Mechanistic aspects of the cytotoxic activity of glufosfamide, a new tumour therapeutic agent

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Summary β-D-Glucosyl-ifosfamide mustard (D 19575, glc-IPM, INN = glufosfamide) is a new agent for cancer chemotherapy. Its mode of action, which is only partly understood, was investigated at the DNA level. In the breast carcinoma cell line MCF7 glufosfamide inhibited both the synthesis of DNA and protein in a dose-dependent manner, as shown by the decreased incorporation of [3H-methyl]-thymidine into DNA and [14C]-methionine into protein of these cells. Treatment of MCF7 cells with 50 μM glufosfamide was sufficient to trigger poly(ADP-ribose) polymerase (PARP) activation, as revealed by immunofluorescence analysis. Both CHO-9 cells, which are O6-methylguanine-DNA methyltransferase (MGMT)-deficient, and an isogenic derivative, which has a high level of MGMT, showed the same cytotoxic response to β-D-glc-IPM, indicating that the O6 position of guanine is not the critical target for cytotoxicity. By contrast, a sharp decrease in survival of cross-link repair deficient CL-V5 B cells was observed already at concentrations of 0.1 mM β-D-glucosyl-ifosfamide mustard, whereas the wild-type V79 cells showed a 90% reduction in survival only after treatment with 0.5 mM of this compound. The therapeutically inactive β-L-enantiomer of glufosfamide also showed genotoxic effects in the same assays but at much higher doses. This was probably due to small amounts of ifosfamide mustard formed under the conditions of incubation. The results indicate that the DNA crosslinks are the most critical cytotoxic lesions induced by β-D-glucosyl-ifosfamide mustard. © 2000 Cancer Research Campaign

Keywords: β-D-gluocosyl-ifosfamide mustard; tumour therapy; poly(ADP-ribose) polymerase; DNA repair deficiency

Most of the clinically used cytostatic drugs have serious side-effects. There is much hope that gene therapy will make tumour therapy more specific, but at present gene therapy is far from being applicable to larger numbers of patients. Therefore, there is an urgent and persistent need for new drugs in cancer therapy. Several attempts have been made to minimize the side-effects of tumour therapeutics. For example, the urototoxic effects of ifosfamide are reduced by co-administration of 2-mercapto-ethane-sulphonate (Mesna) which deactivates acrolein, a metabolite of ifosfamide in the bladder, but Mesna itself can also lead to unwanted effects (Shaw and Graham, 1987). In view of the growing importance of pharmacokinetics in drug design and early clinical trials, new drugs for cancer chemotherapy should have low molecular weight to guarantee a clear pharmacokinetic behaviour (Workman, 1997). The glucose-coupled ifosfamide mustard D19575 (glc-IPM, INN = glufosfamide) (Pohl et al, 1995) is a compound which meets these requirements.

The major toxic effects of ifosfamide are attributed to its metabolites, especially ifosfamide mustard (IPM) and the urotoxic acrolein. In glufosfamide, IPM is coupled to C1 of glucose in an ester-like bond. Because of its glucose moiety the compound is preferably taken up by cancer cells rather than by normal cells. Inside the cell it is cleaved by glucosidases, thus liberating the cytostatic IPM (Seker et al, 1996; Seker, manuscript in preparation).

Glufosfamide shows a lower myelotoxicity and a higher antitumour activity than ifosfamide, as demonstrated with cultured cells and with human tumours grown in immunodeficient mice (Pohl et al, 1995; Stüben et al, 1996; Fiebig, personal communication). In July 1996, glufosfamide entered a clinical phase I trial and will enter phase II in 1999. Its mode of action is still under investigation.

Whereas whole-body autoradiography of the 14C-labelled compounds did not reveal considerable differences in the distribution of the β-D- and the β-L-isomers in rats, radioactivity appeared in brain and tumour only after the administration of β-D-glc-IPM, strongly suggesting that β-D-glc-IPM is able to cross the blood–brain barrier (Stüben et al, 1996; Schaper, manuscript in preparation). Since β-D-glc-IPM is a hydrophilic molecule, it is assumed that this is due to an active transport mechanism, and it has been hypothesized that a specific protein is responsible for this translocation. Recently, it has been reported that a sodium-coupled transporter (SAAT1 or SGLT3) is indeed responsible for the transport of β-D-glc-IPM into the cell (Veyhl et al, 1998). This is an example for drug targeting by employing a plasma membrane transporter.

