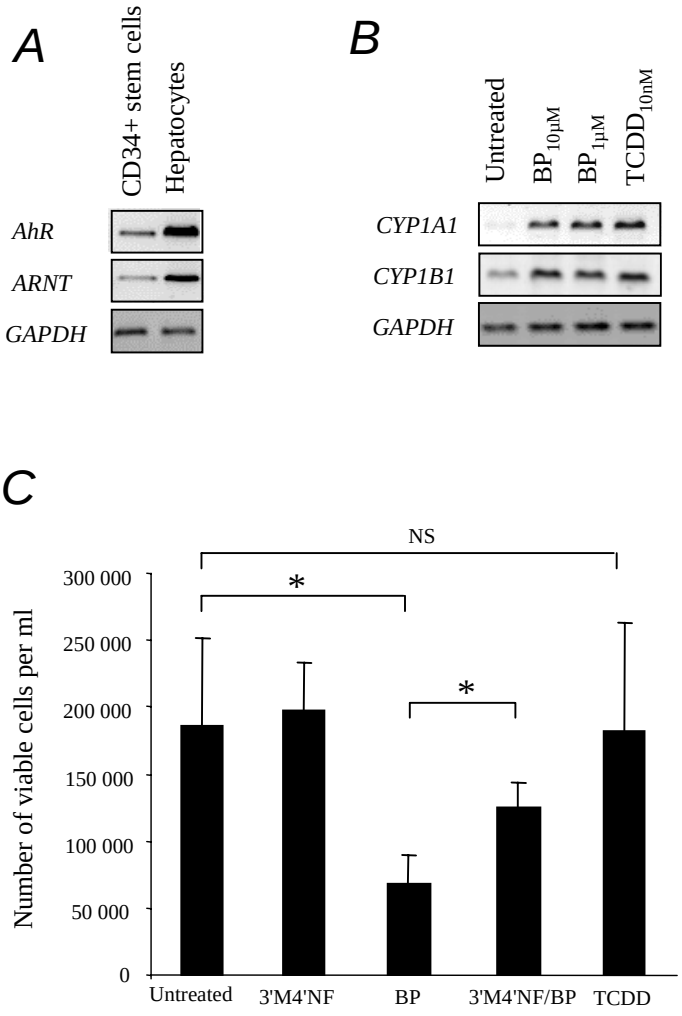

Figure 5

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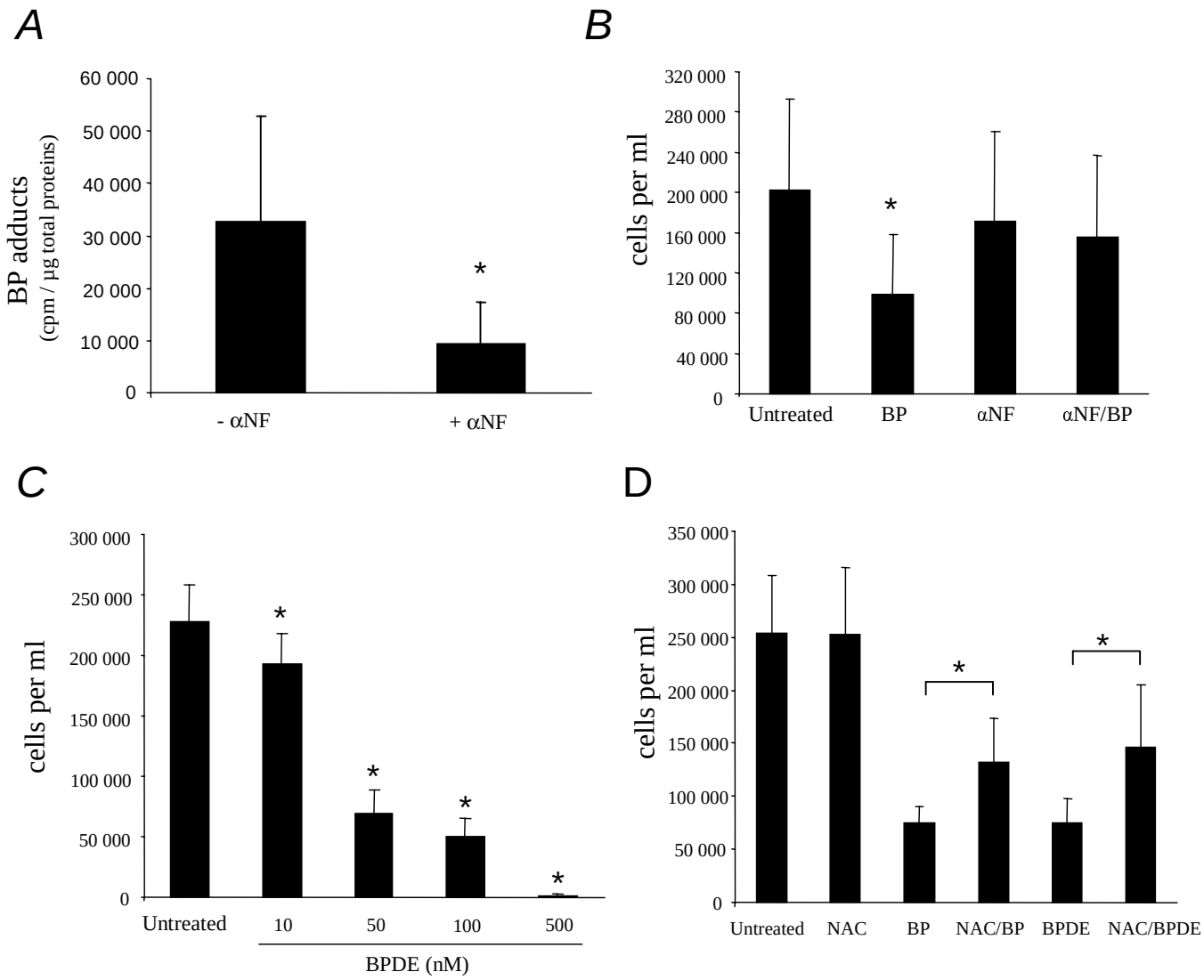

Figure 6