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EFFECTS OF ALKYLATING METABOLITES OF IFOSFAMIDE AND ITS BROMO ANALOGUES ON DNA OF HELA CELLS

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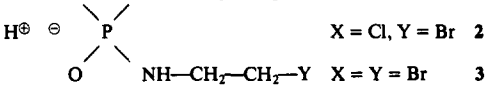
Abstract—The *in vitro* cytotoxicity and mechanism of action of three alkylating compounds: an active metabolite of ifosfamide (**1**, isophosphoramide mustard, *N,N'*-bis(2-chloroethyl)phosphorodiamidic acid) and its bromo substituted analogues, **2** (one chlorine atom replaced by bromine atom) and **3** (two chlorine atoms replaced by bromine atoms), were studied in cultured HeLa cells. Alkaline elution analysis of cellular DNA demonstrated the presence of concentration- and time-dependent interstrand crosslinks, DNA–protein crosslinks and alkali-labile sites (ALSs) in HeLa cells following a 1 hr exposure to the compounds. The bromo analogues were more cytotoxic than 1 and exhibited higher crosslinking potency. The time-course of crosslink formation and removal for the three compounds was similar. ALSs in DNA were produced by all tested drugs but **3** exhibited exceptionally high activity and was able to induce two kinds of alkali-labile lesion (fast- and slow-appearing) whereas **1** and **2** generated only slow-appearing ones. The results suggest that **1** and **2** are more specific in their reaction with DNA in that they produced a lesser variety of lesions than **3**. A potential advantage of **2** over **1** seems to be its higher DNA interstrand crosslinking activity.

The antitumour drug ifosfamide is a member of the oxazaphosphorine cytotstatics exhibiting significant activity against a variety of malignant diseases [1]. Our knowledge of the enzymic and chemical activation of oxazaphosphorines indicates that the transport forms of the drugs are primary metabolites (4-hydroxy-derivatives) whereas the ultimate alkylating species are *N*-mustard-phosphorodiamidic acids [2, 3]. Although the selectivity of oxazaphosphorine drugs *in vivo* seems to be linked to the cytotoxic properties of their activated primary metabolites [3], the specific reactions of their alkylating metabolites with the cellular target molecules (DNA, proteins) may also influence selectivity of the drugs antitumour actions. There are numerous data suggesting that the formation of DNA interstrand crosslinks (ISCs)‡ by the drugs' metabolites is a lethal cellular lesion [4–6]. Therefore, the ability to form ISCs may be a rationale for the design of more cytotoxic compounds [7, 8].

In an effort to find antitumour agents based on their capacity to induce specific DNA lesions, ifosfamide analogues with the chlorine atom replaced by another atom or group undergoing nucleophilic substitutions were synthesized [9]. These compounds were tested *in vivo* against murine L-1210 leukemia. The bromo analogue exhibited a higher therapeutic index than did ifosfomide and cyclophosphamide [9]. The differences in antitumour activity between ifosfamide and its analogues depend on several factors including cellular uptake, rate of metabolic activation, stability of the drug transport and alkylating specificity of the ultimate metabolites. Although the detailed routes of metabolic activation of the new bromo analogues of ifosfamide are not known, it is believed that they undergo activation by the enzymatic system common for other oxazaphosphorines [3] and that the ultimate alkylating metabolites are bromo substituted analogues of **1** (*N,N'*-bis-(2-chloroethyl)-phosphorodiamidic acid): compounds **2** and **3**.



In order to test the concept that alkylators **2** and **3** may react more selectively with DNA than the ifosfamide metabolite **1**, compounds **1–3** were synthesized and DNA of HeLa cells treated with the drugs was analysed by the alkaline elution technique.

MATERIALS AND METHODS

Drugs. Compounds **1**, **2** and **3** of purity exceeding 98% were synthesized according to the procedure described in Ref. 10 from their aziridinyl precursors. All drugs were dissolved in sterile dimethylformamide immediately before cell treatment.

