Effects of Temperature on the Therapeutic Efficacy and Pharmacokinetics of Ifosfamide

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ABSTRACT

The influence of tumor temperature (28, 32, 37, 39, 41, or 43°C for 1 h) on the therapeutic efficacy of i.v. single bolus injections of ifosfamide (IFO) (32, 65, 125, or 250 mg/kg body weight) in human tumor xenografts (MX1 breast carcinoma) grown in nude mice (n = 240) was studied. Tumor temperature was controlled by water bath immersion. Sixty days after treatment the percentage of tumor-free survival was determined. For example, at 37°C IFO in a dose of 65 mg/kg body weight led to 10% tumor-free survival in the treated animals. At 43°C the same dose resulted in 60% tumor-free survival. A clear drug dose- and temperature-dependent increase of the therapeutic efficacy of an active oxazaphosphorine compound was also demonstrated in vitro. The concentrations of IFO and of 4-hydroxyifosfamide in blood and at different body temperatures (controlled by water bath immersion) were determined over 120 min and WBC counts were obtained. The half-lives and the areas under the curve for IFO in blood were not significantly different at 37°C and 41°C. Since the half-life of IFO depends mainly on hepatic metabolism, the similarity of half-lives and of areas under the curve for IFO at 37°C and 41°C indicates a constant activation rate. However, significantly lower plasma concentrations of the activated drug at a liver (body) temperature of 41°C, compared with 37°C, were found, indicating a higher elimination rate. The concentration of the activated drug in the tumors within the first 60 min at 41°C, however, exceeded by >2-fold that at 37°C. The bone marrow toxicity of the same drug dose did not significantly increase with body temperature.

INTRODUCTION

A broad range of human tumors respond to the oxazaphosphorine compound IFO, an analogue of the well established alkylating agent CP. These include malignant non-Hodgkin lymphoma, sarcoma, teratoma, and carcinoma of the bronchus (1-3). IFO, like CP, is a prodrug which undergoes complex metabolism in vivo (4, 5). The initial metabolism of IFO consists of two different pathways; firstly, an enzymatic hydroxylation at carbon-4 forms 4-OH-IFO, which is probably the major biologically active alkylating compound derived from IFO, and, secondly, a side-chain oxidation leads to the formation of chloroacetaldehyde, a compound with possible neurotoxic properties (6). Chloroacetaldehyde is also demonstrated in vitro. The concentrations of IFO and 4-hydroxyifosfamide in blood and at different body temperatures (controlled by water bath immersion) were determined over 120 min and WBC counts were obtained. The half-lives and the areas under the curve for IFO in blood were not significantly different at 37°C and 41°C. Since the half-life of IFO depends mainly on hepatic metabolism, the similarity of half-lives and of areas under the curve for IFO at 37°C and 41°C indicates a constant activation rate. However, significantly lower plasma concentrations of the activated drug at a liver (body) temperature of 41°C, compared with 37°C, were found, indicating a higher elimination rate. The concentration of the activated drug in the tumors within the first 60 min at 41°C, however, exceeded by >2-fold that at 37°C. The bone marrow toxicity of the same drug dose did not significantly increase with body temperature.

Enhanced therapeutic efficacy resulting from combining locoregional hyperthermia with IFO in cancer patients (9) and in human tumor xenografts (10) was observed previously. To date, the cause of the enhanced therapeutic efficacy of oxazaphosphorine alkylating agents at elevated tumor temperature is poorly understood. Waterman et al. (11), with tumor patients, and our group, with xenotransplanted nude mice (10-13), found no indication for a shutdown of tumor circulation under hyperthermia. Obviously, the observed increased cytotoxicity of thermochemotherapy cannot be explained by reductions of the nutritive blood flow, as has frequently been suggested in the past (14, 15). Possible reasons for the increased cytotoxicity of oxazaphosphorine alkylating agents during hyperthermia in vivo may be greater drug uptake into the tumor and higher alkylating rates of the activated drug.

