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Short communication

Stem cell toxicity of oxazaphosphorine metabolites in comparison to their antileukemic activity

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Abstract

The oxazaphosphorine agent cyclophosphamide (CP) is an alkylating agent with a relative low stem cell toxicity. The aim of this study was to further evaluate the stem cell toxicity of the active metabolites of CP and its structural analogue ifosfamide (IFO) in comparison to their antileukemic efficacy. Cells of different malignant hematologic disorders (HL-60, HS-Sultan and THP-1) and CD34+ stem cells were treated with cytotoxic CP-metabolite mafosfamide (MAFO) and IFO-metabolites 4-hydroxy-IFO (4-OH-IFO) and chloroacetaldehyde. The clonogenity of the cells was investigated by using a colony-forming assay. All metabolites reduced the formation of both tumor-derived colonies and stem cell-derived CFU-GMs in a concentration-dependent manner. Our data showed a relative tumor-specific, stem cell protecting action of the substances tested with a higher toxicity against tumor cells (IC_{50} against HS-Sultan: MAFO 1.1 μM; 4-OH-IFO 1.3 μM; CAA 3 μM) than against stem cells (IC_{50} MAFO 14.8 μM; 4-OH-IFO 16.9 μM; CAA 14 μM). However, while the cytotoxic action of 4-OH-IFO corresponded to MAFOs activity, CAAs cytotoxic effect against the hematologic tumor cells was lower. In conclusion, the results confirm the observed cytotoxicity of CAA against solid tumors for cells of malignant hematologic disorders. Although the relative cytotoxic specificity of CAA is lower than for 4-OH-IFO and MAFO, also CAA, like 4-OH-IFO and MAFO, was found to be in part a tumoricidal, stem cell protecting substance.

Keywords: Alkylating agents; 4-Hydroxy-cyclophosphamide; 4-Hydroxy-ifosfamide; Chloroacetaldehyde; Stem cell toxicity; Antileukemic activity

1. Introduction

CP and IFO belong to the group of oxazaphosphorine agents and are structural analogues. Both are prodrugs and require microsomal hepatic activation to the 4-hydroxy-metabolite before they can exert their cancerotoxic activities [1,2]. In contrast to CP, a second metabolic pathway with liberation of chloroacetaldehyde (CAA) and the inactive metabolites 2- and 3-dechloroethyl-IFO is important for IFO. CAA was made responsible for specific side effects of IFO such as neurotoxicity [3–5] and nephrotoxicity [6] only. Brain *et al*. [7] recently proposed modulation of the different liver cytochrome P450 isoenzymes shifting IFO metabolism from CAA to 4-OH-IFO to decrease CAA levels. However, our group was the first who showed a toxic effect of CAA against solid tumor cells (MX-1 mamma carcinoma and S117 thyroid gland sarcoma) *in vitro* and *in vivo* [8,9]. The generation of CAA may explain in part the clinically observed lack of complete cross-resistance between CP and IFO and the higher remission rates achievable by IFO in certain tumor types [10,11]. Suppression of CAA metabolic pathway [7], although it may be beneficial as a means of reducing neurotoxic responses, might also be associated with a reduction in antitumor effect.

Although bone marrow toxicity is a frequent side effect and may be dose-limiting, alkylating agents are commonly used for chemotherapy of several malignancies. CP is an alkylating agent with a relative low toxicity against hematopoietic stem cells [12,13]. Therefore, CP is used for stem cell mobilization in high-dose chemotherapy regimens followed by autologous blood stem cell transplantation. In the past years, MAFO, an active CP derivative, is used

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Abbreviations: CP, cyclophosphamide; MAFO, mafosfamide; IFO, ifosfamide; 4-OH-CP, 4-hydroxy-cyclophosphamide; 4-OH-IFO, 4-hydroxy-ifosfamide; CAA, chloroacetaldehyde; RCS, relative cytotoxic specificity; ALDH, aldehyde dehydrogenase.

