Ibosfamide Cytotoxicity on Human Tumor and Renal Cells: Role of Chloroacetaldehyde in Comparison to 4-Hydroxyifosfamide

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ABSTRACT

The initial metabolism of ifosfamide (IFO) consists of two different pathways, which lead to the alkylating metabolite 4-hydroxy-IFO and to chloroacetaldehyde (CAA). CAA has been reported to cause side effects, such as neuro- and nephrotoxicity, whereas no direct antitumor effect has been described thus far. Therefore, two human tumor cell lines (MX1 and S117) and a renal tubular cell line (Landa Leiden) were exposed to 4-hydroxy-IFO, CAA, and a combination of both. The concentrations used were in the same range as measured in the blood of 10 patients treated with 5 g/m² IFO. The cell survival was measured using the MTT assay. Similar dose-response curves were found for both metabolites. For the MX1 tumor, the IC₅₀ of 4-hydroxy-IFO and CAA were 10.8 and 8.6 μM, respectively. For the reduction of S117 cell survival, higher concentrations of the metabolites were needed (25.0 μM 4-hydroxy-IFO and 15.3 μM CAA). Combination treatment of the cells resulted in an approximately additive effect. Both metabolites exhibited similar toxicity against Landa Leiden cells. Our results indicate that CAA has its own cytotoxic profile against tumor cells. Hence, we conclude that the molecular mechanism of action of IFO seems to be only in part an alkylating effect and that CAA may play an important role in the therapeutic efficacy of IFO.

INTRODUCTION

IFO² belongs to the group of oxazaphosphorine agents and is a structural analogue of CP. The only difference between IFO and CP is a shift of one chlorethyl group from the exocyclic nitrogen to the nitrogen of the oxazaphosphorine ring. This variation is responsible for the distinctive metabolism of the two drugs (1—3). Like CP, IFO is a prodrug that requires biotransformation by hepatic cytochrome P450 isoenzymes at the cyclic carbon 4 position before it can exert its cancerotoxic effects. The resulting active metabolite is 4-hydroxy-IFO. In contrast to cyclophosphamide, a second competitive pathway exists for IFO: side chain oxidation leads to the inactive metabolite 2-dechloroethyl-IFO and 3-dechloroethyl-IFO and liberates CAA (Fig. 1). IFO is reported to exert greater antitumor activities than CP in some experimental (4) and human (5—8) malignancies. It may even be active in CP-pretreated patients because there is only a partial cross-resistance between these two alkylants (6, 9). However, this is not generally accepted (10), because there is no prospective randomized trial comparing CP and IFO treatment. Furthermore, in the studies cited above, the scheduling of CP and IFO during the treatment courses was different (10). Although there are some differences in the metabolism and possibly also in the chemical reaction of the active alkylating metabolites, no molecular basis for a fundamentally different mode of action between these two alkylating agents has been found thus far.

The metabolite 4-hydroxy-IFO is considered to be responsible for the antitumor efficacy of IFO (1, 11), whereas the metabolite of side chain oxidation CAA may produce side-effects such as neurotoxicity (12, 13) and nephrotoxicity (14). Other than glutathione depletion (15—18), which may facilitate the attack of alkylating metabolites (18), no direct antitumor effect of CAA has been described to date.

The aim of this study was to investigate whether CAA, in addition to possessing toxic side-effects, has activity against tumor cells and may even contribute to the efficacy of IFO. Therefore, two human tumor cell lines were treated with different concentrations of CAA or 4-hydroxy-IFO which were in the range of those measured in 10 cancer patients after the administration of 5 g/m² IFO. With respect to the supposed nephrotoxic side effect of CAA, the activities of CAA and of 4-hydroxy-IFO on renal tubular cells were also examined. We demonstrate that CAA has its own cytotoxic profile and may contribute to the antitumor efficacy of IFO.