To obtain more information on the pharmacokinetics of IFO, in the present preclinical study we determined the concentrations of IFO and of activated IFO (4-OH-IFO) in blood and in tumors following i.v. application of IFO in nude mice at different rectal temperatures. In addition, the rate of tumor-free survival of nude mice carrying human MX1 breast carcinoma was determined following treatment with IFO at different tumor temperatures.

MATERIALS AND METHODS

Cell Line and Culture. For in vitro studies the human breast cancer cell line MX1 was used (obtained from Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cell line was cultured as subconfluent monolayers, in 80-cm² culture flasks, at 37°C in a humidified atmosphere with 5% CO₂ (Nunclon). The medium used was RPMI 1640 medium (Boehringer, Mannheim, Germany) supplemented with 2 mm L-glutamine, 10% fetal calf serum (Boehringer), 100 IU/liter penicillin, and 100 µg/liter streptomycin (Boehringer). The cells were fed or subcultured twice each week.

Reagents. MTT (Sigma) was dissolved in 0.9% NaCl solution to 2 mg/ml, sterilized filtered, and stored in aliquots at −20°C until use. Dimethylsulfoxide was purchased from Merck (Darmstadt, Germany). The cyclohexylamine salt of MAF (4-hydroxycyclophosphamide), an active derivative of cyclophosphamide kindly provided by ASTA Medica AG (Frankfurt, Germany), was dissolved in RPMI 1640 medium. Drug solution was freshly prepared for every experiment.

MTT Assay. Single-cell suspensions were obtained by trypsinization of the monolayer cell cultures in the exponential growth phase. Cell counts were performed using a hemocytometer (Sysmex X 1000; Digitana AG). The MTT assay, originally developed by Mosman (16), was performed as described by Carmichael et al. (17) with some minor modifications (18, 19).

With the assay the number of viable cells is indirectly determined by measuring the conversion of the substrate MTT to a violet water-insoluble formazan product, which can be measured spectrophotometrically. The amount of formazan is directly proportional to the number of viable cells.

A linear relationship between cell number and MTT formazan crystal formation was found. The relationship between seeding density, incubation volume, and incubation period was established for the cell line after growth studies; 5000 cells were seeded in each 200-µl well. An incubation time of 4 days after thermochemotherapy was chosen for the experiments. Assays were performed using a 1-h exposure to drug and/or hyperthermia, as in the in vivo experiments.

MAF and cells were mixed in 1.5-ml test tubes (Save-lock; Eppendorf, Hamburg, Germany) directly after solubilization of MAF. Four different drug concentrations and a control (cells without the drug) were used, covering a concentration range from 1 µg/ml to 20 µg/ml, thus including the 50% inhibiting concentration that had been determined by preliminary assays.

The test tubes were incubated for 60 min in a precision-controlled circulating water bath at 37 ± 0.1°C or 42 ± 0.1°C. Cells were centrifuged at 1400 g for 3 min, resuspended in RPMI 1640 medium, and then plated onto flat-bottomed microtiter plates (Nunclon). After 4 days of culture at 37°C, 25 µl of MTT (2 mg/ml) were added to each well and cells were incubated at 37°C for an additional 4 h. The medium was then aspirated from the plates, leaving about 20 µl of medium in each well. Care was taken not to disturb the formazan crystals at the bottom of the wells. The formazan crystals were solubilized by adding 100 µl of 100% dimethylsulfoxide to each well. Imme-
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Diately after resolubilization, all plates were scanned at 540 nm on a scanning multiwell spectrophotometer (Titerrek Multiscan Plus; Flow, Irvine, UK). For each drug concentration and temperature at least 11 experiments were performed, each presenting the average of a minimum of six wells. The cell survival or fractional absorbance at 37°C was calculated by the formula: mean of test samples/mean of untreated samples.

Animals. Female athymic (nude) NMRI mice (5–6 weeks of age; Zentralinstitut für Versuchstierzucht, Hannover, Germany) were maintained in laminar-flow installations. The mice had free access to pelleted food (special nude mouse diet) and drinking water (HCl added to pH 2.5, 1.35 g/liter potassium sorbate). Room temperature was kept at 24 ± 1°C, with a relative humidity of 70%. Makrolon size 2 cages were used, holding up to six mice each.