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for *ex vivo* purging of the autologous bone marrow [12–16] and more recently this strategy was adapted to leukapheresis products [17,18]. The reason for the comparatively low bone marrow toxicity of CP is that hematopoietic stem cells contain high amounts of aldehyde dehydrogenase (ALDH) [19], which inactivate aldophosphamide, the ring-opened tautomeric form of 4-hydroxy-cyclophosphamide (4-OH-CP) [20–24]. Because CP and IFO are structural analogues, the same metabolic pathways might be responsible not only for activating, but also for deactivating both substances.

Different reactions of CAA, which might also cause a cell kill, had been found [25–31], but no direct antitumor effect of CAA had been described prior to the findings of our group [8,9]. However, the concentration of CAA used by Sood and O'Brien [26] to induce a 50% cell kill in hepatocytes, cells with a high content of ALDHs, had been 30-fold higher than in our *in vitro* studies [8]. Furthermore, Sood and O'Brien found out that inhibition of ALDHs even increased the glutathione-depleting action of CAA [32], a well known mechanism to facilitate the attack of alkylating metabolites [33]. Those results indicate that, apart from 4-OH-CP and 4-OH-IFO, also CAA might be deactivated by ALDHs. Thus, CAA might also be a cytotoxic metabolite with a relative low stem cell toxicity.

The aim of this study was to compare the cytotoxic potency of CAA, 4-OH-IFO and 4-OH-CP with the toxicity against hematopoietic progenitor cells. Therefore, cells of three different human hematologic tumor cell lines and human CD34+ stem cells were treated with CAA or with the 4-hydroxymetabolites of CP and IFO.

2. Materials and methods

2.1. Patients and stem cell preparation

The stem cells were obtained from aliquots of 10 leukapheresis products of patients aged 45–58 years (median age 54 years) with different hematologic malignancies, who were treated with high-dose chemotherapy followed by peripheral blood stem cell transplantation. After informed consent, a 1:100 diluted aliquot gathered from the stem cell products for quality assessments such as flow cytometric CD34+ enumeration and short-term culturing were used for these investigations. The mononuclear cells were washed twice in RPMI 1640 (Biochrom) supplemented with 1% L-glutamine and 1% penicillin–streptomycin (both: Bioproducts, Boehringer Ingelheim).

2.2. Cell lines and cell cultures

Three different cell lines of human hematologic malignancies (HL-60: acute promyelocytic leukemia, HS-Sultan: B-lymphocytic Burkitt-lymphoma, THP-1: acute monocytic leukemia) (European Collection of Cell Cultures, CAMR) were used. The cells were cultured in 75 cm^2 culture flasks (Nunclon). RPMI 1640 medium, supplemented with 10% fetal calf serum (PAA Laboratories GmbH), 1% L-glutamine and 1% penicillin–streptomycin was used; for THP-1 2.5 μL mercaptoethanol (Merk-Schuchard)/500 mL medium was added.

2.3. Reagents

4-Hydroperoxy-IFO and MAFO were kindly provided by J. Pohl (ASTA Medica Oncology). After dissolution in RPMI 1640 medium, 4-hydroperoxy-IFO rapidly decomposes to 4-OH-IFO without enzymatic involvement [28], MAFO decomposes to equimolar 4-OH-CP and mesna. Therefore, equimolar mesna was added in CAA and 4-OH-IFO experiments. Mesna (ASTA Medica Oncology) and CAA (Aldrich) were dissolved in a 0.9% NaCl solution before use. All reagents were diluted with RPMI 1640 medium.

2.4. Drug treatment and colony-forming assay

A total of 10 experiments was performed for each concentration of the metabolites tested. An amount of 1 mL cell suspension containing 1×10^7 mononuclear cells with different amounts of CD34+ cells or 1×10^5 tumor cells was centrifuged and the cells were resuspended in medium. CAA, MAFO and 4-OH-IFO were added with (CAA and 4-OH-IFO) or without mesna (MAFO) giving concentrations of 1–100 μmol/L. The incubation of the cells at 37° was stopped after 30 min by chilling to 4° and the cells were washed twice with cooled medium (tumor cells: RPMI 1640, stem cells: IMDM (BioWhittaker)).

