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ORIGINAL ARTICLE

Rainer Preiss · Frank Baumann · Dragan Stefanovic
Ulf Niemeyer · Wolfgang Pönisch · Dietger Niederwieser

Investigations on the pharmacokinetics of trofosfamide and its metabolites—first report of 4-hydroxy-trofosfamide kinetics in humans

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Abstract Trofosfamide (TRO), like cyclophosphamide (CYCLO) and ifosfamide (IFO), is a prodrug oxazaphosphorine derivative that requires hepatic biotransformation to form the cytotoxically active 4-hydroxy derivative (4-hydroxy-TRO). Individual 4-hydroxyoxazaphosphorines and 4-hydroxy-TRO itself have not been demonstrated in humans up to now. For investigation of the principal pharmacokinetics of TRO and its metabolites, six tumour patients (49–65 years of age, Karnofsky index > 70%) with normal liver and renal function were given a single oral dose of 600 mg/m^2 TRO. Plasma was sampled using a bedside technique. Individual 4-hydroxyoxazaphosphorines and TRO together with further metabolites were determined by a specially developed HPLC-UV method and a HPLC-MS method, respectively. With a short apparent half-life (1.2 h) and high apparent clearance (Cl/F 4.0 l/min), TRO was very quickly eliminated from plasma and highly converted to its metabolites, mainly 4-hydroxy-TRO and IFO. In relation to the AUC values of TRO (1.0) the following molar quotients were calculated: 1.59 (4-hydroxy-TRO), 0.40 (4-hydroxy-IFO), 6.90 (IFO) and 0.74 (CYCLO). C_{max} values were in the range 10–13 μmol/l for TRO, 4-hydroxy-TRO and IFO and in the range 1.5–4.0 μmol/l for CYCLO, 2- and 3-dechloroethyl-IFO and 4-hydroxy-IFO. Kinetic data indicate that 4-hydroxy-IFO is formed by both hydroxylation of TRO and exocyclic N-dechloroethylation of 4-hydroxy-TRO. 4-hydroxy-CYCLO was not detected above the quantification limit of the method. Only mild haemodepressive side effects were observed after oral administration of 600 mg/m^2 TRO. In relation to known data for IFO, TRO is much more 4-hydroxylated than IFO. The high 4-hydroxy-TRO/TRO ratio found suggests that TRO is a promising tumourstatic agent.

Keywords Trofosfamide · Ifosfamide · 4-Hydroxy-trofosfamide · Pharmacokinetics · Human

R. Preiss (✉) · F. Baumann · D. Stefanovic
Institute of Clinical Pharmacology,
University of Leipzig, Härtelstraße 16–18,
04107 Leipzig, Germany
E-mail: rainer.preiss@medizin.uni-leipzig.de
Fax: +49-341-9724659

U. Niemeyer
Baxter Oncology GmbH,
Frankfurt am Main, Germany

W. Pönisch · D. Niederwieser
Department of Internal Medicine II,
University of Leipzig, Leipzig, Germany

Introduction

Trofosfamide (TRO), increasingly used for treatment of various tumours [2, 8, 11, 15, 18], is an alkylating agent and belongs to the group of oxazaphosphorines, like cyclophosphamide (CYCLO) and ifosfamide (IFO). Oxazaphosphorines are prodrugs which require hydroxylation at the cyclic carbon 4 position to become cytotoxic. These unstable 4-OH metabolites are in equilibrium with their 4-aldotautomers, which spontaneously decompose by β-elimination to the DNA-alkylating oxazaphosphorine mustards and acrolein (Fig. 1). Exo- or endocyclic nitrogen side-chain oxidation leads to the formation of the tumourstatically inactive 2- and 3-dechloroethyl-IFO and the simultaneous release of chloroacetaldehyde. Some reports [5, 6] suggest that chloroacetaldehyde is associated with neurotoxic side effects.

The bioactivation of oxazaphosphorines is primarily mediated by cytochrome p450 isoenzymes. In human liver microsomes we and others have shown that the 4-hydroxylation of IFO is mainly mediated by CYP3A4, CYP2A6 and CYP2C9, whereas the 4-hydroxylation of CYCLO is primarily mediated by CYP2B6 and CYP3A4 [4, 16, 17]. Investigations in human liver microsomes have indicated that the 4-hydroxylation of TRO could also be mediated by CYP3A4 [14]. However, the method used for measuring the bioactivation of TRO by liberated acrolein did not discriminate between the 4-hydroxy derivatives of IFO, CYCLO and TRO.