Tumors. The antitumor effect of thermochemotherapy with IFO was studied on human-derived MX1 breast carcinoma (obtained from Deutsches Krebsforschungszentrum, Heidelberg, Germany) grown s.c. by serial passage in nude mice. The MX1 tumor line has been included in the drug-screening panel at the Max-Planck-Forschungszentrum, Heidelberg, Germany) grown s.c. by serial passage in nude mice. The MX1 tumor line has been included in the drug-screening panel of the National Cancer Institute since 1972. The human origin of the tumor was confirmed by immunohistochemical methods (i.e., labeling with Ki-1).

Tumor pieces for implantation were obtained from donor mice carrying MX1 tumors s.c. in the neck region. The tumors were excised and cut into standardized pieces of 1–2-mm diameter, which were implanted s.c. into the dorsum of the right hind paw of mice of age 6–7 weeks. After 2 weeks tumors became measurable. When the tumors had attained a volume of approximately 200 mm³ (mean volume, 190 ± 15 mm³; only mice with geometrically well-defined tumors were used), the mice were distributed according to tumor size and randomly assigned to one of the treatment or control groups, each comprising 10 mice.

The median tumor diameter at the beginning of therapy was 8 mm. Tumor growth was recorded 2 or 3 times per week by two-dimensional measurement with vernier callipers. Individual tumor volumes were calculated by the modified ellipsoid formula, 4/3πAB², where A is the longest and B the shortest perpendicular axis of an assumed ellipsoid. As shown previously (10, 13), untreated MX1 tumors demonstrated rapid exponential growth.

For the experiments, 10–12-week-old mice of 26–32 g body weight were used. In the MX1 tumor, hormone receptor assays showed no significant estrogen (<2 fmol/mg) and no significant progesterone (6 fmol/mg) receptor protein. Therefore, no estrogen was supplemented.

Drug Administration. IFO (Asta Medica, Frankfurt, Germany) was dissolved in sterile water and injected i.v. (32, 65, 125, or 250 mg/kg body weight) via the tail vein 6–4 min before the tumor was warmed.

Locoregional/Whole-Body Hyperthermia. Prior to treatment, all mice were anesthetized with pentobarbital (0.4–0.5 ml of a 5 mg/ml solution) by i.p. injection. The anesthetized animals were fixed on a flat perspex holder (Fig. 1), and the tumor-carrying hind paw (local hyperthermia) or the entire mouse (whole-body hyperthermia) was immersed up to the hip or the neck, respectively, in a mechanically agitated, thermostatically controlled, water bath (Julabo V; Julabo, Saalbach, Germany) with a temperature accuracy of ±0.1°C.

In the experiments with local water bath hyperthermia for the measurements of tumor temperature, a chromel-alumel microthermocouple (type K1/2; Philips, Munich, Germany) with a diameter of 250 µm was inserted into the tumor center. The signal from the thermocouple was fed into an electronically ice-point-compensated digital thermometer circuit (type 871A; Keithley). When the temperature-sensitive tip of the thermoprobe was placed at different depths in the tumor tissue, from the periphery to the center, no temperature differences exceeding 0.3°C were observed. The rectal temperature was continuously measured with another semi-micro thermocouple inserted into the rectum and, by adjustment of the location of an IR lamp, kept at 37 ± 0.5°C. In all whole-body hyperthermia experiments the rectal temperature of the animals and the tumor temperature were continuously measured.

Tumor Preparation for Pharmacokinetic Studies. Anesthetized mice were taken 5, 10, 15, 20, 40, 60, 90, and 120 min after i.v. application of IFO, and the tumors were immediately extirpated with a pair of razor blade-armed tongs cooled in liquid nitrogen.

