METABOLISM OF IFOSFAMIDE TO CHLOROACETALDEHYDE CONTRIBUTES TO ANTITUMOR ACTIVITY IN VIVO

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ABSTRACT:

Metabolic activation of ifosfamide (IFO) leads to the active 4-hydroxy-metabolite and to a substantial liberation of chloroacetaldehyde (CAA). CAA has been presumed responsible for side effects of IFO. We recently have shown cytotoxic effects of CAA against human tumor cells in vitro. The aim of this study was to demonstrate antitumor effects of CAA in vivo, and to compare its potency to 4-OH-IFO. Pharmacokinetics of IFO and metabolites were evaluated after infusion of 250 mg/kg IFO in mice. The area under the curve (AUC) for 4-hydroxyifosfamide (4-OH-IFO) and CAA were 138.5 and 102.4 μmol·h/liter, respectively. To compare pharmacokinetics and antitumor effects, the mice received isolated infusion of 4-OH-IFO or CAA in equimolar doses to IFO. Administration of 4-OH-IFO yielded AUC values comparable with those obtained after administration of the parent drug. In contrast, infusion of isolated CAA via tail vein gave a low AUC value of 51.5 μmol·h/liter due to slow flow in the tail vein and rapid degradation. Administration of the parent drug gave highly cytotoxic intratumoral peak concentrations of 25 and 12 μmol/kg tumor weight for 4-OH-IFO and CAA in MX1 xenotransplanted nude mice. Both IFO and isolated 4-OH-IFO led to complete remissions. Administration of isolated CAA (75 mg/kg) delayed tumor growth significantly. The equitoxic dose of isolated 4-OH-IFO was 40 mg/kg. On a molar basis CAA was seven times less potent as 4-OH-IFO. However, on the basis of achieved AUC values, CAA seems to exhibit a similar antitumor activity to 4-OH-IFO.

Ifosfamide (IFO)1 belongs to the group of oxazaphosphorine cytostatics and is a structural analog of cyclophosphamide from which it differs only in the position of one chloroethyl group (Wagner, 1994). This difference leads to quantitative differences in their hepatic biotransformation (Wagner et al., 1981; Sladek, 1988; Kaijser et al., 1994). Although cyclophosphamide is almost completely converted to its active 4-hydroxy-metabolite in humans, up to 50% of IFO is metabolized to chloroacetaldehyde (CAA) by a second pathway (Fig. 1). To date, CAA has been presumed to be responsible for the side effects of IFO such as neuro- (Goren et al., 1986; DiMaggio et al., 1994) and nephrotoxicity (Skinner et al., 1993). Brain et al. (1998) recently proposed modulation of the different liver cytochrome P450 isoenzymes shifting IFO metabolism from CAA to 4-hydroxy-ifosfamide (4-OH-IFO) to decrease CAA levels. However, recently we have demonstrated significant in vitro cytotoxicity of CAA against two human tumor cell lines (MX1, S117) using clinically relevant concentrations (Brüggemann et al., 1997). Generation of CAA may explain in part the clinically observed lack of complete cross-resistance between cyclophosphamide and IFO and the higher remission rates achievable by IFO in certain tumor types (Brade et al., 1985; Bramwell et al., 1987). Moreover, other factors like the greater cross-linking arm length of IFO and its longer half-life may be contributing to this difference (Kamen et al., 1995).

The aim of this study was to show an antitumor effect of CAA in vivo and to further elucidate its contribution to the cytotoxic effect of IFO. We present data of pharmacological studies and tumoricidal effects on nude mice bearing human MX1 xenografts treated with either IFO or isolated 4-OH-IFO or isolated CAA.

Materials and Methods

Animals. NMRI mice (Charles River, Sulzfeld, Germany) were used for pharmacokinetic data and immunodeficient nude nu-nu mice (Bomholm, RV Denmark) with human MX1 xenografts for treatment experiments. All procedures have been carried out in accordance with the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education (New York Academy of Sciences, New York, NY).