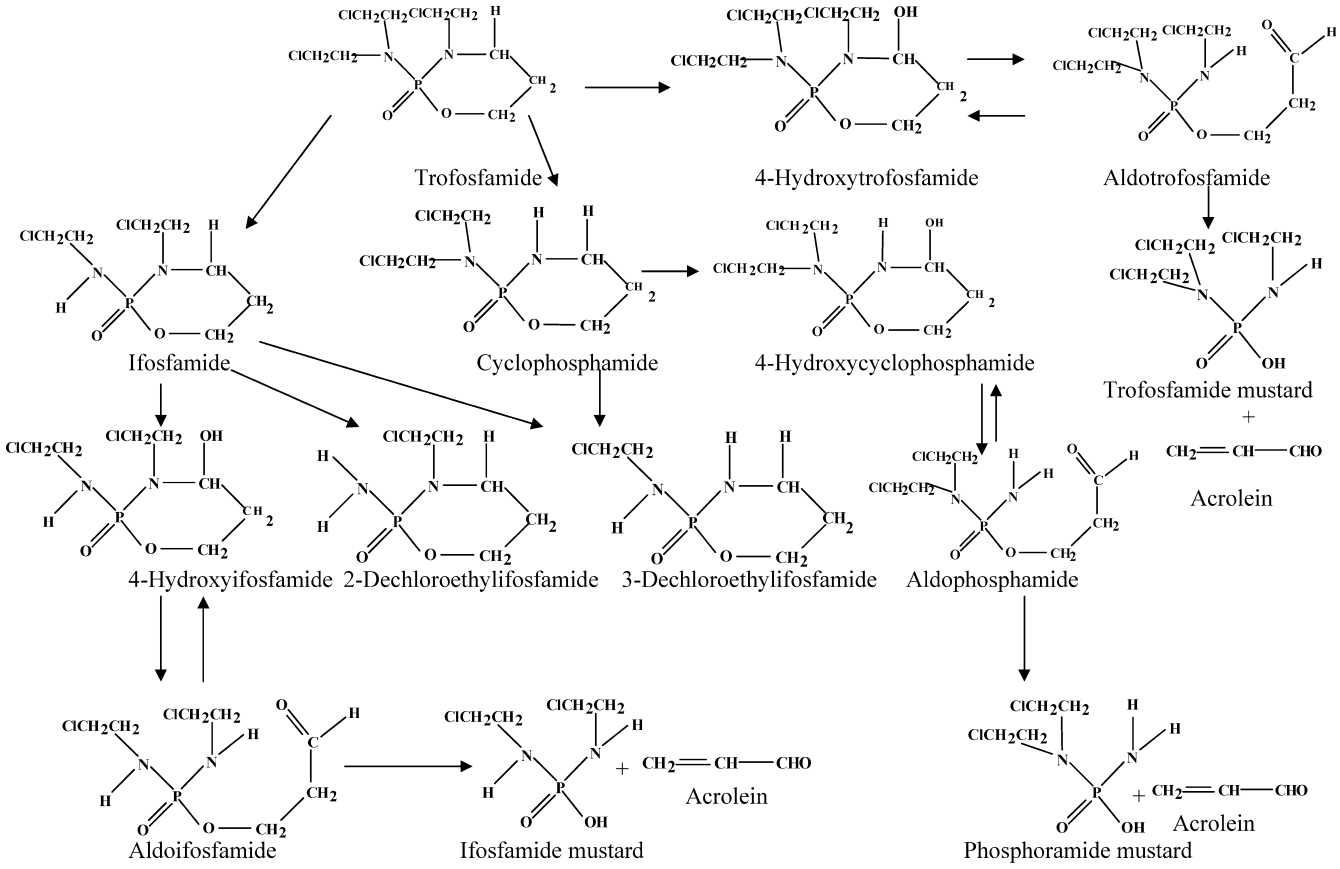


Fig. 1 Metabolic pathway of trofosfamide

Therefore, 4-hydroxylation of IFO and CYCLO formed by side-chain oxidation of TRO could not be excluded.

After oral administration of TRO, Brinker et al. [3] found a considerable amount of 4-hydroxy derivatives in plasma of tumour patients ($AUC_{4\text{-hydroxy derivatives}}/AUC_{TRO} = 0.55$) with virtually identical plasma elimination behaviours for the 4-hydroxy metabolites and TRO. As in the investigations of May-Manke et al. [14], a method based on liberated acrolein was used for the determination of 4-hydroxy derivatives that did not allow a distinction between the 4-hydroxy derivatives of TRO, IFO and CYCLO. Until now, in no study have individual 4-hydroxyoxazaphosphorines in TRO metabolism been determined [1]. The main goal of the present study was to differentiate the metabolic activation of TRO by separate detection of the 4-hydroxy derivatives of TRO, IFO and CYCLO. The results show that TRO underlies considerable bioactivation predominantly in its own 4-hydroxy derivative.

Patients and methods

Patients and study design

Six patients (four female and two male, aged 57.3 ± 6.1 years, range 49–65 years) with plasmacytoma ($n = 3$), lymphoplasmocytic immunocytoma ($n = 2$) and chronic lymphadenosis ($n = 1$) were enrolled in the study after having given their written consent. The study was approved by the local Ethics Committee. The patients did not show marked hepatic or renal dysfunction as measured by standard biochemical parameters prior to the administration of TRO (bilirubin < 20 μmol/l, creatinine < 100 μmol/l; one patient had a value of 162 μmol/l and one a value of 183 μmol/l). At the start of TRO administration the thrombocyte and leucocyte counts were > 80,000/μl (one patient > 50,000/μl) and > 2500/μl, respectively, and the Karnofsky index of all patients was > 70%. None of the patients had received cytostatics or radiotherapy within 4 weeks prior to inclusion in the study.