MX1 tumors shock-frozen with liquid nitrogen were obtained 5, 10, 15, 20, 40, 60, 90, and 100 min after the i.v. application of IFO (n = 6 mice at each time point). The tumors were ground in a mortar and stored in liquid nitrogen. For the IFO assay tumor tissue was handled the same way as blood samples. For the activated IFO assay aliquots of about 5 mg of tumor tissue were extracted with 1 ml of 5% trichloroacetic acid by homogenization with a sonicator. The IFO concentration in tumor tissue is given in nmol/g tumor fresh weight. The IFO concentration in blood is given in nmol/ml blood.

IFO Assay. Blood samples were drawn 0, 5, 10, 15, 20, 40, 60, 90, and 120 min after the i.v. IFO application. For the IFO assay blood samples were drawn from the retrobulbar venous plexus into heparin-coated 10-µl capillary tubes and directly mixed with dichloromethane (20).

For the determination of activated IFO, samples of whole blood were processed as described below. After extraction from blood with dichloromethane, IFO determination was performed by means of N/P flame ionization gas chromatography after derivatization of IFO and the internal standard CP with heptfluorobutyric acid (4, 5, 20).

Assay of Activated IFO. The term “activated IFO,” as used in this paper, denotes the sum of all IFO derivatives [4-hydroxyifosfamide, its acetyl tautomer aldoifosfamide, and 4(SR)-sulfidoifosfamides constituting reversible detoxification metabolites with thiol] that give rise to liberation of acrolein (20). Blood samples (10 µl) were deproteinized with 500 µl of ice-cold 5% (w/v) trichloroacetic acid and shaken vigorously. After centrifugation the supernatant, containing activated IFO, was kept at 95°C for 20 min, resulting in the release of acrolein. Subsequently, under acidic conditions acrolein reacted with 3-aminophenol, forming 7-hydroxyquinoline. The latter can be detected fluorometrically (20). 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 (4, 21, 22).

Ifosfamide used for in vitro experiments was determined by the same assay (20) and used as a standard preparation (4).

Pharmacokinetics, Calculation, and Statistical Evaluation. From the concentration-time profiles the half-lives of IFO and activated IFO were calculated mathematically by using a pharmacokinetic PC program (TOPFIT version 2.0) (23). The areas under the concentration-time curve were calculated by the trapezoidal rule. For comparison of the pharmacokinetic data for different body temperature groups, the Mann-Whitney test was used.

With regard to the often reported inhomogeneous tumor blood supply, in this paper the results of calculations of tumor drug concentrations are denoted as “apparent” concentrations.

RESULTS

Local Water Bath Hyperthermia Alone. Within <3 min after immersion of the tumors, intratumorally controlled hyperthermia at 43 ± 0.3°C was achieved in all cases. In the MX1 tumor hyperthermia at 43°C for 1 h resulted in a transient growth delay (4 days) before regrowth began at approximately the same rate as in untreated controls. In no case did tumor-free survival at day 60 occur; not even minor tumor regression was seen. In addition, the effects of low-grade hyperthermia alone were investigated (39°C for 1 h or 41°C for 1 h). In no case was tumor regression or transient growth delay seen.

Drug Dose and Temperature Dependence of the Therapeutic Efficacy of Ifosfamide. In Fig. 2 the effect of different doses of the oxazaphosphorine compound IFO at different tumor temperatures was investigated...
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The percentage of tumor-free survival at day 60 is plotted for the groups of different tumor temperatures (for 1 h; controlled by water bath immersion of the tumors at a mean rectal temperature of 37 ± 0.5°C) and doses of the oxazaphosphorine compound IFO. In no case did treatment with hyperthermia alone at 39°C, 41°C, or 43°C (for 1 h) result in tumor-free survival at day 60.

(28°C, 32°C, 37°C, 39°C, 41°C, or 43°C, locally controlled by water bath, for 1 h; rectal temperature, 37 ± 0.5°C) on the percentage of tumor-free survival at day 60 is depicted. It can be seen that at a temperature of 37°C (for 1 h) IFO in a dose of 250 mg/kg body weight led to 100% tumor-free survival. At 43°C (for 1 h), however, only half the dose (125 mg/kg body weight) was sufficient to attain the same therapeutic efficacy. At a still lower dose of 65 mg/kg body weight the percentage of tumor-free survival rose from 10% at 37°C to 60% at 43°C. At 28°C and 32°C (both for 1 h) even 250 mg/kg body weight IFO remained virtually without therapeutic effect. The strong temperature dependence is noteworthy.