The optimum number of cells plated into 35 mm dishes and the preferable incubation time for the clonogenity assays were evaluated by microscopic scoring criteria. After flow cytometric quantification, 500 tumor or 1000 CD34+ cells were suspended in 100 μL medium (tumor cells: RPMI 1640; stem cells: IMDM) and plated on 35 mm cell culture dishes. An amount of 1 mL methylcellulose supplemented with different cytokines (MethoCult™ H4434, Stem Cell Technologies Inc.) was added and cells were cultured in a semisolid short-term culture assay in triplicate. The number of colonies was scored on an inverted microscope after an incubation time of 7 (HL-60 and HS-Sultan) or 12 days (THP-1 and CD34+ cells) at 37° and 5% CO_2. Colonies are defined as containing more than 40 cells. In regard to stem cells only CFU-GMs were counted.

3. Results and discussion

In the past, the advantages of the use of IFO instead of CP were not generally accepted [34]. In addition, side chain metabolite CAA was assumed to cause IFO-specific

side effects such as nephro- and neurotoxicity only. Our group was the first who demonstrated cytotoxic activity against human tumor cells (breast carcinoma MX-1 and thyroid gland sarcoma S117) *in vitro* in concentrations as observed in human pharmacokinetics [8]. This effect was confirmed *in vivo* on xenotransplanted nude mice [9]. The present experiments show the demanded head to head comparison of the two drugs [34] with similar cytotoxic effects of the two 4-hydroxy-metabolites. Moreover, also CAA could be shown to be effective against cells of the various hematologic malignancies.

Incubation of the leukemia and lymphoma cells with the three metabolites show a logarithmic concentration-dependent reduction of colonies. CAA, 4-OH-IFO and MAFO in equimolar concentrations exert similar cytotoxic effects against HL-60 (Fig. 1a) and HS-Sultan (Fig. 1b) without significant differences. Concentrations of 100 μmol/L lead to a nearly complete inhibition of colony growth for all metabolites. The results for THP-1 differ in two notable ways (Fig. 1c). In comparison to HL-60 and HS-Sultan, THP-1 demonstrates a reduced sensitivity to the metabolites tested. 4-OH-IFO in a concentration of 2.5 μmol/L shows a 21% inhibition of colony growth for THP-1, however, the development of colonies of HL-60 and HS-Sultan is reduced by about 39 and 55%, respectively. MAFO and CAA lead to similar results. Additionally, THP-1 shows a remarkable concentration-dependent difference in colony reduction between CAA and the 4-hydroxy-metabolites of IFO and CP. At low concentrations of 2.5 μmol/L, the metabolites exert similar effects with a reduction of colonies of 19–21%. In contrast, CAA is less effective than 4-OH-IFO and MAFO when higher concentrations are used. Whereas the colony growth is almost completely suppressed by the use of 50 μmol/L 4-OH-IFO or MAFO, incubation of THP-1 with 50 μmol/L CAA reduces the formation of colonies only to about 50%. In a recent study, we demonstrated cytotoxic activity against human tumor cells. In comparison to 4-OH-IFO, CAAs cytotoxicity had been shown to be as high or even higher and CAA was found to enhance 4-OH-IFOs cytotoxic effect. However, although the new results differ from those of our previous studies, our present experiments expand the observation of a cytotoxic activity against solid tumor cells for cells of malignant hematologic disorders. A possible explanation for those differences between the efficacy of 4-hydroxy-metabolites and CAA against various malignancies could be a distinct mechanism of action of CAA compared to the 4-hydroxy-metabolites.