The aim of the present work was to study the biological consequences of β-D-glc-IPM uptake by cells. Following up a previous study (Schaper, manuscript in preparation) we measured the effect of this new cytostatic drug on DNA and protein synthesis as well as stimulation of PARP activity. Inside the cell, glufosfamide is split mainly by glucosidases into glucose and ifosfamide mustard, but also non-enzymatic hydrolysis contributes to its decay. Since ifosfamide mustard also arises in the metabolism of ifosfamide, we investigated whether glufosfamide behaves like ifosfamide in the reaction with DNA. Therefore, we compared the response of cells
deficient in O6-methylguanine-DNA methyltransferase (MGMT) as well as a DNA cross-link repair deficient strain with the corresponding wild-type cells as to β-δ-glc-IPM induced cell killing (Kaina et al., 1991).

MATERIALS AND METHODS

Chemicals

β-δ-Glc-IPM was synthesized in the Chemical Research Laboratories of ASTA Medica AG, Frankfurt, Germany, according to the method described by Dickes (1988). β-δ-Glc-IPM and β-δ-l-glucopyranosyl-14C-Glc-IPM were synthesized according to a method developed by Wieländer (Veyhl et al., 1998). 14C-label was introduced in the β-chloroethylamine side chain of the compounds; the specific radioactivity was 10 mCi mmol⁻¹. The α/β-anomers were separated by recrystallization and column chromatography. Purity of the substances was > 99%. The absence of isophosphoramid mustard was ascertained by high-performance liquid chromatography (HPLC) and then liquid chromatography (TLC). l-[14C]-methionine, and [3H]-methyl-thymidine were purchased from Amersham (Buckinghamshire, UK).

Cells

The generation of strains CHO-9-neo and TK47-AT17-C3 was described previously (Kaina et al., 1991). CHO-9-neo was derived from transfection of CHO-9-neo with the neo gene only; it is used as a MGMT-deficient control. The strain TK47-AT17-C3, which was transfected with neo plus human MGMT cDNA, expresses MGMT to a high level (720 fmol mg⁻¹ protein).

The mutant CL-V5B was derived from V79 Chinesehamster cells (here designated as V79 wild-type (wt)). The line was originally characterized as mitomycin C hypersensitive and is defective in DNA cross-link repair (Tellellmann et al., 1995). Cells were kindly provided by Dr M Zdienicka, Leiden, The Netherlands. They were grown in Dulbecco’s F12 medium containing 10% fetal calf serum.

RESULTS

In previous experiments, genotoxic activity of β-δ-glucopyranosyl-IPM was shown by using the comet assay (Schaper et al., manuscript in preparation). Here we continued this study by investigating the effects of glufosfamide on proliferation and on cellular DNA monitored by the DNA strand-break-dependent formation of poly(ADP-ribose). Furthermore we studied β-δ-glucopyranosyl-IPM cytotoxicity in repair-deficient cells in order to investigate the relevance of possible O6-alkylating or cross-linking effects of glufosfamide. Figure 1A shows that β-δ-glucopyranosyl-IPM induced a concentration-dependent decrease in proliferation as measured by the incorporation of [3H]-methyl-thymidine into DNA of MCF7 cells. Starting with a 10% decrease after addition of 2.5 μM β-δ-glucopyranosyl-IPM to the cells, the inhibition of DNA synthesis was 50% at 25 μM. The
The effect of β-L-glucosamine was much less pronounced, showing a 25% inhibition of DNA synthesis only at the highest concentration (25 μM) of this isomer.

Figure 1B shows that 10 μM and 25 μM β-D-glucosamine lowered the protein synthesis in MCF7 cells, measured by 14C-methionine incorporation into protein of these cells by 50% and about 70%, respectively. The influence of β-L-glucosamine was again less pronounced, showing a 25% inhibition of protein synthesis only at a concentration of 25 μM.