The cytotoxicity of an activated oxazaphosphorine compound (MAF) (for details, see “Materials and Methods”) on MX1 tumor cells in vitro was plotted against temperature (Fig. 3A). At all concentrations examined, the therapeutic effect of the activated oxazaphosphorine compound on MX1 cells in culture rose with increasing temperature (from 32°C to 42°C).

Thus, in vitro and in vivo temperature dependence of the drug efficacy exists (Fig. 3). However, the therapeutic efficacy increases more steeply with rising tumor temperatures in the in vivo situation. This corresponds to at least a 2-fold increase of the apparent concentration of the activated drug in the tumors with rising tumor and body temperature from 37°C to 41°C (see Fig. 6B).

Effects of Increased Body Temperature on the Pharmacokinetics of Ifosfamide. The time course of the concentration of the inactive parent compound IFO in blood at 37°C and 41°C over a period of 120 min (the animals were immersed in the water bath for 60 min only) is shown in Fig. 4. The concentrations decreased exponentially with time at both body temperatures, without significant differences.

In Fig. 5 is depicted the time course of the apparent concentrations of IFO in the tumor tissue at 37°C and at 41°C. Within the initial 20 min the difference between the IFO concentrations at 37°C and at 41°C reached a maximum; the concentration at 41°C exceeded that at 37°C by about 2-fold (P < 0.05 at 15, 20, and 40 min).

The course of the concentration of the activated compound 4-OH-IFO in the blood of the nude mice at 37°C and at 41°C is shown in Fig. 6A. It can be seen that within the first 60 min, i.e., during the water bath immersion, the concentration of 4-OH-IFO at 41°C was significantly lower than at 37°C (P < 0.05 at 15, 20, and 40 min).

Fig. 6B shows the time course over 120 min of the apparent concentration of 4-OH-IFO in the tumor tissue at 37°C and at 41°C. In the tumor tissue the calculated apparent concentration of 4-OH-IFO, for almost the whole measuring period, remained at a level about twice as high as that at 37°C (P < 0.05 at 5, 10, 15, 20, 40, 60, 90, and 120 min).

Effects of Increased Body Temperature on the Systemic Toxicity of Ifosfamide. The results of WBC counts after administration of 250 mg/kg body weight IFO at 37°C, 39°C, and 41°C body temperature in nude mice are shown in Fig. 7. Within 4 days a minimum count of <3 × 10⁶ leukocytes/liter blood was found. No significant differ-

Fig. 2. The percentage of tumor-free survival at day 60 is plotted for the groups of different tumor temperatures (for 1 h; controlled by water bath immersion of the tumors at a mean rectal temperature of 37 ± 0.5°C) and doses of the oxazaphosphorine compound IFO. In no case did treatment with hyperthermia alone at 39°C, 41°C, or 43°C (for 1 h) result in tumor-free survival at day 60.
DISCUSSION

Our results indicate that in vitro the cytotoxicity of an activated oxazaphosphorine compound on human tumor cells (MX1) is clearly dose and temperature dependent (Fig. 3A). To measure quantitatively the therapeutic efficacy of a given dose of the oxazaphosphorine compound IFO at different tumor temperatures (28°C, 32°C, 37°C, 41°C, and 43°C, each applied for 1 h) (Fig. 2) in vivo, controlled tumor temperature produced by water bath immersion of the tumors was applied. The sole criterion for therapeutic efficacy was the percentage of complete tumor remissions for no less than 60 days. Transient retardations of tumor growth were thus excluded, and a measure of the therapeutic efficacy was applied that is also used in clinical studies.

