Cytotoxic and genotoxic effects of Br-containing oxaphosphole on Allium cepa L. root tip cells and mouse bone marrow cells

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Abstract

The continuous production and release of chemicals into the environment has led to the need to assess their genotoxicity. Numerous organophosphorus compounds with different structures have been synthesized in recent years, and several oxaphosphole derivatives are known to possess biological activity. Such chemical compounds may influence proliferating cells and cause disturbances of the genetic material. In this study, we examined the cytotoxicity and genotoxicity of 4-bromo-N,N-diethyl-5,5-dimethyl-2,5-dihydro-1,2-oxaphosphol-2-amine 2-oxide (Br-oxph). In A. cepa cells, Br-oxph (10^{-9} M, 10^{-6} M and 10^{-3} M) reduced the mitotic index 48 h after treatment with the two highest concentrations, with no significant effect at earlier intervals. Mitotic cells showed abnormalities 24 h and 48 h after treatment with the two lowest concentrations but there were no consistent changes in interphase cells. Bone marrow cells from mice treated with Br-oxph (2.82 x 10^{-3} /c109 g/kg) also showed a reduced mitotic index after 48 h and a greater percentage of cells with aberrations (principally chromatid and isochromatid breaks). These findings indicate the cytotoxicity and genotoxicity of Br-oxph in the two systems studied.

Key words: Allium cepa L. root tip cells, Br-containing oxaphosphole derivative, chromosome aberrations, ICR mouse bone marrow.

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The chemistry of organophosphorus compounds is a subject of increasing interest, and a large number of compounds with different structures, properties and biological activities have been synthesized (Smee and Reist, 1996; Leblond et al., 2002). Heterocyclic organophosphorous compounds are an interesting group of molecules, especially oxaphosphole derivatives that contain oxygen and phosphorus. Enchev et al. (1986) demonstrated that some oxaphospholes affect plant growth at concentrations of 10^{-9} M, 10^{-6} M and 10^{-3} M, these concentrations were also used in the Allium test. The solutions were prepared immediately before use and the cells were incubated with Br-oxph for 3 h and then for a further 24 h and 48 h after treatment with the two lowest concentrations but there were no consistent changes in interphase cells. Bone marrow cells from mice treated with Br-oxph (2.82 x 10^{-3} /c109 g/kg) also showed a reduced mitotic index after 48 h and a greater percentage of cells with aberrations (principally chromatid and isochromatid breaks). These findindsg indicate the cytotoxicity and genotoxicity of Br-oxph in the two systems studied.

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of Br-oxph to assess their ability to recover from possible damage. Distilled water and methyl methanesulfonate (MMS, CAS 66-27-3; 10^{-4} M for 24 h) were used as negative and positive controls, respectively.

Chromosomal aberrations in *Allium* root cells were assessed by light microscopy (Rank, 2003). The roots were fixed in Clarke’s fixative (95% ethanol:acetic acid glacial, 3:1 v/v) for 90 min, hydrolyzed in 3 N HCl for 8 min and in 45% acetic acid for 30 min at room temperature, and stained for 30 min in 1% aceto-orcein. The terminal root tips (1-2 mm) were removed and squashed in 45% acetic acid. The microscopic analysis included calculation of the mitotic index and the scoring of aberrant cells. Each sample consisted of six root meristems with at least 600 cells analyzed in each meristem. The mitotic index was calculated by counting the number of mitotic cells in 100 cells/root. The categories of aberrations scored included chromosomal bridges and fragments, vagrant chromosomes, aberrant metaphases and anaphases in dividing cells, micronuclei in interphase cells, and the presence of binucleate cells.

ICR mice (2n = 40) were obtained from the Base for Experimental Animals at Slivnitza (Bulgaria). All of the experiments were done under permission granted by the Faculty of Natural Sciences of the University of Shumen (Bulgaria) (permission no. 153/02). The mice were housed ad libitum on a 12/12 h light/dark cycle at 24°C (Bulgaria) (permission no. 153/02). The mice were housed ad libitum on a 12/12 h light/dark cycle at 24°C. The lysed cells were then fixed in methanol:acetic acid (3:1, v/v), air dried and stained with 5% Giemsa stain. The mitotic index was calculated by counting the number of mitotic cells in 1000 cells per mouse. Fifty well-spread metaphases per mouse were analyzed for chromosomal aberrations (Preston et al., 1987), using the following categories: chromatid and isochromatid breaks, centromeric and telomeric fusions, and fragments. The number of tetraploid metaphases (as a result of spindle abnormalities), chromosome gaps (defined as achromatic lesions; Ito and Ito, 2001) and apoptotic cells (identified by typical fragmented condensed nuclei; Gorneva et al., 2005) were also determined.

