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ORIGINAL ARTICLE

Pharmacokinetics of oral fenretinide in neuroblastoma patients: indications for optimal dose and dosing schedule also with respect to the active metabolite 4-oxo-fenretinide

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Abstract
Purpose Pharmacokinetic data on fenretinide (4-HPR) are scant, thus limiting the rational use of the drug. We investigated the pharmacokinetics of 4-HPR and its active metabolite 4-oxo-fenretinide (4-oxo-4-HPR).
Experimental design Pharmacokinetics were assessed in 18 children (3 for each dose) with neuroblastoma who received oral 4-HPR once daily for 28 days at the doses of 100, 300, 400, 600, 1,700 and 4,000 mg/m^2/day. 4-HPR and 4-oxo-4-HPR were determined by HPLC in plasma collected up to 48 h after the first and 28th administration.
Results After single administration, 4-HPR mean C_{max} ranged from 0.9 to 6.6 μM and these concentrations roughly doubled at steady state (range 1.6–14.5 μM). 4-HPR mean $t_{1/2}$ was 22 h. 4-HPR pharmacokinetics were linear in the dose range 100–1,700 mg/m^2; less than dose-proportional increase in exposure was found at 4,000 mg/m^2. At steady state, pharmacologically relevant plasma concentrations (range 0.7–10 μM and 0.4–5 μM for 4-HPR and 4-oxo-4-HPR, respectively) were maintained during the 24 h dosing interval in the dose range 300–4,000 mg/m^2.
Conclusions 4-HPR pharmacokinetics supports once-daily dosing. Steady state concentrations of 4-HPR and 4-oxo-4-HPR in children with neuroblastoma are in line with those found to have in vitro growth inhibitory effects in neuroblastoma cells.

Keywords Fenretinide · Retinoids · Pharmacokinetics · Metabolism · Pediatric · Neuroblastoma

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Introduction

Retinoids are natural and synthetic analogs of vitamin A (retinol) that regulate many important cellular processes, including growth, differentiation and apoptosis [1]. Clinical data support the potential of retinoids for the prevention and treatment of various malignant diseases even though the toxicity of these compounds limits their therapeutic use. Fenretinide or *N*-(4-hydroxyphenyl)retinamide (4-HPR) is a synthetic retinoid which has already demonstrated efficacy in preneoplastic [2] and neoplastic conditions [3, 4] and which is well tolerated in humans [3, 5]. The tumor growth inhibitory effects of 4-HPR have been widely studied and several mechanisms of action have been proposed. While classical retinoids often induce differentiation, 4-HPR mainly promotes apoptosis. The concentrations of 4-HPR that are effective in vitro against the growth of tumor cells of different histotypes, including neuroblastoma, range from 0.7 to10 μM [6]. In embryonal carcinoma cell lines 4-HPR has been shown to have a dual effect, i.e., a rapid induction of cell death at 10 μM and a slower induction of differentiation at 1 μM [7]. Therefore,

4-HPR might have different activities depending on concentrations.

The metabolites of 4-HPR identified up to now are *N*-(4-methoxyphenyl)retinamide (4-MPR), and 4-oxo-*N*-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR). 4-MPR, the most abundant metabolite in human plasma [8], is ineffective in vitro in inhibiting the growth of almost all tested cell lines [6, 9]. On the contrary, 4-oxo-4-HPR, a recently identified 4-HPR metabolite with modifications in position 4 of the cyclohexene ring [10], has antiproliferative and proapoptotic effects and is more potent and effective than the parent drug in vitro [6]. The 50% inhibitory concentrations (IC_{50}) of 4-oxo-4-HPR were 2–4 times lower than those of 4-HPR in all tested cell lines, including neuroblastoma and they ranged from 0.4 to 5.3 μM [6].

Clinical studies with 4-HPR were first conducted to assess the preventive activity of this retinoid against the recurrence of breast cancer. A phase III trial was conducted [3] to evaluate the efficacy of a 5-year treatment period with oral 4-HPR at the dose of 200 mg/day, which produced steady state plasma concentrations of approximately 1 μM [8]. At this dose 4-HPR was well tolerated and had a protective effect against second breast cancer in premenopausal women [3] which persisted for up to 10 years after treatment cessation [11]. This study thus suggested that 1 μM plasma concentrations of 4-HPR might be associated with a chemopreventive activity against breast cancer.