Tumor Transplantation and Evaluation. The human undifferentiated breast carcinoma MX1 cell line (Deutsches Krebsforschungszentrum, Heidelberg, Germany) was used for xenotransplants. Tumor pieces of 1 × 1 mm³ were implanted s.c. into the dorsum of the right hind paw of mice aged 5 to 6 weeks as described by Wiedemann et al. (1993). Tumors of 180 ± 20 mm³ had developed after 2 to 3 weeks. Only mice with solid and geometrically well defined tumors without necroses were used for experiments. Tumor growth was recorded 2 times per week, measuring the tumor dimensions [height (A), length (B), and width (C)] with venier calipers and the tumor volume was calculated using the formula (π/6) × A × B × C.

Drug Administration. IFO and 4-OH-IFO were a generous gift of J. Pohl, ASTA Medica AG, Frankfurt, Germany. CAA was obtained from Sigma-Aldrich, Steinheim, Germany. Mice were anesthetized by i.p. administration of ketamine (Rompun; Bayer, Leverkusen, Germany) and pentobarbital-sodium (Sanofi Ceva, Hannover, Germany). IFO was administered as an i.v. bolus, cross-linking arm length of IFO and its longer half-life may be contributing to this difference (Kamen et al., 1995).

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4-OH-IFO and CAA were infused for 150 min via tail vein. During drug administration, the tumor-bearing extremity was kept at 37 ± 0.1°C by local water bath as described earlier (Wiedemann et al., 1993). The control mice received identical infusion volumes of isotonic saline.

**Blood Samples for Pharmacokinetic Data.** Blood samples were drawn from the retrobulbar venous plexus into heparin-coated 10-μl capillary vessels at 5, 10, 15, 20, 40, 60, 90, and 120 min for IFO and 0, 15, 30, 60, 90, and 120 min for CAA and 4-OH-IFO after IFO administration or 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 158, and 165 min after CAA and 4-OH-IFO administration for pharmacokinetic analysis.

**Tumor Preparation for the Measurement of Tissue Drug Levels.** After drug administration (15 and 120 min) the tumors of respective mice were excised with a pair of razor blade-armed liquid nitrogen-cooled tongs. MX1 tumors shock-frozen with liquid nitrogen were ground in a mortar and stored in liquid nitrogen (Wiedemann et al., 1993). For CAA and 4-OH-IFO assays, the tumor tissues were handled the same way as the blood samples.

**IFO Assay.** For IFO determination, the blood samples were centrifuged and the plasma spiked with an internal standard solution containing the oxazaphosphorine derivative trofosfamide. After extraction with dichloromethane, IFO was measured by nitrogen/phosphor flame-ionization gas chromatography, N2 serving as carrier gas (for details, see Kurowski and Wagner, 1993).

**CAA Assay.** To determine CAA concentrations blood samples were rapidly mixed with 100 μl of formaldehyde solution 3.7%. The mixture was shaken vigorously, resulting in the lysis of erythrocytes and other blood cells and centrifuged. Supernatant (80 μl) was mixed with 8 μl of the internal standard (1 μl of chloroform in 100 μl of methanol). The samples were mixed and 2 μl were injected into the gas chromatography system with an electron capture detector, a capillary column (Hewlett-Packard 5, cross-linked 5% phenylmethylsilicone) and helium serving as carrier gas (for details, see Kurowski and Wagner, 1993).

**4-OH-IFO Assay.** For determination of 4-OH-IFO, blood samples were deproteinized with 1000 μl of ice-cold trichloroacetic acid 5% and shaken vigorously. After centrifugation, 500 μl of supernatant containing 4-OH-IFO were added to 600 μl of 3-aminophenol. Samples were kept at 95°C for 20 min. Acrolein, originally present or released during incubation, reacted under acidic conditions with 3-aminophenol, forming the fluorescent 7-hydroxyquinoline (Alarcon, 1968). To eliminate interfering fluorescence and to increase the sensitivity of this assay, the acrolein-derived 7-hydroxyquinoline was extracted from the derivatization mixture and subsequently quantified by high performance liquid chromatography with fluorescence detection (for details, see Kurowski and Wagner, 1993).