It is noteworthy that at 28°C and at 32°C, temperatures not infrequently occurring at aerated sites of patients, IFO at the highest dose used (250 mg/kg body weight) exerted almost no effect. Upon application of the same dose at 37°C, however, complete tumor remissions amounted to 100%. At 43°C, only one half of the aforementioned dose elicited the same maximal response. Still, one fourth of the aforementioned dose was sufficient at 43°C to bring about complete tumor remissions in 40% of the animals.

It is unlikely that the increase of the therapeutic efficacy with rising temperature in the tumor xenografts studied is caused by a decrease of tumor blood supply. Even after exposure of the tumors to 43°C for 1 h, histological examinations showed a largely unaltered tumor vascularization (12). In previous studies we showed that tumor oxygenation (tissue pO₂) and tumor blood flow under these conditions were increased (10, 12, 13) and tumor pH did not change significantly (10, 12, 13).

Virtually identical results were obtained by us from temperature studies with tumor xenografts from the same cell line growing on nude rats (12). Moreover, our data are in good accord with results obtained by Waterman et al. (11) with cancer patients.

Under in vitro conditions the cultured tumor cells are exposed to a uniform concentration of the cytostatic drug. Under this condition a clear temperature dependence of cytotoxicity exists. At a given drug concentration cytotoxicity increases with temperature (Fig. 3A).

Under in vivo conditions at 37°C the calculated drug concentration in the tumor lies far below the concentration in the blood. With rising temperature the calculated apparent concentration of the drug in the tumor increases considerably (2-3-fold). We assume that this increase

Fig. 5. The apparent concentration of IFO in MX1 tumor tissue at 37°C or 41°C body temperature is plotted against time (for each point of measurement, six tumors). At zero time 250 mg/kg body weight IFO was administered i.v. Bars, SEM.

Fig. 6. Concentrations of 4-OH-IFO in blood and tumor. A, concentration of 4-OH-IFO in blood at 37°C or 41°C body temperature plotted against time (for each group, n = 6 animals). At zero time 250 mg/kg body weight IFO was administered i.v. Bars, SEM. B, apparent concentrations of activated ifosfamide (4-OH-IFO) in MX1 tumor tissue at 37°C or 41°C body temperature plotted against time (for each point of measurement, six tumors). At zero time 250 mg/kg body weight was administered i.v. Bars, SEM.

ences occurred between 37°C and 41°C body temperature. The WBC remained at these low values for 2 days.
of the drug concentration in the tumor is the consequence of a rise in the tumor blood flow due to an opening up of formerly closed tumor blood vessels. This assumption is supported by our previously reported data, according to which the tumor blood flow increases with rising temperature up to 43°C for 1 h (10, 12).

Thus, the strong temperature dependence of the cytotoxic action of the cytotoxic drug reported here seems to be the result of the increased drug activity (a qualitative effect) with rising temperature and the increasing drug availability (a quantitative effect) for the tumor cell population.

According to our data, the concentrations of IFO in the blood of the animals decrease with time with the same slope at 37°C and at 41°C (Fig. 4). Also, the areas under the curve for blood IFO are not significantly different between 37°C and 41°C (Table 1). Since the half-life of IFO depends mainly on hepatic metabolism, the similarity of half-lives and of the area under the curve for IFO at 37°C and at 41°C indicates a constant activation rate. However, the blood concentration of 4-OH-IFO within the first 60 min after the beginning of the 41°C treatment was significantly lower than that at 37°C body temperature (Fig. 6A), indicating a faster elimination rate. In spite of the lower blood concentration of 4-OH-IFO, the apparent concentration of this metabolite in the tumor exceeded that in the blood by about 2-3-fold within the first 60 min (Fig. 6B). Regrettably, we were unable to study the time course of blood IFO and 4-OH-IFO concentrations at temperatures above 41°C, because the animals died at body temperatures higher than 41°C.

Our results indicate that an increase of the body (tumor) temperature to 41°C for 1 h significantly improves the therapeutic efficacy of IFO in human tumor cells. Our results from the studies on nude mice encourage further studies in cancer patients treated with IFO and whole-body hyperthermia.

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