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In vitro toxicity of naphthalene, 1-naphthol, 2-naphthol and 1,4-naphthoquinone on human CFU-GM from female and male cord blood donors

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ABSTRACT

In animal models, naphthalene toxicity has been studied in different target organs and has been shown to be gender-dependent and metabolism related. In humans, it is readily absorbed and is metabolised by several cytochrome P450's. Naphthalene and its metabolites can cross the placental barrier and consequently may affect foetal tissues.

The aim of this study was to compare the *in vitro* toxicity of naphthalene and its metabolites, 1-naphthol, 2-naphthol and 1,4-naphthoquinone, on human haematopoietic foetal progenitors (CFU-GM) derived from newborn male and female donors. The mRNA expression of Cyp1A2 and Cyp3A4 was also evaluated. Naphthalene did not affect CFU-GM proliferation, while 1-naphthol, 2-naphthol and particularly 1,4-naphthoquinone strongly inhibited the clonogenicity of progenitors, from both male and female donors. mRNA of Cyp1A2 and Cyp3A4 was not expressed neither at the basal level, nor after naphthalene treatment, while treatment with 1,4-naphthoquinone induced expression of both enzymes in both genders, with Cyp1A2 being expressed four times more than Cyp3A4. Female CFU-GM was significantly more sensitive to 1,4-naphthoquinone than male and after treatment both enzymes were expressed twice as much as in the male precursors. These results suggest that a gender-specific 1,4-naphthoquinone metabolic pathway may exist, which gives rise to unknown toxic metabolites.

1. Introduction

The polycyclic aromatic hydrocarbon naphthalene is used in the production of phthalate plasticizers and resins, azo-dyes, dispersants, and tanning agents in the rubber and leather industries (Preuss et al., 2003). Humans can come into contact with naphthalene particulates and vapours during its production, usage, and combustion processes; most of all from vehicle exhausts, cigarette smoking or contamination of drinking water (Weintraub et al., 2000). It can be absorbed by oral, inhalation, and dermal routes of exposure (NTP, 1992, 2000; Bock et al., 1979; Eisele, 1985; Turkall et al., 1994). Regardless of the route, humans are exposed to naphthalene at sufficient levels so that it circulates within the body, and it has been found in adipose tissue and breast milk (Pellizzari et al., 1982; Stanley, 1986).

The toxicity of naphthalene and its metabolites has been widely studied in animal models, in different target organs. Naphthalene has been shown to produce respiratory-tract tumours in rats and mice of both sexes (Abdo et al., 1992; Abdo et al., 2001; NTP, 1992, 2000). Chronic naphthalene vapour exposure resulted in an increased incidence of pulmonary alveolar/bronchiolar adenomas and carcinomas, but only in female mice (NTP, 1992; Lakritz et al., 1996; Abdo et al., 1992). Differences in response due to sex were also observed after intra peritoneal administration of naphthalene. Female mice have an earlier onset as well as a different pattern and extent of acute airway epithelial injury compared with males at the same concentration (Van Winkle et al., 2002).

In addition, large oral doses (2400 mg/kg total) given to rats on days 7–14 of pregnancy caused reduced live births and reduced survival (Schamhal, 1955).

Limited information is available for humans. Carcinogenicity of naphthalene has been demonstrated in two mammalian species. For this reason, as well as for the abundance of naphthalene in indoor and outdoor air, it has been recently reclassified as a possible human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 2002) and the US Environmental Protection Agency (EPA) (US EPA, 2003).

There is evidence from literature of effects induced by naphthalene in neonates born by mothers exposed to the compound or who "sniffed" or ingested naphthalene (as mothballs) during pregnancy (HEI Air Toxics Reviews Panel, 2007; US EPA, 1986; Hayes,

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1982; Diodovich et al., 2003; Zinkham and Childs, 1958; Anziulewicz et al., 1959). Moreover, it has been reported that prenatal exposure to xenobiotics is associated with a decrease in naive cells in umbilical blood samples collected from neonates from the same population (Belles-Isles et al., 2002). These observations indicate that naphthalene or its metabolites can cross the placental barrier in sufficient amounts to cause foetal toxicity.

The toxicity of naphthalene in animal models is closely related to the metabolism of the compound and resulted gender-dependent. Indeed, susceptibility of mice to naphthalene correlates with the formation of toxic metabolites by CYP2F2 (Buckpitt et al., 1995) and pulmonary sub-compartments from female mice metabolised naphthalene more rapidly and with a greater proportion of dihydrodiol metabolite formation than males (Van Winkle et al., 2002). In these sub-compartments a considerable variability in gene expression for CYP2F2 has been found (Stelck et al., 2005).

Naphthalene is metabolised by Cytochrome P450 isoenzymes to naphthalene-1,2 oxide (NPO), which gives rise to 1-naphthol, 2-naphthol, and subsequently to 1,4- and 1,2-naphtoquinone (NPQs). All these metabolites have demonstrated toxicity on mouse and rats lung cells (Zheng et al., 1997; Chichester et al., 1994).

Cho et al. (2006), identified in human liver microsomes, Cyp1A2 as the most efficient isoform for producing 1-naphthol from naphthalene, and 1,4-naphthoquinone from 1-naphthol in the secondary metabolism, while the Cyp3A4 is the isoform mainly responsible of the production of 2-naphthol from naphthalene.