Cells and DNA labeling. HeLa cells (Flow Laboratories, U.S.A.) were grown in minimum

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‡ Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanosulfonic acid; ISC, interstrand crosslink; DPC, DNA–protein crosslink; ALS, alkali-labile site; SSB, single strand breaks.

essential medium (Wytwornia Surowic i Szczepionek, Poland) supplemented with 10% calf serum (Gibco, Grand Island, NY, U.S.A.) and 0.02 M Hepes buffer (Polfa, Poland) in monolayer cultures. Cells (5×10^5) were seeded in 25-cm^2 tissue culture flasks containing [^{14}C]thymidine (0.02 μCi/mL; Chemapol, Czechoslovakia) and grown for 24 hr. Radioactive thymidine was removed 24 hr prior to DNA damage measurement.

Cytotoxicity assays. Replicating HeLa cells were seeded at 2.5×10^5 cells per 25-cm^2 flask and 24 hr later drug solution was added. Cells were incubated with drug for 1 hr at 37°. After 72 hr of incubation in fresh medium, the cells were trypsinized and counted [11].

Alkaline elution. Alkaline elution procedure was performed essentially as described by Kohn *et al.* [12] except that the pumping rate was 0.1 mL/min and tetraethylammonium hydroxide (Sigma Chemical Co., MO, U.S.A.) was used as a component of the eluting buffer. Fractions of 6 mL were collected at 1-hr intervals for 5 hr. SSBs, ISCs and ALSs in DNA were assayed using polycarbonate filters, pore size 1 μm (Nucleopore, Plaesanton, U.S.A.). For DPC measurement, type BSWP poly(vinyl chloride) filters, pore size 2 μm (Millipore, USA), were used. In the ISC and DPC assays cells were irradiated with γ-radiation to introduce SSBs into DNA. The most commonly used standard doses are 300 and 3000 rad for the ISC and DPC assay, respectively [12]. Although the pumping speed in our experiments was 3-fold higher then in the original method, it was found that the standard doses of 300 and 3000 rad produced the most appropriate elution slopes under these conditions (data not shown). The ISC frequency (P_c), expressed in rad equivalents, was calculated by the formula:

$$P_C = (\sqrt{(1 - R_0)/(1 - R_1)} - 1) \times 300 \text{ rad}$$

where R_0 and R_1 are relative retentions of [^{14}C]-DNA from untreated and drug-treated cells, respectively. The retention end points were taken at the point of elution when 20 mL of eluting solution passed through the filter. The DPC frequency (P_{CD}) in rad equivalents was calculated by the formula:

$$P_{CD} = \left(\frac{1}{\sqrt{1 - R_1}} - \frac{1}{\sqrt{1 - R_0}}\right) \times 3000 \text{ rad}$$

where R_1 and R_0 are the extrapolated fractions of slowly eluting DNA in drug-treated and control cells, respectively [12]. In the ALS assay these lesions were converted, prior to elution, into SSBs by a 3-hr incubation of deproteinized cell lysates in the eluting buffer at pH 12.7. The frequency of SSB (P_{BD}) was estimated according to the formula:

$$P_{BD} = \left(\frac{\log (r_1/r_0)}{\log (R_0/r_0)}\right) \times 300 \text{ rad}$$

where r_0 and R_0 are the relative retentions for unirradiated or irradiated controls, respectively. r_1 is the retention of DNA from drug-treated cells [12].

Colorimetric determination of alkylating activity. The procedure of Acosta and Mitchel [13] was used, with minor changes. The drugs (100 μM) were incubated with 5.8 mg/mL of 4-(4-nitrobenzyl)-pyridine (Sigma) for 2 hr at 37°. Amount of alkylation blue product was expressed as A_{540} units.