TRO was given as a single oral dose of 600 mg/m^2 (50-mg tablets; ASTA Medica Oncology, Germany) on an empty stomach together with 100 ml tea. The first food intake was allowed 6 h after oral administration of TRO. At 0, 4 and 8 h after administration of TRO, 120 mg/m^2 mesna was given intravenously to each patient. By means of heparin monovettes, blood samples were drawn at 0, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 12 and 24 h after intake of the TRO tablets. The samples were immediately (bedside technique) centrifuged at 4°C for 5 min and the plasma samples were stored in 5-ml aliquots at −70°C until analysis.

Substances and equipment for HPLC

TRO, IFO, CYCLO, 4-hydroperoxy-TRO, 4-hydroperoxy-IFO, 4-hydroperoxy-CYCLO, dimethyl-CYCLO, 2- and 3-dechloroethyl-IFO and d_4-CYCLO were gifts from ASTA Medica AWD (Frankfurt am Main, Germany). Acetic acid, methanol (HPLC grade), KH_2PO_4 (Ultrapur), and acetonitrile (Ultra Gradient HPLC grade) were obtained from J.T. Baker (Phillipsburg, N.J.). Dinitrophenyl hydrazine (pro analysi) and ammonium acetate (A.C.S.) were purchased from Merck.

The following instrumentation was used: a TSP AS 3000 Spectra system autosampler, membrane degasser, Consta Metric 4100 pump, and UV-detector Spectra Monitor 3200, and a Finnigan SSQ 7000 mass spectrometer.

Analysis of 4-hydroxyoxazaphosphorines by HPLC-UV

Plasma (0.5-ml aliquots) was thawed carefully and mixed with 0.5 ml chilled acetonitrile. After immediate centrifugation, the supernatant was removed, and for derivatization of 4-hydroxyoxazaphosphorines to the hydrazone derivatives the following substances were added: 100 µl $Na_2S_2O_3$ (5 mg/ml), 70 µl 1 *N* HCl and 100 µl dinitrophenylhydrazine (3.8 mg/ml). The derivatization was carried out for 20 min at 45°C, and the entire quantity of liquid was extracted twice with 2 ml chloroform. The organic phase was vaporized carefully by means of nitrogen. The bottom layer was stored until measurement at −70°C. For measurement the bottom layer was dissolved in 100 µl mobile phase A and chromatographed on an Ultrasep ES Pharm RP 18 column (150×2 mm; Sepserv, Berlin, Germany) using a linear gradient. The following mobile phases were used:

A: 390 ml acetonitrile + replenished bidistilled water to 1 l (containing 2.772 g KH_2PO_4, pH 4.8)

B: 600 ml acetonitrile + replenished bidistilled water to 1 l (containing 2.772 g KH_2PO_4, pH 4.8).

The detection wavelength was 357 nm.

Intra- and interassay precision were determined with three different concentrations in the range 0.6 to 17 µmol/l ($n = 5$). Because the standard deviations of the intraassay determination (< 15%) were lower than those of the interassay determination, the assay was calibrated daily in the range from the lower limits of quantification (LLOQ) to 20 µmol/l. The LLOQs of the 4-hydroxy derivatives were 0.07 µmol/l (4-hydroxy-TRO), 0.08 µmol/l (4-hydroxy-IFO) and 0.05 µmol/l (4-hydroxy-CYCLO).

Stability tests at −70°C over 7 months showed that more than 95% of the 4-hydroperoxyoxazaphosphorines were still available after 2 months. Therefore controls and calibration samples were prepared monthly. The patient samples were stored at −70°C for a maximum of 3 weeks. 4-Hydroxyoxazaphosphorine derivatives were stable for 6 h. The solubilized samples were measured during this time period.

Determination of TRO and its metabolites IFO, CYCLO and 2- and 3-dechloroethyl-IFO by HPLC-MS

For sample preparation, a solid-phase extraction method was used. RP 18-cartridges (Bakerbond spe Octadecyl, J.T. Baker; 100 mg) were conditioned with 1 ml acetonitrile and 1 ml 0.5% acetic acid. Plasma (0.5 ml) and dimethyl-CYCLO solution (20 µl, IS) were added to the cartridges. The cartridges were cleaned twice with 1 ml 0.5% acetic acid and then the substances were eluted three times with 1 ml acetonitrile. Acetonitrile was evaporated by means of nitrogen and the residue was stored at −70°C until measurement. The residue was resolved well in 150 µl 30% methanol/20 m*M* NH_4Ac and 50 µl d_4-CYCLO solution (methanolic solution). Because of strong impairment of the extraction by the high proportion of methanol in the d_4-CYCLO solution, the solution was added after the solid phase extraction. A linear gradient was used. The following mobile phases were used:

A: 90% acetonitrile, 5 m*M* NH_4Ac and 0.1% acetic acid, pH 6.90

B: 10% acetonitrile, 5 m*M* NH_4Ac and 0.1% acetic acid, pH 4.45

A flow rate of 0.3 ml/min and an Ultrasep ES Pharm RP 18 column (125×2 mm; Sepserv) were used.