PATIENTS AND METHODS

Patients. The pharmacokinetics of the IFO metabolites 4-hydroxy-IFO and CAA were determined in a group of 10 patients with non-small cell lung cancer. Treatment consisted of a combination of 5 g/m² IFO, 300 mg/m² carboplatin, and 100 mg/m² etoposide. Mesna (single dose equal to 20% of total IFO dose) was given concomitantly with IFO at 0, 4, and 8 h after the start of IFO infusion. All patients gave their written consent prior to the initiation of therapy.

Pharmacokinetics. Blood samples (10 ml) were drawn at 0, 1, 1.5, 2, 3, 4, 8, and 24 h. For the determinations of 4-hydroxy-IFO and CAA, whole blood samples were processed immediately as described earlier (19). The areas under the concentration-time curve were calculated according to the trapezoidal rule by using the Topfit program (Version 2.0; Ref. 20).

Concentration versus Time Profiles in Vitro. The determination of 4-hydroxy-IFO and CAA values in vitro was performed with 4-hydroxy-IFO- or CAA-containing RPMI 1640. The initial drug concentration was adjusted to the IC₅₀ as determined in the MTT assays. Concentration-time products and half-lives were calculated with the program mentioned above.

Cell Lines and Cell Cultures. Three different human cell lines were used: the breast carcinoma cell line MX1, the thyroid gland sarcoma cell line S117 (both obtained from Deutsches Krebsforschungszentrum, Heidelberg, Germany), and the renal tubular cell line Landa Leiden SV40 TC (kindly provided by Dr. M. R. Daha (Academic Ziekenhuis Leiden, Leiden, the Netherlands; Refs. 21, 22)). The cell lines were cultured as subconfluent monolayers at 37°C in a humidified atmosphere with 5% CO₂. For the tumor cells, 80-cm² culture flasks (Nunclon, Wiesbaden-Biebrich, Germany) and RPMI 1640 supplemented with 10% FCS (Biochrom KG, Berlin, Germany) were used. The tumor cells were supplied with fresh medium or subcultured twice each week (23). For the renal tubular cell line, 250-ml culture flasks (Greiner, Frickenhausen, Germany) and DMEM/HAM’s F-12 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (Biochrom KG, Berlin, Germany), 0.5 ml of epidermal growth factor, 0.5 ml of insulin-transferrin sodium selenite, and 0.5 ml of hydrocortisone, 20 μg of 3,3',5'-triiodo-L-thyronine (all obtained from Sigma Chemical Co., Deisenhofen, Germany), 0.2% penicillin-streptomycin (Life Technologies, Inc., Paisley, Scotland) were used. The tumor cells were supplied with fresh medium daily and subcultured once each week.

Reagents. IFO (Aldrich Chemical Co., Steinheim, Germany) was dissolved in a 0.9% NaCl solution before use. 4-Hydroperoxy-IFO and IFO were kindly provided by J. Pohl (ASTA Medica AG, Frankfurt, Germany). After dissolution in medium, 4-hydroperoxy-IFO rapidly decomposes to 4-hydroxy-...
IFO CYTOTOXICITY: ROLE OF CAA

Fig. 1. Pathways for IFO metabolism. The prodrug IFO is enzymatically (CYP3A4) metabolized either to 4-hydroxy-IFO (path 1) or to 2-3-dechloro-IFO under liberation of CAA (path 2).

IFO without enzymatic involvement (17). The tetrazolium salt MTT (Sigma) was dissolved in aqua dest to 2 mg/ml, sterile filtered, and stored in 10-ml aliquots at −20°C until use. DMSO was obtained from Merck (Darmstadt, Germany).

MTT Assay. The cell survival was measured using the MTT assay. The MTT assay was first described in 1983 by Mosmann (24). Our experiments were performed with minor modifications as described previously (23). One hundred μl of a 5 × 10⁶ (S117/MX1) or 1 × 10⁶ (Landa Leiden) cells/ml cell suspension and 100 μl of a drug solution were added to each well of microtiter plates; 100 μl of cell suspension with 100 μl of medium served as a control. The plates were incubated for 3 (S117) or 4 (MX1/Landa Leiden) days at 37°C (4-hydroxy-IFO). For the CAA experiments, the medium was aspirated after an incubation time of 4 h. Fresh medium was added, and plates were incubated for another 3 (S117) or 4 (MX1/Landa Leiden) days at 37°C. For each drug concentration, at least 10 experiments were performed, each representing the average of three wells. The cell survival was expressed as a percentage.