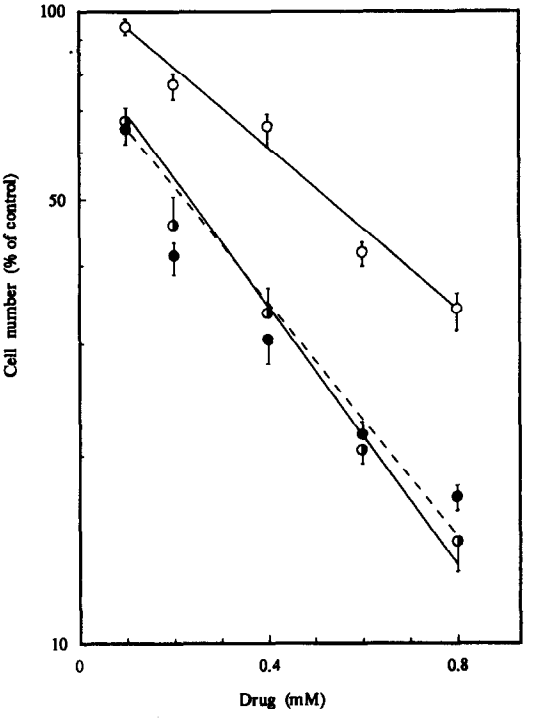

Fig. 1. The effect of isophosphoramide mustards **1**, **2** and **3** on the proliferation of HeLa cells. Cells were treated for 1 hr at 37° in growth medium. Cell number relative to control was determined after 72 hr. The points are means ± SD of three independent experiments. (○) **1**; (◑) **2**, (●) and the dashed line **3**.

RESULTS

Cytotoxicity

The effects of **1**, **2** and **3** on the proliferation of HeLa cells are shown in Fig. 1. The survival curves for **2** and **3** were very similar and both compounds exhibited higher cytotoxicity than **1** (Table 1). As the solubility of *N*-mustard-phosphorodiamidic acids in water is rather low, the drugs were dissolved in dimethylformamide. The highest concentration of the drugs used in cytotoxicity experiments (0.8 mM) corresponds to 2% of dimethylformamide. Because the solvent at concentrations exceeding 2% slightly inhibited cell growth, doses higher than 0.8 mM were not tested.

Alkylating activity

Non-specific alkylating activity of compounds **1**, **2** and **3** is shown in Table 1. Dichloro substituted **1** exhibited the lowest activity. Its bromo analogues **2** and **3** were more active and produced about 3.5 and seven times more alkylated product than **1**, respectively.

Alkaline elution analysis of DNA damage

The presence of lesions in cellular DNA affects

Table 1. Alkylating and cytotoxic activities and DNA damaging properties of isophosphoramide mustards 1–3

	Compound 1	2	3
Alkylating activity (A_{540})	0.020 ± 0.003	0.070 ± 0.014	0.149 ± 0.025
Cytotoxic activity (ED_{50}) (mM)	0.52 ± 0.04	0.23 ± 0.04	0.22 ± 0.3
ISCs*	7.3 ± 6.1	31.3 ± 7.5	29.4 ± 9.1
DPCs*	96 ± 20	123 ± 8	136 ± 7
DNA breaks measured at pH 12.1*	ND	ND	ND
DNA breaks measured at pH 12.7 (ALS)*	20 ± 16	20 ± 23	340 ± 67

* DNA lesion frequencies (expressed in rad-equivalents) were measured immediately after 1 hr incubation of cells with drug (0.8 mM). Data are means ± SD of 3–4 determinations. ND, not detected.

the alkaline elution profiles. Both types of DNA crosslinking (interstrand and DNA–protein) reduce the elution rate whereas it is increased by DNA breaks [12]. In Fig. 2, DNA crosslinking is shown in elution assays performed 3 hr after drug treatment. As cell lysates were deproteinized prior to elution, the observed decrease in elution rate is due to ISCs. All three compounds were able to produce ISCs. The bromo substituted analogues **2** and **3** induced comparable levels of crosslinks and were significantly more potent than **1** (Fig. 2).