IFO, CYCLO, and 2- and 3-dechloroethyl-IFO were quantified by measuring the peak heights in relation to that of d_4-CYCLO with the respective molar masses in the SIM mode (261, 261, 199, 199 to 265). For TRO (323) dimethyl-CYCLO (289) was used as internal standard. The following mass spectrometry parameters were chosen: 4.5 kV ionization voltage, 220°C capillary temperature, 413.6 kPa (60 psi) sheath gas, 68.9 kPa (10 psi) auxiliary gas and 10 V collision-induced dissociation. The LLOQs were 0.30 µmol/l (TRO), 0.13 µmol/l (IFO), 0.25 µmol/l (CYCLO), 0.07 µmol/l (2-dechloroethyl-IFO) and 0.13 µmol/l (3-dechloroethyl-IFO). At various concentrations in the range 2 to 40 µmol/l, intraassay standard derivations of lower than 20% ($n = 5$) and interassay standard derivations of over 20% ($n = 5$) were obtained. Therefore daily calibrations in the range from LLOQ to 20 µmol/l for 2- and 3-dechloroethyl-IFO and CYCLO and from LLOQ to 25 µmol/l for TRO and IFO were used.

Pharmacokinetics and calculation

The maximum plasma concentration (c_{max}) and time to reach c_{max} (t_{max}) were obtained directly from the plasma concentration-time data. The area under the plasma concentration-time curve [$AUC_{0-\infty}$; $AUC_{0-\infty} = AUC_{0-t}$ (linear trapezoidal rule) plus $AUC_{t-\infty}$ (last data point/terminal elimination constant)], apparent terminal elimination half-life ($t_{1/2}$; log-linear regression), apparent clearance (Cl/F; $Cl/F = dose_{TRO}/AUC_{0-\infty}$), and mean resistance time ($MRT_{0-\infty}$; $MRT_{0-\infty} = AUC_{0-\infty}/AUMC_{0-\infty}$, where AUMC is the area under the first moment curve) were calculated using the program TOPFIT [7]. For statistical evaluation Wilcoxon's signed ranks test was used. A probability level of 0.05 was considered to indicate statistical significance. The results are presented as arithmetic means ± SD.

Results

The plasma level vs time profiles of TRO and its six metabolites after oral administration of 600 mg/m^2 TRO are shown in Fig. 2. Considerable interindividual variations were found in the pharmacokinetics of TRO and its metabolites, especially in the values of c_{max} and AUC. The corresponding mean pharmacokinetic parameters of TRO and its metabolites are summarized in Table 1. With a t_{max} value of about 2 h, TRO showed signs of rapid absorption. On the other hand, with an apparent terminal half life and a mean residence time of 1 and 3 h, respectively, and an apparent clearance of about 4.0 l/min, TRO was also very quickly eliminated from plasma. The mean apparent elimination half-lives of the metabolites IFO and CYCLO were 7.4 and 7.3 h (Table 1).

The results presented here show for the first time the kinetics of 4-hydroxy-TRO after administration of TRO. 4-Hydroxy-TRO was rapidly formed from TRO and during its formation phase showed a plasma profile similar to that of its parent drug (Table 1, Fig. 2). However, its elimination from plasma was significantly slower ($P < 0.05$) than that of TRO (apparent $t_{1/2}$ 3.3 vs 1.2 h). Plasma levels of 4-hydroxy-IFO were one-third of those of 4-hydroxy-TRO. No 4-hydroxy-CYCLO could be detected in any of the samples from the six patients above the LLOQ of 0.05 µmol/l. As expected IFO was found as the main metabolite of TRO (Table 1, Fig. 2). However, based on the mean c_{max} values the sum of the tumourstatically active 4-hydroxy metabolites (4-hydroxy-TRO + 4-hydroxy-IFO) was quantitatively in the range of IFO plasma levels. The ratios of the mean molar c_{max} values (Table 1) of 4-hydroxy-TRO, 4-hydroxy-IFO, IFO and CYCLO in relation to that of TRO were 1.03, 0.41, 1.36 and 0.16, respectively.

Similarly, the ratios for the AUC values were 1.59, 0.40, 6.90 and 0.74, respectively. In all patients low levels of 2- and 3-dechloroethyl-IFO were found. Whereas the t_{max} values of both metabolites were in the range of those of IFO and CYCLO, the elimination of both metabolites from plasma was found to be considerably slower than that of IFO and CYCLO (Table 1, Fig. 2).

With regard to toxic side effects, anaemia (patients 1, 2 and 4), leucopenia (patients 1, 3 and 5) and loss of appetite (patients 3, 5 and 6) of WHO grades 1–3, and thrombocytopenia (patients 3 and 4) of WHO grades 1–2 were observed during the 3-week observation period.

Discussion

There are two previous reports of the plasma kinetics of TRO and its metabolites IFO and CYCLO in tumour patients after oral administration of TRO [3, 9]. The mean oral doses of TRO were 450 and 177 mg/m^2, respectively, in these studies, i.e. somewhat lower than in our investigation (600 mg/m^2). Regarding IFO and CYCLO, in all three studies an identical pattern of TRO metabolites was found. The c_{max} molar ratios for IFO and CYCLO in relation to TRO were in the ranges 1.0–2.1 and 0.2–0.4, respectively, and the molar ratios for AUC were in the ranges 6.0–7.5 and 0.7–1.2, respectively, in all three studies. Moreover, in all three studies the elimination half-life of TRO was about 1 h. We and Hempel et al. [9] found an apparent clearance (Cl/F) of TRO in the range 3–4 l/min and we and Brinker et al. [3] found elimination half-lives of IFO and CYCLO in the range 4.5–7.4 h. Above all, the results of the three studies not only show a good accordance in conversion of TRO into its metabolites IFO and CYCLO, but also give some indication of a dose-linear, not capacity-limited exo- and endocyclic dechloroethylation of TRO in the investigated dose range of 170–600 mg/m^2.