Poly(ADP-ribosyl)ation is a post-translational modification of nuclear proteins which is triggered by DNA strand-breaks and therefore can serve as a marker for genotoxic effects (Figure 2 A–H). The method developed by Bürkle et al (1993) is based on the fact that DNA strand-breaks, as induced directly by ionizing radiation or arising in the course of DNA base excision repair, induce activation of poly(ADP-ribose) polymerase. The treatment of MCF7 cells with γ-rays and subsequent immunofluorescence assay of poly(ADP-ribose) formation induced fluorescence signals in a dose-dependent manner, proving the reliability of this test system in MCF7 cells and the inducibility of poly(ADP-ribose) polymerase. Figure 2 h shows the effect of a 10-min irradiation with 8.4 Gy min⁻¹. The effects of an incubation of MCF7 cells with 50 μM, 250 μM and 750 μM glucosamine for 24 h are shown in Figure 2 B,C,D. It is evident that PARP activity was triggered by β-D-glucosamine at concentrations as low as 50 μM. The β-L-isomer led also to an induction of PARP-activity but only at a concentration of 750 μM (Figure 2F).

Survival of MGMT-deficient and MGMT-proficient CHO cells (strains CHO-9-neo and Tk47-AT17-C3 respectively) upon treatment with glucosamine is shown in Figure 3A. There was no difference in survival between both strains indicating that O6-alkylguanine, which is subject to repair by MGMT, is not formed at significant amounts after treatment with the drug, or alternatively, that this lesion does not persist long enough, perhaps due to the rapid conversion of the monoadduct into a DNA cross-link. On the other hand, the cross-link repair-deficient cell line CL-V5B was clearly hypersensitive to glucosamine as compared to V79 wt, which is indicative of cross-link formation by the agent. It should be noted that CHO-9 cells were more resistant than V79 wt. The reason for this strain difference is unknown, but it is tempting to speculate that either cross-link repair is more efficient, or the uptake by glucose transporter occurs less efficiently in CHO than in V79 cells. A further explanation could be that the content of β-glucosidases in CHO cells necessary for metabolic activation of glucosamine is possibly lower than that of V79 cells.

DISCUSSION

The molecular basis for the neoplastic behaviour of advanced tumours is largely undefined (Monks et al, 1997). Notably, the target for drug intervention is unclear. Here we concentrated on effects of the cytostatic compound glucosamine on DNA in MCF7 cells concerning proliferation and repair. Toxic effects of glucosamine on DNA and on protein synthesis in MCF7 cells, indicative of a genotoxic activity of this compound, were observed already at low doses. The effects on DNA became apparent between 2 and 5 μM, shown by an impaired incorporation of [3H-methyl]-thymidine into DNA of these cells. The L-isomer exerted similar effects but at higher concentrations. We suppose that this effect is due to partial hydrolysis of β-L-glucosamine liberating the IPM-moiety, because cells were incubated for 24 h after which time hydrolysis has already begun. The inhibition of DNA and protein syntheses is probably due to alkylating properties of glucosamine. IPM, which arises in the metabolism of glucosamine, is a powerful alkylating agent (Hemminki, 1986) and may react with cellular components involved in protein synthesis like ribosomes, ribosomal RNA or proteins involved in translation processes (Monks et al, 1997), thus leading to an impairment thereof.

One of the first responses of eukaryotic cells to some types of DNA damage is the covalent modification of nuclear proteins with poly(ADP-ribose). Poly(ADP-ribosyl)ation is catalysed by the nuclear enzyme poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) which utilizes NAD⁺ as substrate (for review see: DeMurcia and Ménissier-de Murcia, 1994; Lindahl et al, 1995; Oci et al, 1997). The two zinc fingers in the aminoterimal DNA-binding domain of PARP mediate the recognition of single- or
Figure 2. Immunodetection of poly(ADP-ribose) induced by various concentrations of β-D-glucose-IPM in MCF7 cells. Cells were treated for 24 h. β-L-Glc-IPM which has no cytostatic activity has been used as control substance. (A) β-D-glucose-IPM 0 μM; (B), 750 μM; (C), 500 μM; (D), 50 μM; (E), β-L-glucose-IPM 0 μM; (F), 750 μM; (G), 500 μM; (H), positive control (10 min at 8.4 Gy min⁻¹)
double-strand-breaks in DNA, thus triggering the activation of the catalytic centre in the carboxyterminal NAD$^+$-binding domain. As a consequence, exposure of cells to certain chemical or physical DNA-damaging agents (including reactive oxygen species, alkylating agents, and γ-radiation) induces a dose-dependent stimulation of cellular poly(ADP-ribose) synthesis.

