Genotoxicity of cyclopentha[*c*]phenanthrene and its two derivatives based on an *in vitro* micronucleus test

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ABSTRACT

Environmental pollution causes a variety of health problems, including cancer. Many known pollutants have carcinogenic properties and polycyclic aromatic hydrocarbons (PAH) belong to this group. In this study, an *in vitro* culture of V79 cells of the Chinese hamster was subjected to three tested PAHs: 5-amino-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (ACP[*c*]Ph), 5-amino-9-methoxy-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph) and cyclopenta[*c*]phenanthrene (CP[*c*]Ph). The *in vitro* micronucleus (MN) assay was applied in order to evaluate the genotoxic properties of the studied compounds. The highest genotoxic effect was observed for AMCP[*c*]Ph in a concentration of 0.02μg·ml^{-1}. The genotoxic effect of the other two compounds was slightly lower.

INTRODUCTION

Many environmental contaminants have mutagenic properties and carry a potential risk of cancer among individuals and a risk of genetic disease for future human generations. The occurrence of cancer depends largely on the quality of the environment. It was found that about 80% of cancers are environmentally conditioned (Wise et al. 1986).

Genotoxic chemicals exert their adverse influence through interaction with the genetic material (DNA) of cells. Genotoxicity tests are designed to detect chemicals (or their mixtures) which can induce genetic damage, directly or indirectly, by various mechanisms. Compounds identified as genotoxic in these tests have the potential to be human carcinogens or mutagens and ultimately may induce hereditary defects.

Polycyclic aromatic hydrocarbons (PAH) are common constituents of the air, water and soil. Fossil fuels such as coal, petroleum and shale oils are the main sources of these compounds, releasing them directly or *via* combustion into the environment (Marrocchi et al. 1996). They are persistent in the environment and have the potential to bio-accumulate (Shaw and Connell 1994).

The *in vitro* micronucleus test is a short-term screening method widely used to detect genotoxic effects (Villarini et al. 1998). It is one of the simplest, most reliable and rapid screening systems for both clastogenic (chromosome breakage) and aneugenic (chromosome lagging) screening (Ahmad and Jaber Saleh 2010).

Clastogenic and aneugenic effects can be identified on the basis of the micronucleus size and the presence or absence of kinetochore (Heddle et al. 1991; Kirsch-Volders et al. 2011). Micronuclei formation can occur in any of the dividing cells of any species (Fenech 1993; Heddle et al. 1983).

The main goal of this study was to calibrate the laboratory for cell culturing and an MN test which was recently established in the Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn (Poland). In this pilot experiment, we studied the genotoxic effect of three PAHs: 5-amino-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (ACP[*c*]Ph), 5-amino-9-methoxy-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph)

and cyclopenta[*c*]phenanthrene (CP[*c*]Ph). We followed methodology recommended by ISO 21427-2:2006 with slight modifications concerning the duration of cell culturing, filtering of the medium and staining duration of the cell preparations. We have demonstrated that the laboratory and the applied methodology enable obtaining reproducible and scientifically sound MN test results.

MATERIAL AND METHODS

Chemistry

5-amino-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (ACP[*c*]Ph), 5-amino-9-methoxy-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph) and cyclopenta[*c*]phenanthrene (CP[*c*]Ph) (Figure 1) were synthesized from their respective naphthalenecyclopentanones (Góra et al. 2005). Their structure was confirmed using spectroscopic methods. The purity of the compounds was designated by gas chromatography and NMR spectroscopy and its level was >95% (Łuczyński et al. 2007). The compounds tested in this study were synthesized because of a lack of their commercial equivalents.

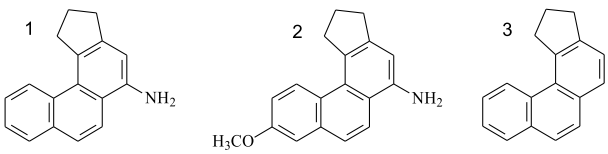

Figure 1. Structure of the investigated chemicals. 1) 5-amino-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (ACP[*c*]Ph), 2) 5-amino-9-methoxy-2,3-dihydro-1*H*-cyclopenta[*c*]phenanthrene (AMCP[*c*]Ph), 3) cyclopenta[*c*]phenanthrene (CP[*c*]Ph).

In vitro micronucleus test

The methodology of the test was based on ISO 21427-2: 2006. Water Quality-Evaluation of Genotoxicity by Measurement of the Induction of Micronuclei-Part 2: Mixed Population Method Using the Cell Line V79.