The results presented here demonstrate for the first time the kinetic pattern of 4-hydroxy-TRO in humans. After oral administration of TRO, its 4-hydroxy derivative is detectable in considerable amounts in plasma, and represents, beside IFO, a main metabolite in the conversion of TRO. Its plasma kinetics are similar to those of TRO. 4-Hydroxy-TRO is very rapidly formed from TRO, a process which is mainly mediated by hepatic cytochrome p450 isoenzymes. The high lipophilicity of TRO and its consequential rapid penetration into hepatic cells and intracellular sarcoplasmic structures could be partly responsible for this reaction. However, its elimination from plasma proceeds more slowly than that of TRO itself.

Besides 4-hydroxy-TRO, we also detected 4-hydroxy-IFO as an active oxazaphosphorine derivative after oral administration of TRO, whereas 4-hydroxy-CYCLO was not detectable above the LLOQ of the method (0.05 μmol/l). The molar quotients for 4-hydroxy derivatives (sum of 4-hydroxy-TRO and 4-hydroxy-IFO) in relation to TRO were 1.44 for c_{max} and 1.97 for AUC. Brinker et al. [3] first demonstrated 4-hydroxy metabolites after oral administration of TRO. However, their methods did not differentiate between the 4-hydroxy derivatives of TRO, IFO and CYCLO. Furthermore, in the study of Brinker et al. [3] the molar 4-hydroxy derivative/TRO quotients were only 0.31 for c_{max} and 0.55 for AUC. On the other hand the dose standardized c_{max} and AUC values of TRO were in the same order in the both our study and that of Brinker et al. We cannot give a clear explanation as to why we found fourfold higher plasma levels of 4-hydroxy derivatives than Brinker et al. [3]. However, to measure the very unstable 4-hydroxyoxazaphosphorines we used a bedside technique, centrifuged the plasma samples at 4°C and stored the samples at −70°C until analysis. A detailed description of the method used in the study by Brinker et al. [3] is not given. However, it appears that the plasma samples were centrifuged at room temperature for an unspecified time and the samples were stored at −20°C until analysis.

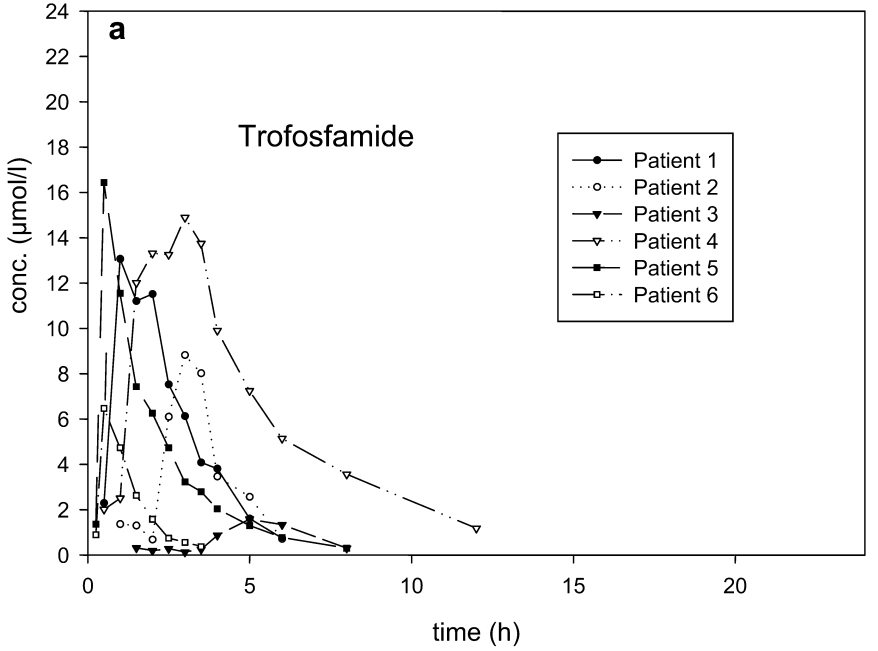
According to the results presented here, TRO seems to be much more hydroxylated to the tumourstatically active 4-hydroxy derivatives than IFO. It should be emphasized that on a standardized dose of 1 mmol/m^2, we found mean AUC and c_{max} values of 4-hydroxy derivatives (sum of 4-hydroxy-TRO and 4-hydroxy-IFO) after administration of TRO about 20- and 30-fold higher, respectively, than those found by the group of Wagner [12, 13] after administration of IFO in 23 tumour patients (29.77 μmol·h/l and 7.47 μmol/l vs 1.55 μmol·h/l and 0.25 μmol/l, respectively).

The results presented here indicate that 4-hydroxy-IFO is formed not only by hydroxylation of IFO but also by exocyclic dechloroethylation of 4-hydroxy-TRO.