**Calculation and Statistical Evaluation.** The areas under the curve (AUCs) and apparent half-lives of IFO, 4-OH-IFO, and CAA were calculated from the concentration-time profiles using the trapezoidal rule (TOPFIT version 2.0; Heinzel et al., 1993). Significance in tumor growth delay was calculated with the Mann-Whitney U-test.

**Results**

**Pharmacokinetics of IFO, 4-OH-IFO, and CAA after IFO Administration.** The pharmacokinetics of IFO are shown in Fig. 2a. The elimination profile of IFO followed a first order kinetic. Whereas concentration-time curves of CAA and 4-OH-IFO were similar, peak concentrations and AUC were higher for 4-OH-IFO (Fig. 2b). Measured half-lives of 4-OH-IFO and CAA after IFO bolus injection were mainly dependent on the long half-life of their parent drug (Fig. 2). Concentrations of 4-OH-IFO and CAA were measured in blood and in the MX1 tumors at 15 and 120 min after IFO bolus injection to investigate whether both metabolites reach relevant cytotoxic intratumoral levels (Fig. 3, a and b). At the time of blood peak levels (15 min) intratumoral concentrations of 4-OH-IFO and CAA were approximately 30% of the corresponding blood concentrations. At 120 min, there was only a small difference between blood and tumor tissue drug concentrations.

**Pharmacokinetics of 4-OH-IFO and CAA after Isolated Infusion.** Mice were treated with 4-OH-IFO and CAA in equimolar doses to 250 mg/kg IFO (280 mg/kg 4-OH-IFO, 75 mg/kg CAA) to compare the isolated effects. Half-lives of CAA and 4-OH-IFO are in a range of 4 and 10 min, respectively. However, the exact half-lives could not be calculated because it was not possible to draw more blood samples from the retrobulbar plexus within this short period of time. Due to these short half-lives, it was necessary to administer both metabolites by infusion to simulate AUC values observed after administration of the parent drug (Fig. 4). We chose an infusion time of 150 min for both metabolites because after IFO bolus injection AUC$_{0\rightarrow150}$ of both CAA and 4-OH-IFO reached approximately 80% of AUC$_{0\rightarrow\infty}$. An unexpected drop in values was observed after 75 min. A possible explanation might be the reinjection of xylazine/pentobarbital-sodium, which preceded this drop by approximately 10 to 15 min. Whereas the AUC of 4-OH-IFO (280 mg/kg) was 25% higher after isolated infusion as compared with its AUC after IFO bolus injection, the AUC of CAA was halved (Table 1).

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**Fig. 2. Pharmacokinetics of IFO (•, n = 10, a), 4-OH-IFO (■, n = 5), and CAA (△, n = 6, b) after administration of 250 mg/kg IFO.**
Antitumor Effect on MX1 Xenografts. MX1 xenografted nude mice were treated with 250 mg/kg IFO (Fig. 5). In control animals, tumor size increased until day 30. Tumors in animals treated with IFO shrank, and complete remissions were achieved in three of six animals lasting throughout the observation period of 30 days.

To test therapeutic implications of pharmacokinetic results, MX1 xenografted nude mice were treated with 280 mg/kg 4-OH-IFO or 75 mg/kg CAA, which are equimolar doses to 250 mg/kg IFO. Isolated infusion of 4-OH-IFO over 150 min at day 1 led to complete remission in five of seven animals. CAA treatment did not achieve a remission but only a growth delay that reached significance in comparison with the control group (Fig. 5). The time to endpoint was 30 days for both the treated mice and the controls. The tumor size at day 30 was 36.6 ± 35.6 mm³ for 280 mg/kg 4-OH-IFO, 841.2 ± 71.3 mm³ for 75 mg/kg CAA, and 1289.1 ± 165.0 mm³ for the control group.