Biometrics. For comparison of the results, the Mann-Whitney U test was used.

RESULTS

Pharmacokinetics. Pharmacokinetic data of 10 patients treated with the IFO, carboplatin, and etoposide schedule were determined for the assignment of the in vitro tests. Fig. 2 shows the concentration-time curves after the administration of 5 g/m² IFO. Peak levels and the AUC of CAA were 2–3-fold higher than the corresponding values of 4-hydroxy-IFO, whereas the time profiles of blood levels and the terminal half-lives were in a similar range.

Effects of CAA and 4-Hydroxy-IFO on Tumor Cells. As shown in Fig. 3, for the MX1 tumor, CAA at a concentration of 5 nmol/ml leads to a nearly complete reduction of MX1 cell survival during the 4-day incubation period. 4-hydroxy-IFO seems to be less effective at the same concentration tested on MX1 cells. However, due to the slow decomposition rate of CAA in vitro (half-life in RPMI, 20.2 h), this result is obviously an overestimation of the CAA cytotoxicity. To adapt the cell exposure with CAA to the in vivo conditions, the incubation time was reduced and the drug was washed out after 4 h. By this adjustment, CAA cytotoxicity decreased to values observed with 4-hydroxy-IFO. 4-Hydroxy-IFO has a short half-life of 1.3 h in vitro in the presence of FCS. Almost all of the cytotoxic effects should be exerted within the first four hours. Nevertheless, the cytotoxicity became lower if the drug was washed out after 4 h (Fig. 3). This may be due to a washout of reversible protein-bound 4-hydroxy-IFO. Furthermore, we have to consider that 4-hydroxy-IFO is not the ultimate alkylating metabolite and exerts no direct cytotoxic effect. Metabolites such as phosphoramidon mustard, which were not measured by us, may be still present at 4 h and removed by the washout procedure. Thus, in the following experiments, we incubated the cells with 4-hydroxy-IFO for the entire duration, whereas the direct cytotoxic and long lasting CAA was removed after 4 h.

Under these conditions, a close dose-response relationship for both metabolites against the MX1 and S117 tumor was found (Fig. 4), whereas the parent drug, IFO, was without any effect. The activity of CAA is at least as high as that of 4-hydroxy-IFO. In comparison to
Table 1: Comparison of IC_{50} and the corresponding concentration vs. time values for 4-hydroxy-IFO and CAA as determined with the MX1 and S117 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>4-hydroxy-IFO IC_{50} (µmol × 1^{-1})</th>
<th>CAA Concentration vs. time (µmol × h × 1^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX1</td>
<td>10.8</td>
<td>10.6</td>
</tr>
<tr>
<td>S117</td>
<td>25.0</td>
<td>50.2</td>
</tr>
</tbody>
</table>

The concentration vs. time products were measured in cell culture medium (see "Patients and Methods"). Initial concentrations of 4-hydroxy-IFO and CAA were adjusted to the corresponding IC_{50} values. As in the MTT assay for cell survival, experiments with CAA were stopped at 4 h.

MX1, the sarcoma cell line needs higher concentrations of both metabolites for the reduction of cell survival. Table 1 summarizes the results with regard to the IC_{50}s. For a better comparison of these data with the in vivo situation, the concentration versus time product (with the IC_{50} as initial concentration) was measured.

Because in the course of metabolism, CAA and 4-hydroxy-IFO are simultaneously present in the blood, the combined effect of both metabolites on tumor cell killing was investigated in addition. Fig. 5 shows that the combination results in an increased efficacy against MX1 tumor cells; this effect is approximately additive.