DPCs were assayed by means of the high-γ-ray-dose method [12] which allows the effect of ISCs on elution rate to be eliminated. All tested compounds behaved in a similar fashion and produced similar frequencies of DPCs (Fig. 3 and Table 1). The

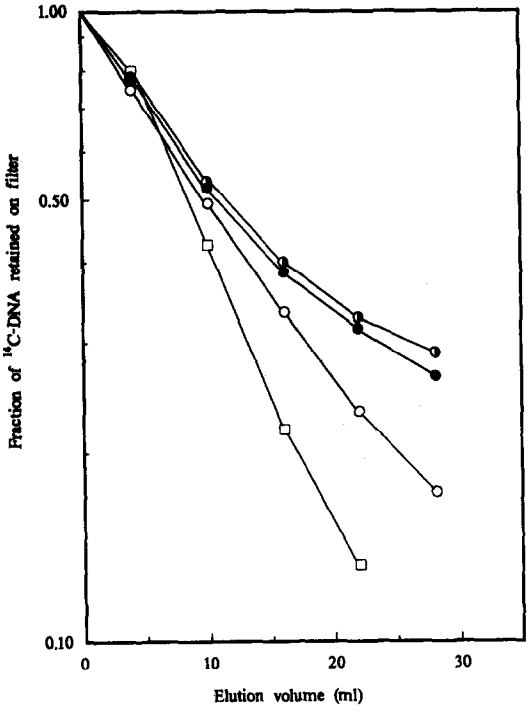

Fig. 2. Alkaline elution assay for DNA interstrand crosslinking. HeLa cells were treated with 0.8 mM drugs for 1 hr at 37° and then incubated in drug free medium for 3 hr. Control and drug-treated cells were harvested and irradiated on ice with 300 rad of γ-radiation. The cell lysates were deproteinized by proteinase K digestion prior to elution. (□) DNA from control cells; (○) **1**, (◑) **2**, (●) **3**.

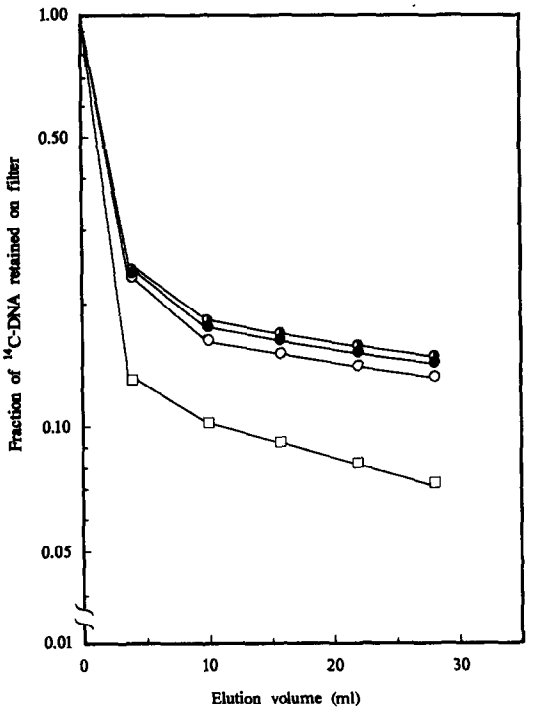

Fig. 3. DNA–protein crosslinking in HeLa cells treated with isophosphoramide mustard. Cells were treated as described in the legend to Fig. 2. Assay was performed at 3 hr after drug treatment. Control and drug-treated cells received 3000 rad of γ-radiation prior to elution. (□) DNA from control cells; (○) **1**; (◑) **2**; (●) **3**.