It was not possible to increase the dose of isolated CAA to a level that would yield an AUC comparable with that of 4-OH-IFO due to frequent local irritation of the tail vein endothelium and subsequent extravasation with higher concentrated CAA infusion.

To compare the relative antitumor potencies of CAA and 4-OH-IFO with respect to the achieved AUC, we tried to establish the dose of 4-OH-IFO with equitoxic effects to 75 mg/kg CAA on MX1 xenografts. Dose-response experiments (data not shown) yielded equivalent antitumor effects of 40 mg/kg 4-OH-IFO as compared with 75 mg/kg CAA (Fig. 6). At these dose levels the measured AUC of 4-OH-IFO was 1.4-fold higher than the AUC of CAA (Table 1). The endpoint tumor size for 40 mg/kg 4-OH-IFO was 812.0 ± 121.3 mm³.

Discussion
IFO is metabolized in vivo to the active 4-OH-IFO and to CAA. The latter is considered to be responsible in part for unwanted nephro- and neurotoxic side effects (Skinner et al., 1993; DiMaggio et al., 1994). The subject of the contribution of CAA to the antitumor efficacy of IFO was never directly addressed. This is somewhat astonishing with regard to demonstrated alkylating properties of CAA in mutagenesis and carcinogenesis studies (Singer et al., 1984; Spengler and Singer, 1988; Kuchenmeister et al., 1998). We first showed a strong in vitro cytotoxicity of CAA against human tumor cells in a concentration range equivalent to values measured in patients treated with 5 mg/m² IFO (Brüggemann et al., 1997). This study expands this observation to the in vivo situation in a mouse model.

The pharmacokinetic results show that IFO metabolism yields systematically detectable amounts of CAA in mice, which mimics the metabolic pattern in human cancer patients. Therefore mice may serve as a comparable experimental model, simulating IFO pharmacokinetics in humans. Because the drug concentrations in tumors could

<table>
<thead>
<tr>
<th>After IFO Administration</th>
<th>AUC</th>
<th>After Isolated Infusion of</th>
<th>AUC</th>
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<tbody>
<tr>
<td>IFO</td>
<td>1300.5 ± 439.1</td>
<td>4-OH-IFO (280 mg/kg)</td>
<td>173.6 ± 55.0</td>
</tr>
<tr>
<td>4-OH-IFO</td>
<td>138.5 ± 31.5</td>
<td>4-OH-IFO (40 mg/kg)</td>
<td>72.5 ± 11.7</td>
</tr>
<tr>
<td>CAA</td>
<td>102.4 ± 35.1</td>
<td>CAA (75 mg/kg)</td>
<td>51.5 ± 11.2</td>
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**FIG. 3.** Comparison of CAA (a, n = 3) and 4-OH-IFO concentrations (b, n = 3) in blood and tumor tissue at 15 and 120 min after administration of 250 mg/kg IFO.

**FIG. 4.** Pharmacokinetics after isolated infusion of 280 mg/kg 4-OH-IFO (a, n = 7) and 75 mg/kg CAA (b, n = 6) over 150 min.

**FIG. 5.** Tumor volume of MX1 xenografts in nude mice after treatment with 250 mg/kg IFO ( ), 75 mg/kg CAA ( ), or 280 mg/kg 4-OH-IFO ( ) in comparison with controls ( ); n = 8). *P < .05 (i.v. administration at day 1).
substantially differ from those in the blood, it was important to measure whether CAA levels reached significant intratumoral cytotoxicity. Intratumoral levels of CAA reached one-third of the blood peak levels, in a range which was cytotoxic to MX1 cells in vitro in our previous studies (Brüggemann et al., 1997). To investigate whether these intratumoral CAA levels exert antitumor effects in vivo, thus contributing to the antitumor effect of IFO, we mimicked the pharmacokinetics of 4-OH-IFO and CAA and studied their cytotoxic effects after isolated infusion. 4-OH-IFO reached higher AUC values after isolated infusion than after IFO bolus injection. Treatment of the MX1 xenografted mice led to complete remissions. This observation confirms the common opinion that 4-OH-IFO possesses a high antitumor efficacy (Connors et al., 1974; Sladek, 1988).