The results were expressed as the mean ± standard deviation (SD) and statistical comparisons were done by using Student’s *t*-test, with *p* < 0.05 indicating significance.

Table 1 shows that there was no significant change in the mitotic index of *Allium* root cells immediately after (0 h) and 24 h after a 3 h incubation with Br-oxph. In contrast, there was a significant reduction in the mitotic index 48 h after a 3 h incubation with the two highest concentrations (10^{-6} M and 10^{-3} M) of Br-oxph. Br-oxph induced a

| Recovery time (h) | Sample  | Mitotic index (%) | Abnormalities in mitotic cells (% total) | Abnormalities in interphase cells (% total) |
|------------------|---------|-------------------|----------------------------------------|------------------------------------------|
| 0                | NC      | 6.79 ± 2.31       | 1.94 ± 1.13                            | 0.16 ± 0.26                              |
|                  | 10^{-9} | 6.54 ± 1.31       | 12.64 ± 8.06**                         | 0.50 ± 0.32                              |
|                  | 10^{-6} | 7.72 ± 2.00       | 4.71 ± 3.64                            | 0.62 ± 0.55                              |
|                  | 10^{-3} | 7.56 ± 2.35       | 4.24 ± 3.02                            | 0.11 ± 0.14                              |
| 24               | NC      | 7.83 ± 2.60       | 2.01 ± 2.61                            | 0.09 ± 0.09                              |
|                  | 10^{-9} | 6.10 ± 1.29       | 9.45 ± 5.66*                           | 0.25 ± 0.39                              |
|                  | 10^{-6} | 6.24 ± 2.18       | 10.34 ± 4.25**                         | 0.43 ± 0.63                              |
|                  | 10^{-3} | 5.63 ± 2.67       | 4.25 ± 4.37                            | 0.54 ± 0.48*                             |
|                  | PC      | 3.86 ± 1.49**     | 20.30 ± 11.27**                        | 1.11 ± 0.73**                            |
| 48               | NC      | 6.61 ± 1.08       | 1.46 ± 1.74                            | 0.22 ± 0.29                              |
|                  | 10^{-9} | 6.03 ± 0.99       | 12.39 ± 0.55***                        | 0.45 ± 0.54                              |
|                  | 10^{-6} | 4.14 ± 2.41*      | 13.24 ± 4.23***                        | 0.19 ± 0.48                              |
|                  | 10^{-3} | 3.51 ± 1.64**     | 11.11 ± 12.87                          | 0.06 ± 0.09                              |

The results are expressed as the mean ± SD. *p* ≤ 0.05, **p* ≤ 0.01 and ***p* ≤ 0.001 compared to the corresponding negative control (NC; distilled water). PC - positive control (methyl methanesulfonate, 10^{-3} M).
A variety of chromosomal aberrations in mitotic cells of *A. cepa* L. root tips (Figure 1) in which anaphases with spindle abnormalities and anaphases and telophases with vagrant chromosomes were the most frequent alterations; anaphase/telophase fragments and bridges and C-mitoses were less frequent.

The treatment with $10^{-9}$ M Br-oxph significantly increased the percentage of chromosomal aberrations in mitotic cells (by ~7-fold) compared to the controls (Table 1), but there were no significant changes with the two higher concentrations. During the 24 h recovery period after the 3 h incubation with $10^{-9}$ M and $10^{-6}$ M Br-oxph, the percentage of chromosomal aberrations was ~5-fold higher than in the control cells; the highest concentration ($10^{-3}$ M) did not significantly affect the number of these aberrations. Similar responses were seen after the 48 h recovery period, although the increases were greater. Abnormalities (the presence of micronuclei and binucleate cells) were also seen in interphase cells (Figure 1). There were no significant changes in the percentage of abnormal interphase cells immediately after the 3 h incubation or during the 24 h and 48 recovery periods, except for an increase with $10^{-3}$ M Br-oxph after 24 h.

Br-oxph triggered apoptosis in bone marrow cells 3 h after administration. Light microscopy revealed typical signs of apoptosis, *i.e.*, nuclear fragmentation and condensation (Figure 2), and this apoptotic effect persisted up to 48 h after treatment. Chromosomal aberrations were scored only when the frequency of mitotic cells was enough for determination of at least 50 well spread metaphases per animal. When apoptosis was very extensive, it was impossible to determine the mitotic index and chromosomal aberrations. For this reason, Table 2 shows only the effect of the lowest dose of Br-oxph tested ($2.82 \times 10^{-3}$ μg/kg; 24 h and 48 h after the treatment). This dose significantly reduced the mitotic index by 55% 48 h after the treatment, and there

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**Figure 1** - Aberrations induced by Br-oxph in *Allium cepa* root tips: A - normal metaphase, B - normal anaphase, C - normal telophase, D, C - mitosis, E, F - Spindle abnormalities in anaphase, G - vagrant chromosome in anaphase, H, I - vagrant chromosome in anaphase-telophase, J - anaphase bridge, K - anaphase-telophase bridge, L - anaphase-telophase with fragment, M - normal interphase cell, N - binucleated cell, O - micronuclei in interphase cell.
was a significant increase (2-fold) in the level of chromosomal aberrations in mitotic cells 24 h and 48 h after the injection of Br-oxph (Table 2). Chromatid breaks were the most frequent aberrations, but isochromatid breaks and centromeric and telomeric fusions were also observed; fragmentation was seen only 48 h after treatment. There was no change in the percentage of cells with gaps or in the number of tetraploid cells.