The methodology of the test was modified for use in the laboratory for cell culture in our department.

The stock Chinese hamster lung fibroblasts (V79) cells were originally obtained from the DSMZ Institute, Germany. Cells were maintained with a Minimum Essential Medium (MEM) with the addition of L-glutamine at 37°C, 95% humidity and 5% carbon dioxide atmosphere. Cells were tested for mycoplasma contamination and were routinely cultured to maintain their exponential growth.

An *in vitro* micronucleus test was conducted on the V79 cell line. It was performed on 4-well plates (1ml) and consisted of four stages. The test conditions for all the steps were the same: temperature 37°C, humidity 95% and 5% carbon dioxide atmosphere. For all steps, the culture medium consisted of 90% of MEM, 10% of FBS (Fetal Bovine Serum) and a 1% antibiotic solution (Amphotericin B, Penicillin, Streptomycin). The S9 microsome fraction (from rat liver) is induced with Aroclor 1254 (Trinova Biochem GmbH Germany) and is able to stimulate those eukaryotic processes that cannot be conduced by studied cells themselves.

Stage I pre-incubation of cell cultures was conducted (MEM supplemented with 10% of FBS, 1% antibiotics). At the beginning of the test, the cell density was $5 \cdot 10^4$ cells per well. Incubation was carried out for 48 hours.

Stage II was divided into two variants. In the variant without an S9 metabolic fraction (-S9), the cell cultures after pre-incubation were covered with fresh culture medium supplemented with appropriate concentrations of test substances (maximal volume of tested chemical if dissolved in DMSO should be 1% of volume of the well), plus positive and negative controls. The cells were then incubated for 24 hours. The second variant was preceded by incubation of the test substances, plus positive and negative controls with an addition of S9 (+S9) (in this variant, FBS should not be added to culture medium) and an appropriate volume of culture medium for 1 hour. After incubation, the solutions obtained were filtered on sterile syringe filters (0.22μm). Cell cultures were covered with solutions of test substances. Incubation was performed for 24 hours.

In **stage III**, the culture medium was removed from the solutions of test substances. The cultures were then covered with fresh medium supplemented with cytochalasin B in the concentration of 3$\mu g \cdot ml^{-1}$ and incubated for 24h.

Stage IV was carried out by:

- fixing of cells with methanol (20 minutes),
- staining cells with Giemsa dye (20 minutes) (for compilation of staining methods with selection of the best staining procedure for V79 cell culture see: Rudnicka and Krzyżewski 2011),
- washing the cells with a pH 6.8 phosphate buffer.

Positive controls were needed to affirm the metabolic capability of the S9 preparation and for drawing conclusions on the clastogenic or aneugenic mechanisms of micronuclei formation. The positive controls should employ known inducers of micronucleus formation. We used benzo[*a*]pyrene as a positive control (for testing clastogenic properties) and mitomycin C (for aneugenic properties).

In cytochalasin B-treated cultures, the micronucleus frequencies were analyzed at 2,000 bi-nucleated cells per concentration (1,000 bi-nucleated cells per culture; two cultures per given concentration of tested chemical). Cells with irregular shapes or with two nuclei differing greatly in their size were not counted as bi-nucleated. Cells containing more than two main nuclei were not analyzed for micronuclei, as the baseline micronucleus frequency may be higher in these cells (for guidebook of types of cells with micronuclei as they appear in our laboratory see: Żelazna et al. 2011).

The following criteria were taken into account by scoring the cells in the test (Fenech 1993):

- maximum size of the micronucleus should be at least 30% of the size of a normal nucleus,
- micronucleus and nucleus should have the same appearance,
- micronuclei should be clearly separated from the nuclei,
- only cells with good cytoplasmatic outlines should be counted.