First, 4-hydroxy-IFO was formed rapidly in parallel with increasing plasma concentrations of TRO and its 4-hydroxy derivative (Fig. 2). The mean t_{max} values of

Table 1 Mean values of pharmacokinetic parameters from six patients after oral administration of 600 mg/m^2 TRO (Ixoten)

Drug/metabolite	t_{max} (h)	c_{max} (μmol/l)	AUC (μmol·h/l)	$t_{1/2}$ (h)	$MRT_{0-\infty}$(h)	Cl/F (ml/min)	V_z/F (l)
TRO	2.33 ± 1.72	9.66 ± 5.36	27.5 ± 25.3	1.24 ± 0.67	3.39 ± 1.62	3984 ± 3222	350 ± 265
4-Hydroxy-TRO	2.58 ± 2.85	9.91 ± 4.02	43.7 ± 33.0	3.33 ± 1.78	–	–	–
IFO	5.75 ± 3.94	13.18 ± 3.84	189.5 ± 87.4	7.44 ± 2.46	–	–	–
4-Hydroxy-IFO	2.08 ± 1.77	3.97 ± 2.11	10.9 ± 12.4	2.33 ± 1.37	–	–	–
CYCLO	5.50 ± 3.55	1.53 ± 0.57	20.4 ± 18.6	7.25 ± 5.22	–	–	–
2-Dechloroethyl-IFO	5.75 ± 3.81	3.62 ± 1.74	133.8 ± 75.3	27.5 ± 15.5	–	–	–
3-Dechloroethyl-IFO	5.75 ± 3.81	3.72 ± 1.05	330.5 ± 226.1	69.3 ± 65.5	–	–	–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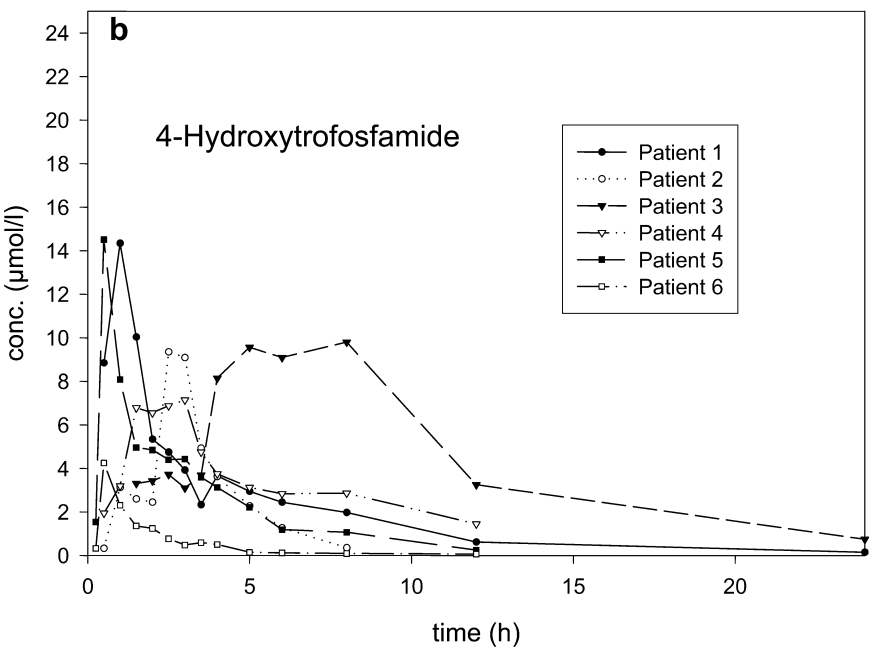
Trofosfamide
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
conc. (µmol/l)
time (h)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Patient 5
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conc. (µmol/l)
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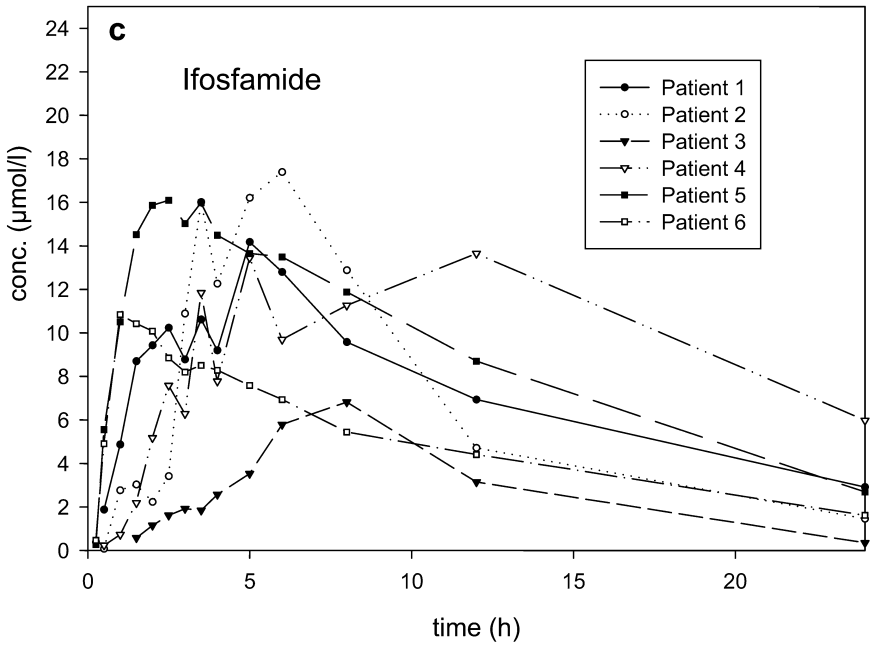
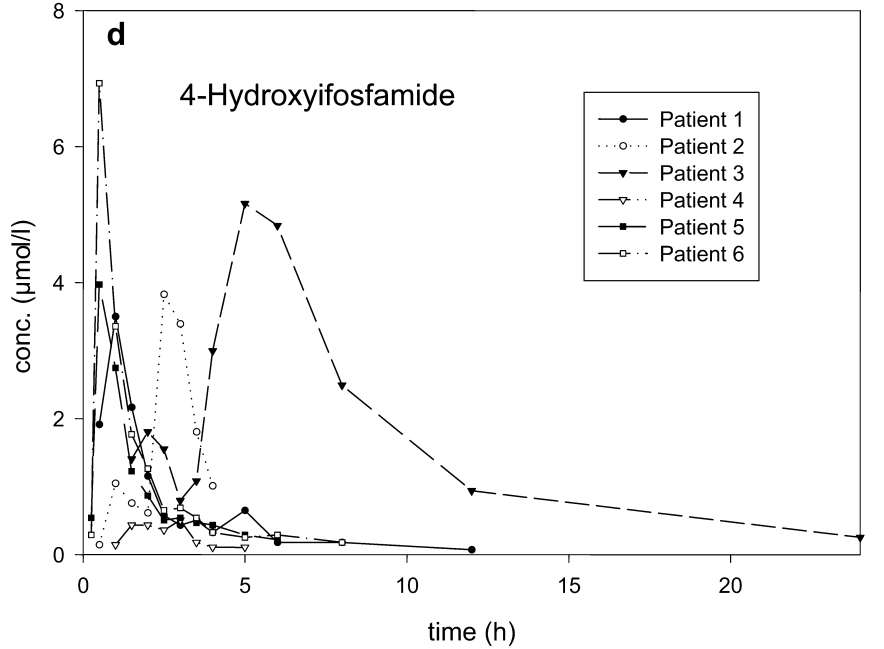