**Effects on Renal Tubulus Cells.** Although in the renal tubular cell line the cytochrome P450 isoenzyme 3A4 could be demonstrated by the alkaline phosphatase anti-alkaline phosphatase method (not shown), and thus this cell type should be able to activate IFO, the parent drug alone was not cytotoxic against the Landa Leiden cells (Fig. 6). As with the tumor cells, both metabolites exhibited a dose-dependent cytotoxic effect on the Landa Leiden cells. At a concentration of 25 nmol/ml both CAA and 4-hydroxy-IFO led to a nearly complete cell kill.

**DISCUSSION**

Although the oxazaphosphorine IFO seems to be a simple analogue of CP, a major difference with respect to their metabolic pattern exists (1, 3). In addition to the formation of the 4-hydroxy metabolite, side chain oxidation with liberation of CAA is a possible method of IFO metabolism (2, 19, 25). Whereas only small amounts of CAA are released in CP metabolism (14, 26), an up to 100-fold higher CAA formation was calculated for IFO metabolism (27). Between 15 and 50% (25, 28, 29) of a given IFO dose is metabolized in humans by this metabolic pathway. Therefore, side effects such as neuro- and nephrotoxicity, which were not observed in CP treatment, are supposed to be caused by the metabolite CAA (12, 30, 31). Nephrotoxicity of IFO became a limiting factor in high-dose chemotherapy and in some pediatric protocols concerning the long-term survival of children (14, 32). In our in vitro studies, CAA leads to a considerable reduction in cell survival of the renal tubular cell line Landa Leiden and all tested tumor cell lines. It is noteworthy that the toxic CAA concentrations are in the range of the CAA blood levels observed in vivo after a common therapeutic dose of IFO (see Fig. 2). Concerning different doses of IFO, CAA levels measured in the present study are consistent with previous results of our group (19) and comparable to CAA concentrations under continuous IFO infusion (33). On the other hand,
Goren et al. (12) reported considerably higher blood CAA concentrations of 100 μM and Cerny and Küpfer (34) reported CAA plasma levels of up to 210 μM. The latter CAA levels are far above the CAA concentrations needed to exert cytotoxic effects against renal and tumor cells in this study. In addition, these high CAA concentrations also exceeded values obtained for the stable dechloroethyl metabolites, nearly reaching concentrations achieved by the parent drug (19). Thus far, a conclusive explanation of the remarkable differences in the reported plasma or whole blood concentrations of CAA is not possible (3).

Although 4-hydroxy-IFO causes toxic effects equivalent to those of CAA on the renal cells, we need to take into account that the blood levels and the AUC of 4-hydroxy-IFO measured in patients are considerably lower than those of CAA. Our data are in accordance with results of Mohrmann et al. (35), who demonstrated cell damage with protein loss and an inhibition of thymidine incorporation into renal tubular cells by CAA. Furthermore, a renal proximal tubule dysfunction was observed by Zamlauski-Tucker et al. (36) following the perfusion of isolated rat kidneys with CAA. All of these results support the assumption that CAA may be at least in part responsible for the nephrotoxic side effect of IFO treatment. Additionally, due to the presence of the IFO-activating isoenzyme CYP 3A4 in the proximal renal tubule (37), these cells should also be able to generate small amounts of CAA and 4-hydroxy-IFO, which might act as a suicide mechanism.

Despite the fact that CAA can also act as an alkyllating agent inducing interstrand cross-links (38) and inhibiting DNA synthesis (39), different molecular mechanisms of this chemically reactive compound are known to cause cytotoxic effects (40). Thus, CAA leads to a glutathione depletion of several cell types in vitro and in vivo (16, 17). The glutathione depletion could be caused by the formation of irreversible conjugates with CAA, and such glutathione-depleted cells are more susceptible to the CAA toxicity (40).