These results show that in both systems Br-oxph depressed cellular proliferation (mitosis) 24 h and 48 h after treatment. Interestingly, Br-oxph (10^{-6} M and 10^{-3} M) appeared to stimulate cell division in A. cepa root tips immediately after a 3 h treatment, but this effect was transitory and was not seen after 24 h and 48 h. The decrease in the mitotic index indicates that Br-oxph can arrest cell growth. The suppression of mitotic activity is often used to assess cytotoxicity (Smaka-Kincl et al., 1996). The ability of Br-oxph to induce chromosomal aberrations in A. cepa root tips and bone marrow cells after treatment for 3 h and during 24 h and 48 h of recovery agrees with the findings of Williams and Omoh (1996) and Miyamae et al. (1997), who observed DNA damage after a 3 h exposure to other compounds.

Br-oxph was generally less genotoxic than the positive control (MMS). The abnormalities caused by Br-oxph showed little concentration- or time-dependence. Interestingly, 10^{-3} M Br-oxph caused fewer aberrations in A. cepa mitotic cells 48 h after treatment with 10^{-6} M and 10^{-3} M Br-oxph correlated with the inhibition of cell division. A number of factors, such as compound solubility, rate of transport and biodistribution, and concentration at the target site (which is influenced by time and cellular permeability), can modulate the time of occurrence of chemically-induced aberrations (McFee and Tice, 1990). In addition, there was marked individual variation in the responses to Br-oxph, which meant that in some experiments the changes observed were not significant.

There were differences in the chromosomal aberrations caused by Br-oxph in the two test systems. The occurrence of abnormal anaphases and C-mitosis in A. cepa indicated that spindle formation was adversely affected (El-Ghamery et al., 2000). According to Rank (2003), vagrant chromosomes are also indicators of spindle poisoning. In bone marrow cells, chromatid breaks were the most frequent aberrations, whereas the number of tetraploid metaphases in bone marrow cells was unaffected by the treatment. These findings suggest a plant-specific action of Br-oxph on spindle formation.

Fusion between chromatids can be initiated by the simultaneous breakage of two chromatids or by the loss of telomere capping (Gilley et al., 2005). The bridges seen in A. cepa cells were also probably formed by breakage and fusion of chromosomes and chromatids (Türköglu, 2007). The relatively low percentage of bridges and fragments in A. cepa root tips agreed with the relatively low percentage of cells with micronuclei (Krishna and Hayashi, 2000). The detection of a binucleate condition in Allium indicated that Br-oxph solutions inhibited cytokinesis.

Apoptosis is an energy-dependent, genetically controlled process by which unnecessary or damaged cells die (Martin, 1993; Earnshaw, 1995). DNA damage can induce cell death and thus, the occurrence of apoptosis in mouse bone marrow cells was another indication of the cytotoxicity of Br-oxph.
xicity of Br-oxph. Light microscopy showed the presence of apoptotic nuclei with an altered morphology with typical nuclear fragmentation and condensation, as described by others (Kam and Ferch, 2000; Gorneva et al., 2005). According to Grishin et al. (2001), genotoxic stresses activate intracellular signaling molecules, which lead to growth arrest, DNA repair, and/or apoptosis. Although several of the pathways linking DNA damage to mitochondria-dependent and -independent mechanisms of death have been elucidated, the connectivity of these pathways is subject to regulation by various other poorly understood factors (Borges et al., 2008).

In conclusion, the results of this study indicate cytotoxicity and genotoxicity of Br-oxph in A. cepa root tip cells and ICR mouse bone marrow cells, with the effects being observed up to 48 h after treatment for 3 h. Chromosomal aberrations provide a sensitive endpoint for assessing the genotoxicity of chemicals (Topashka-Ancheva et al., 2003) and, as shown here, A. cepa may be a sensitive biosensor for screening the genotoxicity of oxaphospholes. On the other hand, our data are in accordance with observation that rodent bioassays are useful for investigating the pharmacokinetics, mechanisms of action, and differential toxicity of various chemicals (Roldan-Arjona et al., 1991).

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