On the basis of its in vitro growth inhibitory effects, 4-HPR was proposed also as therapeutic agent and it was thus tested at higher doses in patients with tumors of different histotypes [5, 12–14]. Phase I–II trials were conducted with 4-HPR administered in cycles of 7 days, repeated every 3 weeks. In adults, 4-HPR, administered up to 1,800 mg/m^2/day divided into 2 or 3 daily doses [5, 12, 13], caused no major toxicities but it was minimally or not effective and thus it was proposed that the drug should be tested at higher doses in future trials. In children with high-risk solid tumors, including neuroblastoma, the maximal tolerated dose was 2,475 mg/m^2/day, divided into 2 or 3 daily doses [14]. At this dose, the mean steady state plasma concentrations of 4-HPR averaged approximately 10 μM, i.e., a concentration similar to that found to be effective in inhibiting tumor cell growth in vitro [6]. Similar steady state plasma concentrations were achieved, without major signs of toxicity, with the dose of 4,000 mg/m^2/day, in another phase I trial in children with neuroblastoma in which the drug was administered once daily for 28 days followed by a 7-day interruption [15]. The two phase I trials in neuroblastoma patients described above were conducted with different dosing schedules and thus provided safety, tolerability and efficacy data that are difficult to compare. However, in both studies prolonged disease stabilization and regression of some lesions were reported, warranting future trials of 4-HPR in these kind of patients.

Even though 4-HPR tolerability and efficacy have been investigated in several clinical trials [2, 3, 5, 12–15], information on 4-HPR pharmacokinetics in man are still limited [16]. The data so far reported were mainly obtained by measuring 4-HPR and/or 4-MPR concentrations in plasma samples collected at only single time points [8, 12–14, 17–23] and no information is available regarding the plasma concentration of the pharmacologically active metabolite 4-oxo-4-HPR. The aim of this study was to investigate the pharmacokinetics of 4-HPR and its metabolites 4-MPR and 4-oxo-4-HPR, in a rigorously and specifically designed study, in order to provide useful information for a rational dose and dosing schedule selection to be applied in future clinical trials. In addition, to provide further indication for dosing schedule selection, we tested the in vitro growth inhibitory activity of 4-HPR and its active metabolite 4-oxo-4-HPR in neuroblastoma cells by adopting treatment protocols that resemble continuous vs intermittent dosing schedules.

Patients and methods

Drug, patients, protocol and sample collection

4-HPR was supplied as 50 and 100 mg capsules by Cilag Pharmaceutical Division (Shaffausen, CH). Patient characteristics and the design of the phase I study have been previously described in detail [15]. The study was conducted in patients that had advanced (resistant or relapsed stage 4 or stage 3 with early relapse) neuroblastoma. The study protocol and other related material was approved by the Ethical Committees of the two Institutions where patients were enrolled (Istituto Gaslini, Genova and Istituto Nazionale Tumori, Milan, Italy). Patients were enrolled for blood drug levels measurements if they accepted to be hospitalized for serial blood sampling. A written informed consent was obtained from the patient's parents before drug treatment and the study was conducted according to the Helsinki declaration for clinical investigations and subsequent revisions. 4-HPR was administered with the following schedule: orally, once a day, for 28 consecutive days with a 7-day drug interval after each course for a maximum of six courses. Patients received doses ranging from 100 to 4,000 mg/m^2/day. Doses higher than 4,000 mg/m^2/day could not be administered due to the unacceptably high number of capsules needed. The doses administered and the number of patients participating in the pharmacokinetic study at each dose level were: 100 mg/m^2, six patients; 200 mg/m^2, five patients; 300 mg/m^2, four patients; 400 mg/m^2, three patients; 500 mg/m^2 five

patients; 600 mg/m^2, four patients; 700 mg/m^2, two patients; 1,000 mg/m^2, six patients; 1,300 mg/m^2, three patients; 1,700 mg/m^2, three patients; 2,300 mg/m^2, two patients; 3,000 mg/m^2, three patients and 4,000 mg/m^2, four patients (total 50 patients).