c
Ifosfamide
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
Patient 6
conc. (µmol/l)
time (h)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4-Hydroxyifosfamide
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Patient 4
Patient 5
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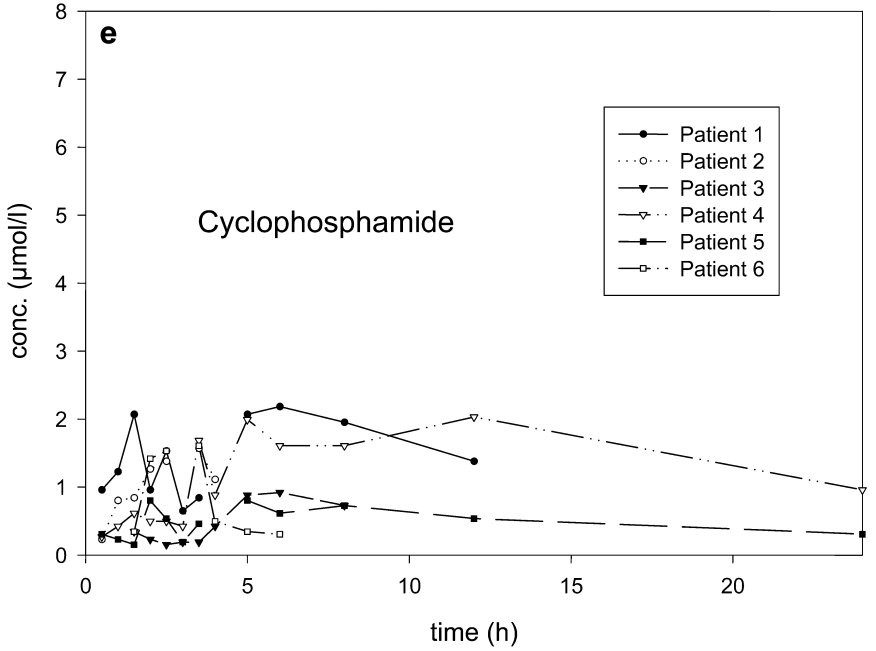
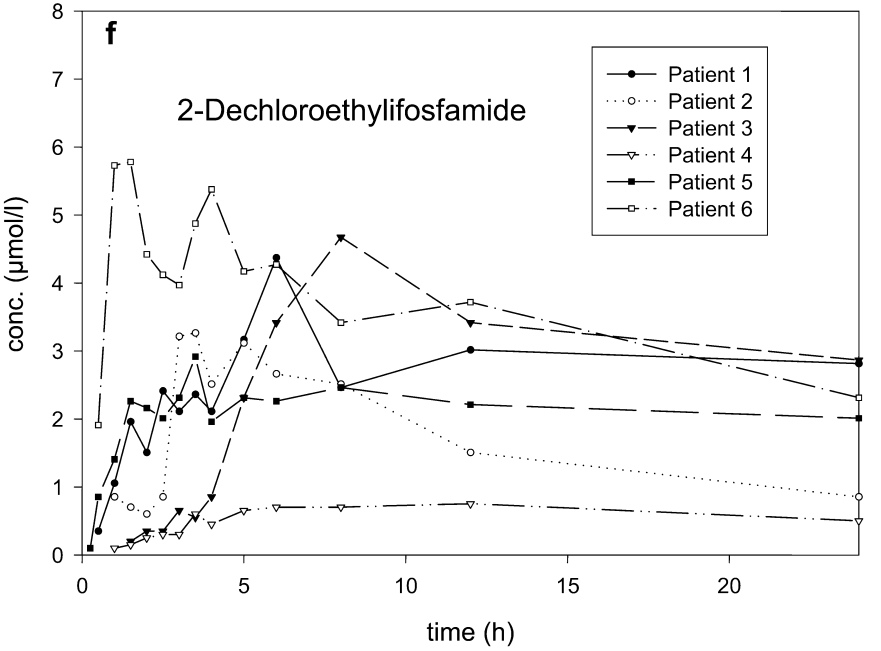
conc. (µmol/l)
time (h)