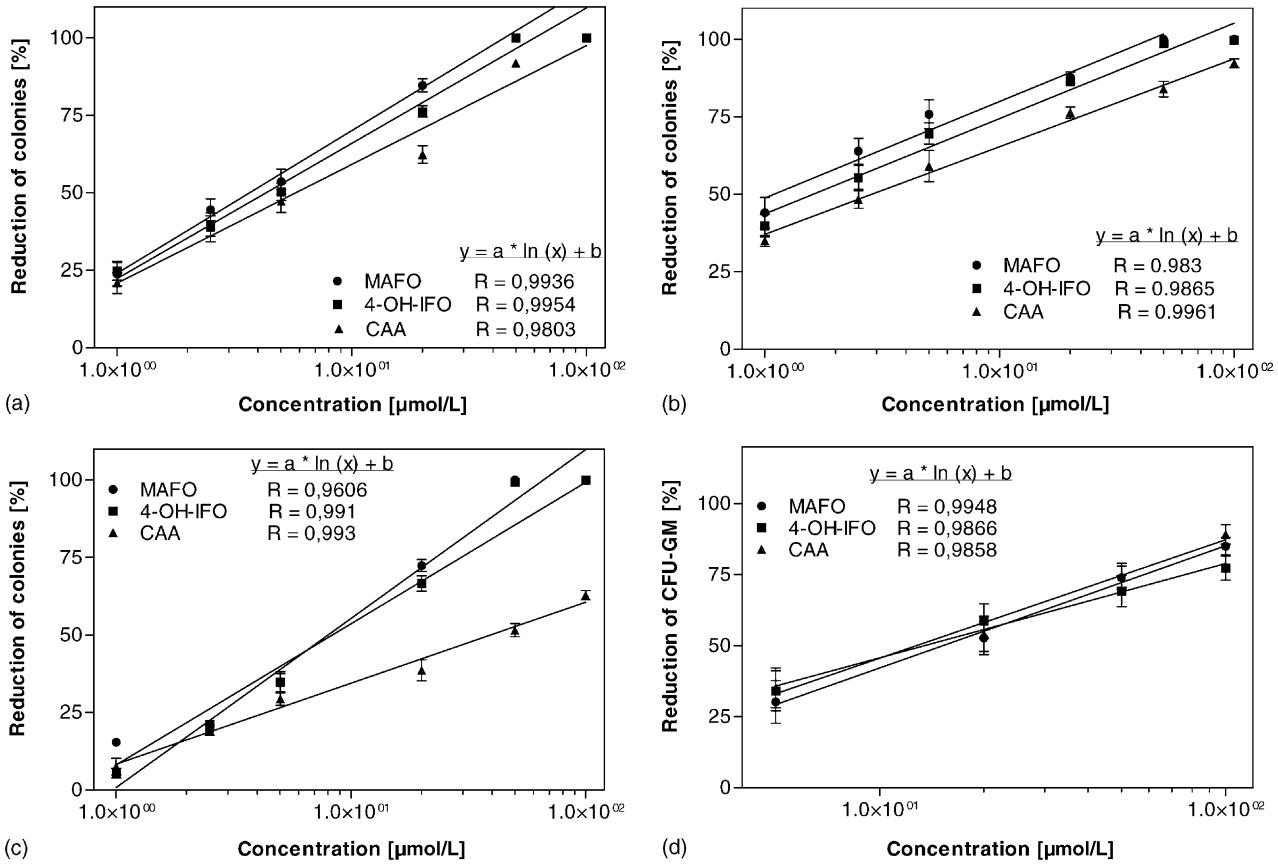

Fig. 1. Reduction of colony-formation of the cell lines (a) HL-60; (b) HS-Sultan; (c) THP-1; (d) CD34+ expressing hematopoietic progenitor cells. Cells were incubated for 30 min with MAFO, 4-OH-IFO and CAA in concentrations of 1–100 μmol/L. The number of colonies was scored after an additional incubation period of 7 days at 37° and 5% CO_2. Mean ± SEM; $n = 10$.

Table 1
Comparison of IC_{50} and RCS of MAFO, 4-OH-IFO and CAA for three different tumor cell lines and CD34+ expressing hematopoietic progenitor cells

	Cell line			
	HL-60	HS-Sultan	THP-1	CD34+
IC_{50} (μmol/L)[a]				
MAFO	3.7**	1.1**	8.3*	14.8
4-OH-IFO	4.3**	1.3**	8 (ns)	16.9
CAA	5.8*	3**	39.3 (ns)	14
RCS				
MAFO	4	13.5	1.8	
4-OH-IFO	3.9	13	2.1	
CAA	2.4	4.7	0.4	

Cytotoxic effects on tumor cells vs. CD34+ cells; ns: not significant.
[a] RCS = IC_{50} CD34+ cells/IC_{50} tumor cells.
* $P < 0.05$; ** $P < 0.01$.