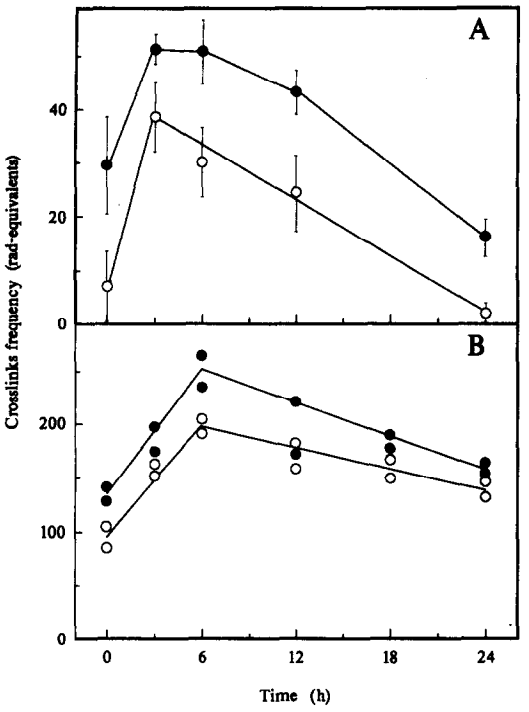

Fig. 4. ISC and DPC frequency as function of time after treatment of HeLa cells with 0.8 mM isophosphoramide mustards **1** and **3**. (A) Interstrand crosslinking; (B) DNA–protein crosslinking. In (A) the points are means ± SD of three experiments. In (B) the points represent data of two independent experiments. (○) **1**; (●) **3**.

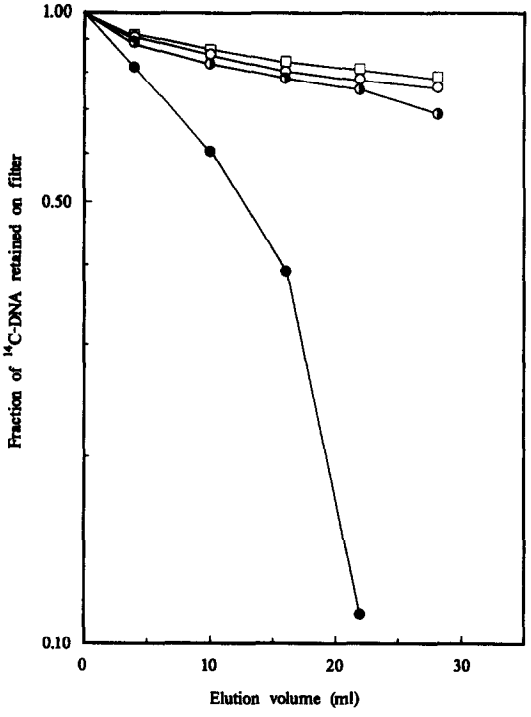

Fig. 5. Alkaline elution assay for ALSs in DNA. Cells were exposed to 0.8 mM **1**, **2** and **3** for 1 hr at 37° and harvested immediately. Cells were lysed and DNA adsorbed on the filters was deproteinized by proteinase K treatment. Filters with DNA were incubated in darkness for 3 hr at room temperature in eluting buffer pH 12.7 prior to elution. (□) control; (○) **1**; (◑) **2**; (●) **3**.

dependence of ISCs and DPCs on time after drug treatment is shown in Fig. 4. ISC frequency peaked at 3 hr after a 1-hr exposure to **1** and **3**, after which the number of crosslinks decreased (Fig. 4A). ISCs caused by a 0.8-mM solution of **1** were repaired almost completely 24 hr after drug removal, whereas about 30% of the crosslinks induced by a 0.8-mM solution of **3** remained unrepaired. The time-course of ISCs produced by a 0.8-mM solution of **2** was very similar to that produced by that of **3** (data not shown). The kinetics of DPC formation and removal were similar for **1** and **3** (Fig. 4B) and for **2** (data unshown). The highest level of DPCs was found 6 hr after drug removal. The decrease in DPCs was slower and at 24 hr high DPC frequencies were still observed.