While the biological functions of PARP have not yet been fully elucidated at the molecular level, cellular poly(ADP-ribose) formation may be used as a marker for the infliction of at least some types of DNA damage, and this can be conveniently assessed in situ by immunofluorescence (Bürkle et al., 1993), using a monoclonal antibody directed against poly(ADP-ribose) (Kawamitsu et al., 1984). Together with previous results on genotoxic effects of glufosfamide as revealed by the comet assay (Schaper et al., manuscript in preparation), the data presented here strongly suggest DNA alkylating properties of glufosfamide. However, in the comet assay the concentration needed to induce detectable DNA damage was very high (10 mM). By contrast, in the present work we could demonstrate that PARP activity, as detected by an immunofluorescence assay (Bürkle et al., 1993), was induced already at a much lower concentration of glufosfamide (50 μM). The question of whether this DNA-damage was mainly due to O$^-$-alkylation processes or to cross-links produced by glufosfamide was addressed by using repair-deficient cell strains. It became apparent that cross-linking was much more toxic than O$^-$-alkylation. The concentrations of glufosfamide needed to produce DNA damage either in the PARP or in the cross-linking assay were in the μM range. In contrast, in the comet assay the appropriate concentrations were in the mM range. We suppose that this discrepancy is a result of the strong cross-linking effects of the metabolite ifosfamide mustard, preventing the DNA-pieces from forming the characteristic comet at concentrations which show activation of PARP. Similar results regarding cross-linking agents investigated in the comet assay have been described (Pfuhler and Wolf, 1996). Experiments are underway to investigate whether suppression of PARP by appropriate inhibitors may increase the therapeutic potency of glufosfamide. The L-isomer led also to an activation of repair mechanisms but the concentrations needed were more than ten times higher than with the β-iso-isomer. The reason is again seen in the formation of IPM which is hydrolytically formed after 24 h of incubation. Results from our comparison of repair-deficient and proficient strains showed that glufosfamide obviously did not induce DNA O$^-$-alkylation which can be repaired by MGMT, but led to cross-link formation. Together with other results reported here, this shows a certain similarity of glufosfamide to ifosfamide suggesting that its cytotoxic effects are mainly due to IPM, the metabolite of both compounds. Yet the toxicity of glufosfamide in white blood cells, colony forming units and spleen colony forming units is considerably lower as compared to ifosfamide (4). Another exciting difference between ifosfamide and glufosfamide was demonstrated by Volm and colleagues who were able to provoke resistance of cells upon continuous drug treatment against ifosfamide but not against glufosfamide in SKOV $^3$ cells (Volm, personal communication).

A necessary requirement for the therapeutic efficacy of saccharide-conjugates is their activation in the target cells. We suppose that the conjugates have to be split by glucosidases to liberate the therapeutic moiety. In a forthcoming paper we will report on the correlation between the content of β-glucosidases in different cell lines and their sensitivity against glufosfamide.

In conclusion, we have shown that the cytostatic compound glufosfamide impaired protein and DNA synthesis in MCF7 cells and that it triggered the activation of PARP, an enzyme specifically sensitive DNA-strand-breaks, at concentrations of 5 μM and 50 μM respectively. The major cytotoxic lesions are probably due to cross-linking effects rather than monofunctional alkylations exerted by glufosfamide, as shown by comparison of isogenic cell-lines deficient and proficient of cross-link repair. In view of the minute amounts of glufosfamide taken up by the cells (Schaper et al., manuscript in preparation), it is surprising that very low concentrations of the drug can lead to the observed striking effects at the DNA level. Further investigations on this issue are in progress.

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