Whereas the highest cytochrome P450 specific activities in mammalian tissues are normally found in the liver, naphthalene metabolism has also been demonstrated to occur in other tissues (notably eye and lung tissue) (Wells et al., 1989; Xu et al., 1992; Buckpitt and Franklin, 1989). Various studies on bronchial and olphactory epithelial cells in mice, rats and hamsters (O'Brien et al., 1985, 1989; Tong et al., 1981; Plopper et al., 1992; Raunio et al., 1999; Ding and Kaminsky, 2003) strongly indicate that metabolic activation in target tissues plays a dominant and possibly exclusive role in site-specific naphthalene cytotoxicity.

Metabolic detoxification processes have been identified in murine bone marrow stromal cells (Gribaldo et al., 1999a; Naughton et al., 1992), as well as myeloperoxidases able to bioactivate the phenolic metabolites of benzene which have been found in murine lineage-negative progenitors and human CD34+ progenitors from bone marrow (Ross et al., 1996). However, the metabolic activity of human cord blood progenitors is still poorly investigated.

The in vitro colony forming assays (CFU) have been used to investigate the pathogenic mechanisms of drug-induced blood disorders. These tests are based on the ability of progenitors to proliferate in vitro in response to growth factors and cytokines, as they occur physiologically in vivo (Gribaldo et al., 1999b). Depending on the specific mixture of cytokines present in the medium, progenitors give rise to phenotypically distinct colonies (clonogenicity). Examination of colony formation following exposure to various agents has proven to be a sensitive indicator of myelotoxicity, as described also in pharmacology reviews on alternative in vitro systems (Parent-Massin, 2001; Parchment, 1998).

Our goal in this study was to determine the *in vitro* toxicity of naphthalene and its metabolites, 1-naphthol, 2-naphthol and 1,4-naphthoquinone, on the populations of granulocytes and macrophages present in human umbilical mononuclear cord blood cells (by CFU-GM assay), derived from male and female donors, in order to evaluate the potential gender-specific toxicity. We also evaluated the presence of tissue specific metabolism of the compounds, looking at mRNA expression of Cyp1A2 and Cyp3A4 cytochromes in these cells.

2. Materials and methods

2.1. Chemicals

Naphathalene (MW 128.16, purity 99.8%), 1-naphthol (1 N) (MW 144.17, purity 99.5%), 2-naphthol (2 N) (MW 144.17, purity 99%) and 1,4-naphthoquinone (1,4NQ) (MW 158.16, purity 98.5%) were purchased by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions of naphthalene (1 M) and its metabolites (1 M 1-naphthol and 2-naphthol; 0.5 M 1,4-naphthoquinone) were prepared in dimethylsulfoxide (DMSO). All solutions were stored at −20 °C.

2.2. Source of human progenitors cells

The mononucleated cell fractions, isolated from human umbilical cord blood (CB), were supplied frozen by Biopredict International (France), indicating on each vial the sex of the baby donor (male or female) and stored in liquid nitrogen. Immediately before use, the cells were thawed quickly at 37 °C, swirling gently for 1–2 min. After wiping the outside of the vial with 70% ethyl alcohol on absorbent paper, the cell suspension was transferred, drop wise, to 10 ml of warmed Iscove Modified Dulbecco's Medium (IMDM) + Glutammax (Gibco, Italy), containing 10% Foetal Bovine Serum (FBS) (Gibco, Italy). It was than centrifuged for 10 min at 300*g* at room temperature. The supernatant was removed and the cells gently resuspended in IMDM 30% FBS and counted using Trypan blue to assess the cell viability.

2.3. Human CFU-GM assay

Cord blood cells were seeded in MethoCult-H4534 (StemCell Technologies, Vancouver, BC, Canada). This medium is without erythropoietin and contains methylcellulose (1%), FBS (30%), Bovine Serum Albumin (BSA) (1%), 2-mercaptoethanol (10^{-4} M), glutamine (2 mM), Interleukin-3 (IL-3) (10 ng/ml), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) (10 ng/ml) and Stem Cell Factor (SCF) (50 ng/ml).

Briefly, 22 μl of 200X compound solutions or of solvent (DMSO), 78 μl of medium, and 300 μl of cells (1.1×10^6 cells/ml) were added to tubes containing 4 ml of methylcellulose. Finally, 1 ml methylcellulose cell suspension was seeded in 35 mm dishes and cultures were incubated at 37 °C in 5% CO_2 for 14 days.

All the chemical dilutions were prepared 200X, in order to obtain the final fold dilution in the dishes and a maximum DMSO concentration of 0.5%. After a pre-screening experiment that assessed the range of toxicity, all the experiments for the IC determinations were performed testing eight dilutions per each chemical (1:5 for naphthalene and 1:3 for 1 N, 2 N and 1,4NQ) starting from 5000 μM for naphthalene, from 2500 μM for 1 N and 2 N, and from 60 μM for 1,4NQ. Naphthalene concentrations were selected on the basis of urinary 1 N concentrations found in subjects occupationally exposed to polycyclic aromatic hydrocarbons (PAHs) (from 5 μM to 500 μM) (Preuss et al., 2003; Bieniek et al., 1993).

2.4. Colonies scoring

Human CFU-GM colonies were scored after 14 days of incubation using an inverted microscope.

A CFU-GM colony was defined as an aggregate containing 50 or more cells (Pessina et al., 2001). Morphologically, four classes of CFU-GM colonies can be observed: compact, diffuse and spread, multicentric, and multifocal colonies. A compact colony presents a dense, central nucleus and a peripheral halo. Diffuse and spread