Reaction of alkylating agents withe DNA often leads to the formation of SSB and ALSs. The latter DNA lesions correspond to sites at which a damaged base has been removed and/or to alkylated phosphate groups [14]. SSBs can be detected by alkaline elution carried out at pH 12.1. For detection of ALSs DNA should be eluted at pH 12.7 to convert these lesions into SSB [12]. When DNA from HeLa cells treated for 1 hr with **1**, **2** and **3** was eluted at pH 12.1 no SSBs were found immediately or 24 hr after drug treatment (data not shown). A very high level of ALSs was demonstrated in cells treated with compound **3** and which were assayed at pH 12.7 immediately after the drug treatment (Fig. 5 and Table 1). Elution profiles obtained for DNA from cells incubated with **1** and **2** were very close to that obtained for control DNA (Fig. 5). Very fast elution of DNA (low DNA retention on filter) was reproducibly observed with **3** also during 24 hr post-drug incubation (Fig. 6A). In the case of compounds **1** and **2** the retention was unchanged up to 6 hr after drug removal and then decreased gradually (Fig. 6A). Because the initial frequency of ALSs induced by 0.8 mM **3** was too high to observe possible changes during post-drug incubation, the time-course of ALSs was assayed at a lower concentration of the drug (Fig. 6B). It can be seen that during the first 4 hr following drug treatment elution slowed down (disappearance of ALSs). A significant increase in elution rate began at 6 hr. The results presented in Fig. 6 may indicate that **3** induced two kinds of ALS. One of them was observed immediately after drug treatment and it was repairable over a few hours. The second, which seems to be common for all tested mustards, appeared at significant frequencies after 6 hr.

The greater reactivity of bromo mustards when compared to the chloro analogues may lead to the decrease in specificity in their reaction with DNA. Therefore, it seems possible with bromo compounds that the actual crosslink frequency might be lower

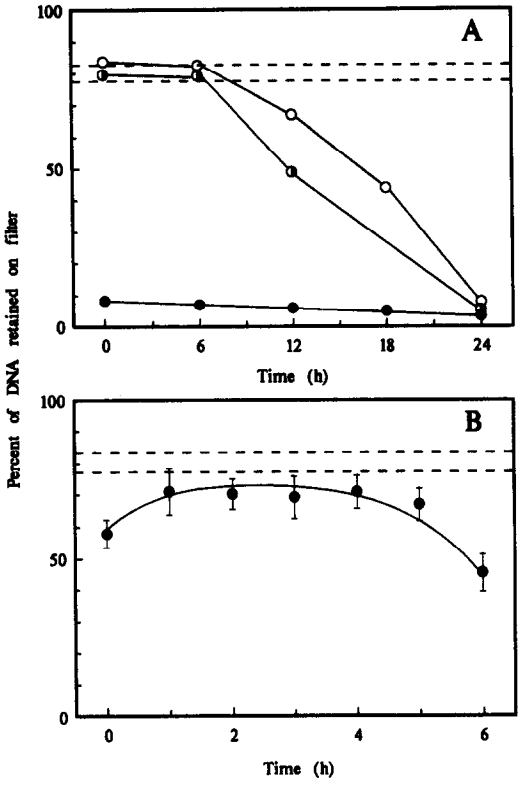

Fig. 6. Changes in DNA retention observed at different times after treatment of HeLa cells with isophosphoramide mustards. (A) Cells treated with 0.8 mM **1**, **2** and **3**; (B) cells treated with 0.2 mM **3**. Points are means ± SD of 4-6 experiments of two independent determinations. The dashed lines show the range of retentions of control DNA. Elutions assays were performed as described in the legend to Fig. 5. The retentions were read at the point of elution when 20 mL of eluting solvent passed through the filter. (○) **1**; (◑) **2**; (●) **3**.

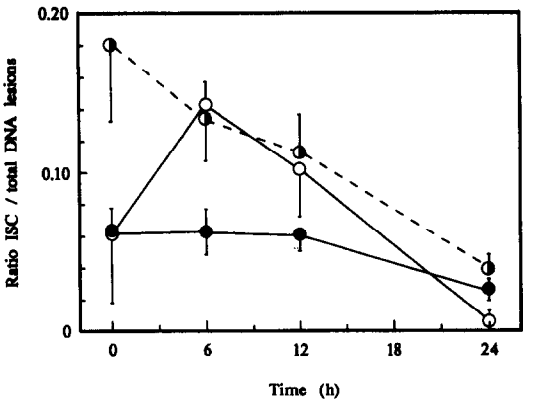

Fig. 7. ISC/total DNA lesions ratio as function of time after treatment of HeLa cells with 0.8 mM isophosphoramide mustards **1**, **2** and **3**. Total DNA lesions = ISCs + DPCs + ALSs. Data are means ± coefficient of variance. (○) **1**; (◑) and the dashed line **2**; (●) **3**.

than with compound **1**. Changes in the ISC/total DNA lesions ratio as a function of time after drug treatment are shown in Fig. 7. Compound **2** exhibited the highest ratio immediately after drug treatment. At 6 hr and after this ratio was very similar for **1** and **2**. Ratio ISCs/total DNA damage for analog **3** was significantly lower than that for **1** and **2**.