4-HPR was administered after an overnight fast. Blood samples (1–2 ml for each sample), drawn through a venous catheter, were collected into heparinized tubes immediately before the first and 28th drug intake (time 0) and 1, 3, 4, 6, 9, 12, 16, 20, 24, 36 and 48 h thereafter. The total blood volume withdrawn after the first and 28th drug intake for pharmacokinetic analysis ranged from approximately 10 to 3% of total in youngest and oldest patients, respectively. The repeated dose treatment period started after the collection of the 48 h blood sample. Samples were protected from light by wrapping the collection tubes in aluminum foil. The bioanalytical methods used in the present investigation have been validated according to the currently adopted FDA and EU guidelines. Therefore, the adopted procedures for sample collection, handling, storage and analysis were supported by the validation data results. Plasma was rapidly separated from blood by centrifugation and stored at −20°C for a maximum of 30 days when they were analyzed.

In order to provide interpretable data, the pharmacokinetic analysis was performed only for those patients who had blood samples collected at all planned time points after both the first and the 28th administration and the actual sampling times did not deviate by >5% from the nominal collection times. In addition, the pharmacokinetic analysis was performed only at those dose levels for which the data, collected as described above, were available from at least three patients. These requirements were met by three patients each at the following doses: 100, 300, 400, 600, 1,700 and 4,000 mg/m^2. Therefore, the total number of patients included in the pharmacokinetic analysis was 18 (3 for each of the 6 doses).

Analytical method

Aliquots of 200 μl plasma were added to 400 μl CH_3CN containing 125 μg/ml of butylated hydroxytoluene (Sigma, St Louis, MO, USA) as anti-oxidant, vortex mixed and centrifuged. The concentrations of 4-HPR, 4-MPR and 4-oxo-4-HPR in the recovered supernatants were simultaneously determined by high-performance liquid chromatography as previously described [8, 10]. Briefly supernatants were analyzed on a liquid chromatograph (Perkin Elmer, Norwalk, CT, USA) fitted with a C18 (5 μm) reverse-phase column (150 mm × 4.6 mm) and a C18 precolumn (Perkin Elmer, Milan, Italy). The mobile phase consisted of CH_3CN:H_2O:CH_3COOH (75:23:2, vol/vol/vol) delivered at a flow rate of 2 ml/min. Detection was performed with a Perkin Elmer LC95 absorbance detector at 340 nm. *N*-(4-ethoxyphenyl)-retinamide (EPR) was used as internal standard. The reference standards for 4-HPR, 4-MPR and the internal standard EPR were supplied by the RW Johnson Pharmaceutical Research Institute; 4-oxo-4-HPR was kindly provided by R.W. Curley (The Ohio State University Columbus, OH, USA). All the procedures were performed with the samples protected from light. The limits of quantification were 3, 5 and 20 ng/ml (corresponding to 0.008, 0.012 and 0.05 μM) for 4-HPR, 4-MPR and 4-oxo-4-HPR, respectively. 4-HPR, 4-MPR and 4-oxo-4-HPR could be accurately and precisely quantified in the concentration range of 0.008–20, 0.012–20 and 0.05–20 μM, respectively. The intra and inter-assay accuracy for the three compounds were included in the 90–115% range and the intra- and inter-assay precision were ≤15%.

Pharmacokinetic analysis

For each subject, 4-HPR, 4-MPR and 4-oxo-4-HPR plasma concentration vs time data were analyzed by noncompartmental pharmacokinetic methods using WinNonlin (version 2.0; Pharsight, Mountain View, CA, USA). The maximum plasma concentrations (C_{max}) and the corresponding times (t_{max}) were taken directly from the raw data. The area under the plasma concentration-time curve up to 24 h after dosing ($AUC_{0-24\ h}$) was calculated using the linear trapezoidal rule. The area under the plasma concentration-time curve was extrapolated to infinity (AUC) assuming monoexponential decay and by adding the portion C_{last}/λ_z where C_{last} was the last measured concentration and λ_z was the elimination rate constant of the terminal linear phase of plasma concentration-time curve. The terminal elimination half-life ($t_{1/2}$) was estimated by linear regression analysis of natural log concentration against time ($t_{1/2} = \ln 2/\lambda_z$). The choice of the terminal phase was based primarily on visual inspection of the concentration-time plots. In any case the selection of the terminal phase was accepted only if the percentage of the total area extrapolated was ≤20%. After repeated treatments, at steady state, the area under the plasma concentrations vs time curve within a dosing interval (24 h) was denoted as AUC_{ss} and the average concentration over a dosing interval ($C_{ss,av}$) was calculated as AUC_{ss}/24 h. Individual accumulation factors were calculated based on repeated dose/single dose ratios of C_{max} and $AUC_{0-24\ h}$ and denoted, respectively, $R_{ac,C_{max}}$ and $R_{ac,AUC_{0-24}}$ and according to the following formula: accumulation ratio = $1/(1 - e^{-\tau \ln 2/t_{1/2}})$ where τ is the dosing interval equal to 24 h [24]. The metabolite-to-parent drug AUC ratios (AUC_m/AUC_p) were calculated to afford a comparison of the relative abundance of the two metabolites in circulation.