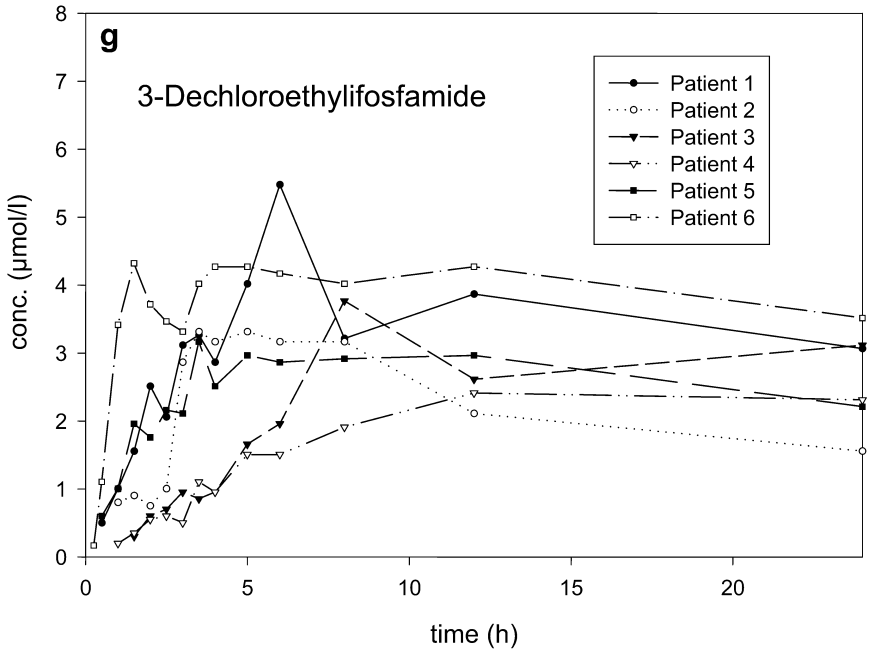


Fig. 2a–g Plasma levels of TRO (**a**), 4-hydroxy-TRO (**b**), IFO (**c**), 4-hydroxy-IFO (**d**), cyclophosphamide (**e**), 2-dechloroethyl-IFO (**f**) and 3-dechloroethyl-IFO (**g**) in six tumour patients after oral administration of 600 mg/m^2 trofosfamide (Ixoten)

4-hydroxy-IFO, 4-hydroxy-TRO and TRO all occurred in a narrow range of 2.1–2.6 h, whereas the mean t_{max} value of IFO occurred at 5.8 h (Table 1). Second, the plasma elimination of 4-hydroxy-IFO mainly conformed with those of 4-hydroxy-TRO and TRO. The apparent elimination half-life of 4-hydroxy-IFO (2.3 h) was in the range of those of 4-hydroxy-TRO and TRO (1.2–3.3 h) but was significantly ($P < 0.05$) lower than that of IFO (7.4 h) (Table 1). Third, after administration of IFO, the 4-hydroxy-IFO/IFO quotients have been found to be in the ranges 0.004–0.009 and 0.005–0.013 for c_{max} and AUC, respectively [10, 12, 13], whereas in the present investigations after administration of TRO much higher values of these quotients were found (0.301 for c_{max} and 0.058 for AUC). Finally, in in vitro experiments in human liver microsomes using 4-peroxy-TRO and an energy delivering system we found that 4-hydroxy-TRO and 4-hydroxy-IFO were generated, whereas 4-hydroxy-CYCLO could not be detected (data not shown).

TRO was well tolerated at the oral dose of 600 mg/m^2 in the six tumour patients investigated. Toxic side effects, including anaemia, leucopenia and loss of appetite of grades 1–3, were observed in each of three patients and thrombocytopenia of grades 1–2 in two patients. In particular, neurotoxic side effects did not occur, although the formation of the IFO metabolites (CYCLO, 4-hydroxy-IFO and 2- and 3-dechloroethyl-IFO) should result in the appearance of considerable amounts of chloroacetaldehyde in plasma.

In summary, the present results demonstrate for the first time the pharmacokinetics of 4-hydroxy-TRO in humans. TRO is mainly metabolized to its 4-hydroxy derivative and IFO and in smaller amounts to 4-hydroxy-IFO, CYCLO and further metabolites. The high 4-hydroxy-TRO/parent drug ratio suggests that TRO is a promising tumourstatic agent. TRO was well tolerated after an oral dose of 600 mg/m^2.

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