DISCUSSION

The aim of this study was to compare the cytotoxicity and DNA damaging properties of *N,N'*-bis-(2-chloroethyl)phosphorodiamidic acid, **1**, which is the ultimate alkylating metabolite of ifosfamide [19] and its two bromo substituted analogues **2** and **3**. Cytotoxic effects of compounds **1**, **2** and **3** (ED range 0.22–0.53 mM) are rather low in comparison with data obtained for other bifunctional crosslinkers (e.g. chloronitrosoureas, *cis*-diamminedichloroplatinium [15–17]. However, this seems to be a common feature of the alkylating metabolites relative to the parent drugs as a similar ED_{50} value for phosphoramide mustard can be read from the data of Crook *et al.* [6]. The low cytotoxicity of phosphoramide mustard type compounds may be due to their poor capacity to penetrate the cell membrane. This is consistent with the hypothesis that the active alkylating metabolites of oxazaphosphorines must be released intracellularly as a result of hydrolytic [2] or enzymatic [18] cleavage of the activated transport form, probably the 4-hydroxy derivative [3].

On the other hand Struck *et al.* [19] reported an antitumor activity of **1** comparable with that of ifosfamide and suggested that the cytotoxicity of ifosfamide *in vivo* is controlled by extracellular **1**. Therefore, an alternative explanation, i.e. that the low cytotoxicity of phosphoramide mustard congeners is caused by their fast decomposition in growth medium, is also possible.

Tested drugs possess two 2-haloethyl groups which may be engaged in bifunctional alkylations of target molecules. thus, ability of these compounds to crosslink DNA was expected. Indeed, in cells treated with **1**, **2** and **3** ISCs as well as DPCs were detected (Figs 2 and 3). The kinetics of the formation and removal of both types of DNA crosslink (Fig. 4) was found to be in general agreement with the findings of other authors in studies with 4-sulfidocyclophosphamides [4], hepatocytes-activated cyclophosphamide [6], 4-hydroxycyclophosphamide [20], phosphoramide mustard [4, 6] and cyclophosphamide *in vivo* [21]. The delay in ISC and DPC formation observed in our experiments suggests that tested alkylators produce these lesions by two-step processes as has been postulated for many other crosslinking agents [7, 8, 22]. Compounds **1**, **2** and **3** exhibited different interstrand crosslinking activity (Fig. 2 and Table 1) which was dependent on the kind of halogen atom present in the molecule. Replacement of the chlorine atom in one of the two *N*-(2-chloroethyl) chains of **1** by bromine (compound **2**) caused an enhancement of interstrand crosslinking potency. When there was such a replacement in both

mustard chains no subsequent increase in the crosslinking activity was observed. DNA–protein crosslinking activity seems to be less dependent on the kind of halogen atom substituting into drug molecule because similar DPC frequencies were produced by compounds **1**, **2** and **3** (Fig. 3 and Table 1).

An interesting difference between tested drugs was found in their capacity for form ALSs in DNA. Compounds **1** and **2** produced ALSs (Fig. 6B), which appeared several hours after drug treatment (slow-appearing ALSs), whereas the dibromo analogue **3** induced a high frequency of ALSs (Figs. 5 and 6) immediately after drug treatment (fast-appearing ALSs). This type of ALS was repairable, although complete repair was difficult to observe as slow-appearing ALSs began to accumulate at 6 hr after drug treatment (Fig. 6B). Several types of DNA damage can be responsible for ALSs detected by alkaline elution. Although there is no direct evidence, it seems reasonable that fast-appearing ALSs are phosphotriesters caused by the alkylation of phosphate groups in DNA [13]. Only compound **3** which exhibited high non-specific alkylating activity in the test with nitrobenzylpyridine (Table 1) was able to form this type of ALS. The slow-appearing ALSs were induced by all tested compounds and they probably represent secondary DNA lesions (base free sites) due to the action of repair enzymes and/or the spontaneous loss of alkylated bases. This is further supported by the observation that ISC and DPC disappearance was correlated with the accumulation of slow-appearing ALSs (Figs 4 and 6).