Statistical analysis

The effect of dose on C_{max}, AUC and $t_{1/2}$ was tested by analysis of variance after log transformation; C_{max} and AUC values were normalized to the dose of 1 mg (C_{max}/dose, AUC/dose) before the analysis. In case of a statistically significant result, multiple comparisons were carried out using the Tukey's test. Differences in $t_{1/2}$ among doses were analyzed using the same test but without dose normalization. Differences in t_{max} were evaluated by nonparametric tests and without dose normalization. For comparison of repeated-administration vs single-administration pharmacokinetic parameters, at the six dose levels, the AUC after a single administration was compared with the AUC_{ss} after the last administration as a measure of accord with expectation from linear superimposition [24] using the *t*-test for paired data. Differences in C_{max}, $t_{1/2}$ and t_{max} following single and repeated administrations were also tested using the *t*-test for paired data. In all the analysis, a *P* value of less than 0.05 was considered statistically significant.

In vitro cell proliferation assay

Two human neuroblastoma cell lines, SK-N-BE and SK-N-MC (American Type Culture Collection (ATCC), Manassas, VA, USA), were tested for their sensitivity to short term (3 days followed by a washout period of 3 days) or continuous treatment (3 days followed by additional 3 days) with 4-HPR and 4-oxo-4-HPR. Cells were cultured in monolayer in RPMI 1640 medium (Cambrex, Walkersville, MD, USA) containing 10% fetal bovine serum. 4-HPR and 4-oxo-4-HPR were dissolved at 10 mmol/l in DMSO and then diluted in the culture medium at the tested concentrations; control cells were treated with the same amount of DMSO as treated cells. Cells treatment started 24 h after seeding to allow cell adhesion. Culture medium with or without drugs was replenished every 3rd day. Cell number was determined by trypsinized cell count using a Z2 counter (Beckman Coulter Fullerton, CA, USA). The antiproliferative activity of 4-HPR and 4-oxo-4-HPR in each cell line was tested in three independent experiments with three replicate wells for each analysis. Differences in cell number after continuous or short treatments were evaluated by the Student's *t*-test.

Results

The demographic characteristics of the patients included in the present study are reported in Table 1. Of the 18 patients, 11 were males and 7 were females; the median age was 7.5 years (range 4–19 years) and the median body mass index (BMI) was 16.2 kg/m^2 (range 12.7–25.4 kg/m^2). Two of the patients were adults (older than 16 years) whereas all the others had to be considered children (aged 2–12 years) [25]. Details regarding the disease staging have been reported earlier [15]. Briefly, all except patient no. 17 who had stage three neuroblastoma, had stage four disease.

Table 1 Patients demographic characteristics

Dose (mg/m^2)	Patient[a] (*n*)	Sex	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)
100	2	M	6	122	24.0	16.1
	3	F	19	162	51.0	19.4
	6	F	6	113	21.7	17.0
300	13	M	9	126	30.0	18.9
	14	M	8	126	23.0	14.5
	15	M	8	131	28.0	16.3
400	16	F	8	133	26.9	15.2
	17	M	5	114	19.0	14.6
	19	F	19	167	54.0	19.4
600	27	F	4	104	17.4	16.1
	28	M	10	143	38.8	19.0
	29	M	5	112	19.4	15.6
1,700	42	M	9	128	23.7	14.5
	43	M	5	108	20.0	17.1
	44	F	7	119	18.0	12.7
4,000	51	M	6	124	39.0	25.4
	53	M	10	133	29.0	16.4
	54	F	6	117	20.4	14.9
M/F = 11/7						
Median			7.5	125	23.9	16.2
Range			4-19	104-167	17.4-54.0	12.7-25.4

BMI = body mass index

[a] All patients, except patient no. 17, had stage 4 neuroblastoma; patient no. 17 had stage 3 neuroblastoma