Statistical analyses

The data from experiments was analysed using a t-test for the number of micronuclei observed in 1,000 or 2,000 cells at each concentration of the tested chemical. A type I error rate of 0.05 was used in the statistical analyses. A positive genotoxic effect was declared for a chemical when the *p*-value was less than or equal to 0.05

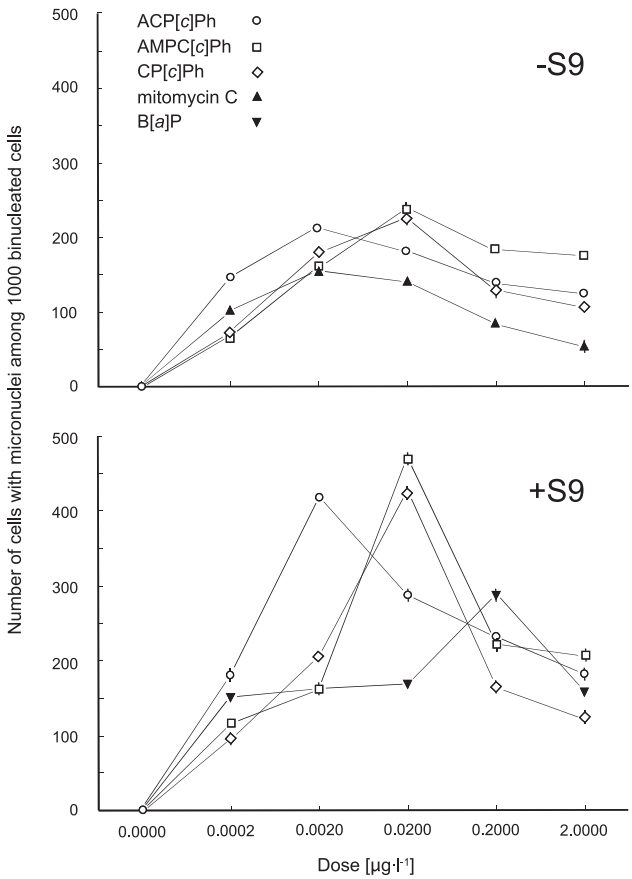

Figure 2. Genotoxicity of ACP[*c*]Ph, AMC[*c*]Ph, CP[*c*]PH and two reference chemicals B[*a*]P and mitomycin C in the absence (-S9) and presence (+S9) of metabolic activation system in the V79 cell line. The results are presented as a mean ±S.E. of duplicated culture wells (spontaneous micronuclei rate was around 3 cells among 1000 binucleated cells).

RESULTS

All examined compounds (ACP[*c*]Ph, AMC[*c*]Ph, CP[*c*]PH) showed genotoxic activity in both the variant with (+S9) and without (-S9) metabolic activation (Figure 2). The results obtained for tested chemicals were higher than those from positive controls (B[*a*]P and mitomycin C). The most significant increase in the number of binucleated cells with micronuclei was observed for the variant with metabolic activation. Exposure to AMC[*c*]Ph and CP[*c*]PH showed a significant increase in the concentration of 0.0200μg·ml^{-1} and for ACP[*c*]Ph in 0.0020μg·ml^{-1}. Both ACP[*c*]Ph and AMC[*c*]Ph showed higher genotoxic activity than the unsubstituted CP[*c*]Ph.

DISCUSSION

The three investigated compounds were highly genotoxic for cells (both with and without metabolic activation). This suggests that V79 lung fibroblasts have genes responsible for metabolic activation of xenobiotics. This should be investigated in the future. The cell system applied in investigating the genotoxicity effect produced similar results in a bacterial Ames test, except that we observed a higher activity of the studied compounds exerted on mammalian cells (Coombs et al. 1973).

The investigated compounds have shown a genotoxic effect on fibroblast cells, both with and without metabolic activation, although the effect was lower in the variant without metabolic fraction (S9). This effect is related to the characteristics of the V79 cell line as the lung fibroblasts have a low cytochrome P450 activity and they cannot metabolize PAHs very efficiently (Doehmer 1993).

The characteristics of the derivatives investigated in this study, when compared to the results obtained for phenanthrene (basic compound), were very different. The phenanthrene has lower genotoxic and mutagenic effect on bacteria and mammalian cells (Wood et al. 1979). This difference in the type of interaction may result from differences in the molecular structure of the studied compounds. Substituents in the molecule may influence interactions between the studied chemical molecule and the cell genetic material.

The genotoxic effects of the studied compounds may be correlated with their molecular structure. The metabolism of amino-PAHs may include the oxidation of the amino function by cytochrome P450 enzymes to form hydroxylamine, which can be transformed into *N*-esters in the enzyme-catalyzed reaction. *N*-hydroxyl, *N*-acetoxy ester as well as *N*-sulfonate ester can subsequently form the nitrenium ion, a potent electrophile. It is possible that both the epoxidation of ring A and oxidation of the amino function may occur. Both metabolites are probably able to form a covalent bond with the bases within DNA (Łuczyński et al. 2007).

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