The alkylating activity of the tested drugs was found to be dependent on the alkylator structure. Compound **1** with chlorine atoms in both chains was a weak alkylator (Table 1). Replacement of the chlorine atoms in the molecule of **1** by bromine atoms enhanced consecutively the non-specific alkylating activities of the derivatives **2** and **3**. Dichloro substituted **1** was the weakest alkylator and crosslinking agent and exhibited the lowest cytotoxic effect. Compound **3** produced about twice as many alkylations as **2** but both compounds induced a similar frequency of ISCs and were almost equitoxic (Table 1). This result is consistent with the hypothesis that the cytotoxicity of phosphoramide mustard and relative compounds correlates with their crosslinking activity [4–8].

A higher crosslinking potency of **2** and **3** over **1** cannot be explained on the basis of alkylating activity. Increase in alkylating activity was accompanied by an increase in crosslinking potency only when one chlorine atom in the molecule of **1** was replaced by bromine. A substitution of the second chlorine atom by bromine caused an increase in the alkylating activity but no change in the crosslinking potency (Table 1). The mechanism of DNA crosslinking of the tested compounds is thought to resemble that of phosphoramide mustard [23]. Prior to the first step of the crosslinking event the 2-chloroethylamino group undergoes cyclization to form aziridinium ion. The same cyclization occurs when the second mustard chain of the drug molecule monofunctionally bound to DNA forms an ISC or DPC. The rate of cyclization should affect the alkylating activity of the drugs. In fact, the rate of aziridine ring formation by the *N*-(2-bromoethylamino) chain of **2** and **3** was found to be higher than that for the chloro substituted chain* and this is consistent with the known high reactivity of mustard chains bearing a bromine atom as a leaving group [24]. Thus, it would be expected that the more active alkylators **2** and **3** may induce higher levels of monoadducts and ISCs then those induced by **1**. However, it is difficult to assess why enhancement of the alkylating activity of the second chain in a molecule of **3** did not increase interstrand crosslinking activity. The simplest explanation is that **3**, the most reactive compound, alkylates more target sites in DNA than **1** and **2** and produces more types of monoadduct. This is further supported by the observation that **3** induced a very high level of fast-appearing ALSs (possibly alkylated phosphate group) whereas **1** and **2** were inactive in this respect. Although it remains unclear as to what are the local determinants for DNA crosslinking (e.g. favorable DNA sequence, steric accessibility of the guanine N7 atoms) by compounds tested in this work, the induction of numerous types of monoadduct seems to be an undesirable feature of any crosslinking drug. Because the alkaline elution technique can not be used for direct measuring of monoadducts the ISC/monoadduct ratio was not estimated and the number of ISCs was calculated as a proportion of total DNA lesions. As shown in Fig. 7, ISC/total DNA lesions ratio for **3** was significantly lower than that observed during post-drug incubation for **1** and **2**. It can be concluded that the actual ISC frequency induced by the most reactive **3** is lower than those induced by **1** and **2**.

As mentioned earlier the bromo analogue of ifosfamide (one chlorine atom replaced by a bromine atom) was more effective *in vivo* than ifosfamide and cyclophosphamide [9]. It is interesting that **2**, which is believed to be an alkylating metabolite of the above oxazaphosphorine, induced in DNA the same types of DNA damage as did compound **1** (ifosfamide metabolite) but exhibited a higher interstrand crosslinking activity. This result suggests that some improvement in therapeutic efficacy of bromofosfamide versus ifosfamide may be related to the enhancement of the interstrand crosslinking potency of its alkylating metabolite.

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