Glutathione depletion also facilitates the attack of the alkyllating metabolites in highly proliferating normal cells and tumor cells as well (15, 41). However, no direct antitumor effect of CAA has been described thus far. On the other hand, distinct, specific reactions of CAA, which might also cause a cell kill, have been found. CAA leads to an inhibition of mitochondrial respiration, ATP depletion, and induction of damaging lipid peroxidation (40). Therefore, we concluded that CAA would also exhibit a remarkable direct toxic effect against tumor cells. Indeed, as with 4-hydroxy-IFO, a similar and also dose-dependent cell kill of MX1 and S117 tumor cells by CAA in cell culture was found. Due to the differences of decomposition rates in vitro or stability of CAA and 4-hydroxy-IFO, it was difficult to simulate in vivo conditions, and a direct comparison of the efficacy of both metabolites may be crucial. In this context, we also have to consider that in vivo intratumoral concentrations of the active IFO metabolites could be significantly lower than in blood. This has been previously demonstrated by us with the MX1 tumor in the nude mouse model. Concentrations of 4-hydroxy-IFO measured during the whole course of metabolism in the xenograft tumor were approximately one-third of the corresponding blood values (23). Nevertheless, even treating the less chemosensitive sarcoma cell line S117, we could demonstrate that the CAA-IC50 and the corresponding cell exposure within the incubation time (AUC) were below the peak levels of CAA and the AUC measured in human pharmacokinetics after administering 5 g/m² IFO (for comparison, see Table 1 and Fig. 2).

Because simultaneous treatment of the cells with 4-hydroxy-IFO and CAA resulted in an increased cytotoxicity, our results emphasize a cytotoxic action of CAA in the presence of 4-hydroxy-IFO and indicate a combined effect without negative interaction of both metabolites. Thus, despite different in vivo and in vitro conditions, our results give strong evidence that CAA contributes to the cytotoxic action of IFO, probably by an independent molecular mechanism.

Interestingly, in a study of Boddy et al. (42), it was reported that in breast cancer patients after IFO treatment, the AUC and urine recovery of dechloroethylated metabolites correlated positively with survival, whereas this was not the case with the AUC of isophosphamide mustard. The authors concluded that the results, although far from conclusive, give some insight into a possible mechanism of action of IFO and that some other species other than isophosphamide mustard, as measured systemically, is responsible for the pharmacological effects of this drug. Regarding our results and the fact that a high AUC and urine recovery of the dechloroethylated metabolites should be accompanied by an equally high CAA liberation, in our opinion CAA is this candidate.

The question has to be raised whether CAA toxicity is nonspecific or whether it exhibits a relative tumor-specific action sparing critical normal tissues, such as bone marrow progenitor cells. It is well known that aldophosphamide, an intermediate of CP metabolism on the way to the ultimate alkylating metabolite phosphoramide mustard, can be oxidized to the inactive carboxyphosphamide by aldehydedehydrogenases (1). Several studies (43–50) have shown that aldehydedehydrogenase content is an important determinant of cell sensitivity against CP toxicity. Certain human tissues with high aldehydedehydrogenase activity (e.g., CD34 + progenitor cells; Ref. 50) were less influenced by CP, and also some resistant tumor cell lines showed elevated aldehydedehydrogenase activity (46, 47, 51). Because the urinary excretion of carboxy-IFO is very low when compared with CP metabolism (52–54), this metabolic pathway of deactivation is obviously not of importance for IFO metabolism. On the contrary, as with acetaldehyde, CAA should be oxidized to chloroacetic acid by aldehydedehydrogenase, which does not generally imply deactivation. The formation of chloroacetyl-CoA from chloroacetic acid may disturb CoA-dependent mechanisms by "lethal synthesis" (55) as it was also described for fluoroacetic acid (56). However, it should be noted that 30-fold higher concentrations of CAA (0.3 mM), as in our study with tubular renal cells and tumor cells, were required to induce a 50% cell kill in hepatocytes (40). This may be due to the higher concentration of aldehydedehydrogenase in the liver when compared with the kidney and several tumor cell lines. Other cell type-dependent differences in the metabolic inactivation of CAA may exist or predominate. To get a better view of the potential clinical applications of our findings we started investigations on nude mice with MX1 and S117 xenografts.

In summary, CAA has its own cytotoxic profile against tumor cells and may contribute to the therapeutic efficacy of IFO. CAA may also act in nonproliferating cells with the mitochondria as its target. Thus, the molecular mechanism of action of IFO seems to be different from CP, only part of which is an alkyllating effect on DNA.

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