In contrast, it was not possible to achieve an AUC of CAA after an isolated infusion equivalent to that observed after IFO administration. This may be due to the slow blood flow in the tail vein downstream from the puncture needle and to a very short half-life of this highly reactive metabolite CAA in blood. To precisely mimic the situation of CAA after the puncture needle and to a very short half-life of this highly reactive metabolite CAA in blood. To precisely mimic the situation of CAA after an isolated infusion, we used a central i.v. infusion could be realized in comparison to the AUC of CAA after isolated infusion. 4-OH-IFO reached higher AUC values after isolated infusion than after IFO bolus injection. Treatment of the MX1 xenografted mice led to complete remissions. This observation confirms the common opinion that 4-OH-IFO possesses a high antitumor efficacy (Connors et al., 1974; Sladek, 1988).

To compare the antitumoral potencies of 4-OH-IFO and CAA with respect to the achieved AUC values by isolated infusion, we titrated 4-OH-IFO dosages in the treatment experiments to find an equitoxic dose. 4-OH-IFO (40 mg/kg) demonstrated a similar growth inhibition of MX1 xenografts as 75 mg/kg CAA (Fig. 6). Hence, compared on a molar basis, 4-OH-IFO is seven times as potent as CAA in this in vivo experiment. These results are contrary to our previous in vitro findings where CAA was at least as cytotoxic against tested tumor cells as 4-OH-IFO on a molar basis (Brüggemann et al., 1997). A possible explanation for this discrepancy might be the fact that in our experimental setting only a very low AUC of CAA after isolated infusion could be realized in comparison to the AUC of CAA after metabolism of an equimolar dose of the parent drug IFO. If we compare the cytotoxicity of 4-OH-IFO and CAA on the basis of achieved AUC values we assume that the contribution of CAA to the antitumor efficacy of IFO is much higher. This view is further supported by alternative interpretation of the data of Yu et al. (1999). These authors treated 9L gliosarcoma tumor-bearing rats with cyclophosphamide alone or in combination with both the P-450-3A-specific inhibitor troleandomycin and the P-450-2B inducer phenobarbital to suppress the generation of CAA and to stimulate the production of 4-hydroxycyclophosphamide. By doubling the plasma AUC of 4-hydroxycyclophosphamide, no improvement of the antitumor efficacy was observed. Yu et al. (1999) found no stringent explanation for this surprising result. We propose that the lack of antitumoriciud improvement in spite of higher 4-hydroxycyclophosphamide levels might be due to a 50% decrease of CAA with a consequent loss of CAA contribution to the antitumor cytotoxicity.

The cytotoxic mode of action of CAA inhibiting tumor growth is still unclear. CAA can act as an alkylating agent, producing interstrand cross-links and strand breaks in DNA (Spengler and Singer, 1988). Additionally, CAA was shown to inhibit mitochondrial function blocking the oxidative phosphorylation (Sood and O’Brien, 1993). Whether the in vivo cytotoxicity of CAA in MX1 tumor cells is a result of DNA alkylation or possibly inhibition of oxidative metabolism in mitochondria remains unclear and will be the object of additional experiments.

In conclusion, CAA plays a role in antitumor efficacy of IFO and is not only responsible for side effects of IFO. Generation of CAA may explain differing antitumoral activities of IFO and cyclophosphamide and also the lack of complete cross-resistance between these drugs. Suppression of CAA metabolic pathway (Brain et al., 1998), although it may be beneficial as a means of reducing neurotoxic responses, may also be associated with a reduction in antitumor effect.

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