The semilogarithmic plots of mean plasma concentration vs time curves of 4-HPR, after the 1st and 28th oral administration of the drug are reported in Fig. 1a, b, respectively, whereas the derived pharmacokinetic parameters are shown in Table 2. The plasma concentrations of 4-HPR were above the limit of quantitation of the bioanalytical method in all treated patients at all investigated times. This allowed an accurate pharmacokinetic analysis in all the selected patients. The pharmacokinetic profiles of the two metabolites 4-MPR and 4-oxo-4-HPR are reported in Fig. 1c, d and Fig. 1e, f, respectively, and the derived pharmacokinetic parameters are summarized in Tables 3 and 4. 4-MPR was detectable in all the samples, except in samples collected 1 h after the first administration from

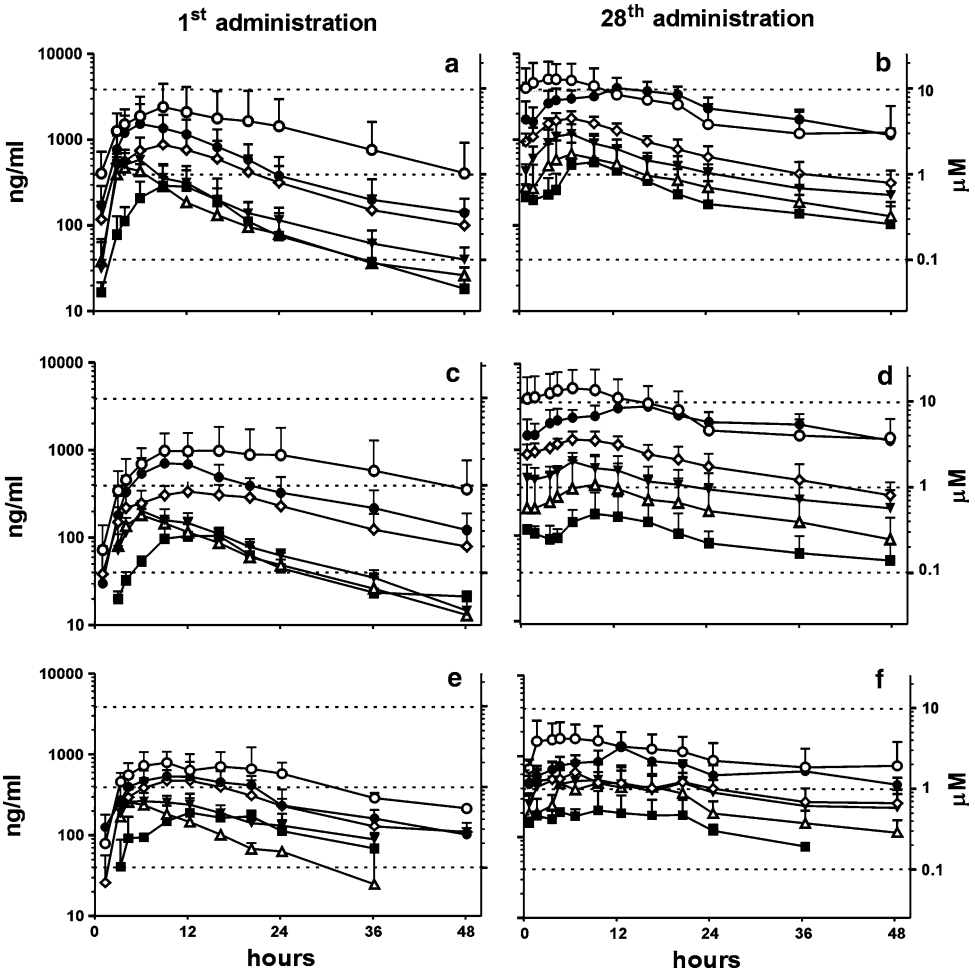

Fig. 1 Semilogarithmic plots of mean plasma concentrations vs time curves of 4-HPR (**a**, **b**), 4-MPR (**c**, **d**) and 4-oxo-4-HPR (**e**, **f**) after the first and 28th administration of 4-HPR. The drug was administered at the following doses: 100 (*dark filled square*), 300 (*open triangle*), 400 (*inverted dark filled triangle*), 600 (*open diamond*), 1,700 (*dark filled circle*), and 4,000 (*open circle*) mg/m^2/day. Plasma concentrations (mean ± SD) are expressed in ng/ml (*left axis*) and in μM (*right axis*)

patients treated with 100, 300 and 400 mg/m^2. 4-oxo-4-HPR was detectable in all the samples except in the samples collected at 1 and 48 h after the first administration of 100, 300 and 400 mg/m^2 and 48 h after repeated administrations of 100 mg/m^2. Table 5 summarizes the most relevant pharmacokinetic parameters for 4-HPR, 4-MPR and 4-oxo-4-HPR providing a convenient comparison across parent drug and metabolites.