As with the tumor cells, the three metabolites lead to a concentration-dependent reduction of the colony growth of human hematopoietic CD34+ progenitor cells. Fig. 1d shows comparable cytotoxic effects of CAA, 4-OH-IFO and MAFO. CAA in a concentration of 100 μmol/L reduces the formation of CFU-GMs by about 89%; 4-OH-IFO and MAFO lead to a reduction of colony growth of 77 and 86%, respectively.

Table 1 summarizes the results with regard to the IC_{50} calculations and the relative cytotoxic specificity (RCS) of the metabolites on all cell lines. The IC_{50} values indicate that HS-Sultan are the most, the CD34+ cells the least sensitive cells with HL-60 followed by THP-1 in between. This ranking holds true for all metabolites with the exception of the IC_{50} of CAA on THP-1, which is three times the IC_{50} of CD34+ cells. RCS is quite similar for MAFO and 4-OH-IFO for all cell lines, being highest for HS-Sultan, lowest for THP-1. Whereas CAA is less specific for HS-Sultan and HL-60 than the alkylating metabolites MAFO and 4-OH-IFO, no tumor-specific activity could be found for CAA on THP-1.

A possible explanation for the tumor-specific, stem cell protecting action of MAFO is that stem cells contain high amounts of ALDHs, which inactivate aldophosphamide, the ring-opened tautomeric form of 4-OH-CP to carboxyphosphamide [19,20,35,36]. Several studies [20–24] have shown that the ALDH content is an important determinant of cell sensitivity against CP toxicity. Certain human tissues with high ALDH activity, e.g. CD34+ progenitor cells [20,35,36], were less influenced by CP than those with a lower content of ALDHs like several tumors [36,37]. This observation is in line with the use of MAFO as a purging drug for bone marrow or peripheral blood. Additionally, elevated ALDH activity has been discussed as being responsible for resistance of several tumor cell lines against CP [22,24,37,38]. Although urinary excretion of inactive carboxy-IFO is comparatively low [39–41], the similar structure, metabolism and efficacy of 4-hydroxy-metabolites of IFO and CP suggest that ALDHs inactivate aldophosphamide not only in CP but also in IFO metabolism. As with 4-OH-IFO and MAFO, CAA is less cytotoxic against hematopoietic progenitor cells than against the tumor cells HL-60 and HS-Sultan. The observation that the same concentrations of CAA lead to a similar effect against stem cells as the 4-hydroxy-metabolites supposes that also CAA might be inactivated by the ALDHs of the hematopoietic progenitor cells. Moreover, hepatocytes containing high amounts of ALDHs were found to be resistant against CAA; inhibition of ALDHs was shown to enhance CAAs cytotoxicity [26,32]. However, in contrast to CP, the influence of ALDHs on IFO metabolism remains unclear. Other cell type-dependent differences in the metabolic inactivation of CAA may exist or predominate.

In summary, 4-OH-IFO exerts a relative tumor-specific activity comparable to MAFO. In addition, also CAA was found to be a tumortoxic stem cell protecting substance confirming our previous finding of a cytotoxic activity of CAA against solid tumor cells *in vitro* and *in vivo* for leukemia and lymphoma cells. CAA had also been shown to enhance the cytotoxic activity of 4-OH-IFO in solid tumor cells [8]. Thus, apart from the possibility of a different mechanism of cytotoxic action of CAA and consequently of IFO compared to CP, which is the subject of further investigations, CAA might contribute to 4-OH-IFOs efficacy against cells of hematologic malignancies. As a conclusion, our head to head comparison of CP and IFO shows that IFO is not just a simple analogue of CP.

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