Single administration

After the first administration, 4-HPR t_{max} occurred between 4 and 8 h (Fig. 1a and Table 2) with no statistically significant difference among doses. Over the dose range investigated, 4-HPR C_{max} and AUC increased with the administered dose: mean C_{max} increased from 0.9 to 6.6 μM and mean AUC from 13.9 to 170.2 μM h. However, when analysis of linearity was performed, the dose-adjusted C_{max} values after the highest tested dose of 4,000 mg/m^2 resulted significantly lower ($P \leq 0.05$) than those observed after the lowest tested doses of 100 and 300 mg/m^2. No statistically significant differences were found with regard to the dose-adjusted AUC values after the 4,000 mg/m^2 dose. The mean terminal elimination half-life ranged from 10 to 14 h (mean 12 ± 2 h, see Table 5) with no statistically significant difference among doses. Thus, the results indicate that the pharmacokinetics of 4-HPR were linear (dose-independent) in the dose range 100–1,700 mg/m^2 and that the increase in the plasma concentrations after 4,000 mg/m^2 was less than proportional based on the dose increase.

4-MPR C_{max} (Fig. 1c and Table 3) occurred within 6 and 12 h postdosing, i.e., 2–4 h later than that of the parent drug. No statistically significant differences were seen with regard to t_{max} and the dose-adjusted C_{max} and AUC values, with the only exception of the mean C_{max} value after 4,000 mg/m^2 that resulted significantly lower ($P \leq 0.01$) than those observed after 100 mg/m^2. The C_{max} and AUC of 4-MPR were lower (2–3 times) than those of the parent drug. The apparent mean elimination half-life of 4-MPR ranged from 11 to 18 h (mean = 15 ± 4 h, see Table 5) and it was comparable with that of 4-HPR.

The mean t_{max} of 4-oxo-4-HPR (Fig. 1e and Table 4) were similar to those of 4-MPR and ranged from 5 to 11 h.

Table 2 Mean 4-HPR pharmacokinetic parameters (±SD) after

1st Administration						28th Administration							
Dose (mg/m^2)	C_{max} (μM)	t_{max} (h)	AUC_{0-24} (μM h)	AUC (μM h)	$t_{1/2}$ (h)	C_{max} (μM)	$R_{ac,C_{max}}$	t_{max} (h)	AUC_{ss} (μM h)	$R_{ac,AUC_{0-24}}$	$C_{ss,av}$ (μM)	AUC (μM h)	$t_{1/2}$ (h)
100	0.9 ± 0.5	8 ± 4	10.5 ± 6.8	13.9 ± 9.1	10 ± 2	1.6 ± 1.4	1.7	7 ± 2	21.8 ± 20.2	2.0	0.9 ± 0.8	43.7 ± 30.6	18 ± 7
300	1.5 ± 0.1	4 ± 1	12.8 ± 0.9	16.8 ± 0.7	13 ± 2	1.9 ± 1.3	1.4	8 ± 3	28.9 ± 19.2	2.2	1.2 ± 0.8	50.1 ± 30.1	19 ± 2
400	1.5 ± 0.4	5 ± 1	17.1 ± 5.0	22.6 ± 7.0	12 ± 2	3.2 ± 0.9	2.3	6 ± 3	46.2 ± 10.6	3.0	1.9 ± 0.4	87.8 ± 34.7	24 ± 11
600	2.6 ± 0.9	8 ± 3	34.8 ± 14.9	50.6 ± 22.1	14 ± 3	4.9 ± 0.9	2.0	5 ± 1	75.5 ± 14.3	2.4	3.1 ± 0.6	128.0 ± 38.2	19 ± 7
1,700	4.3 ± 2.3	6 ± 2	54.1 ± 26.1	75.1 ± 40.1	12 ± 2	9.6 ± 2.4	2.5	10 ± 3	178.5 ± 76.2	3.4	7.4 ± 3.2	403.5 ± 76.5	27 ± 12
4,000	6.6 ± 4.9*	7 ± 3	103.0 ± 97.1	170.2 ± 186.1	12 ± 3	14.5 ± 7.9	2.6	4 ± 1	238.0 ± 146.6	3.0	9.9 ± 6.1	504.8 ± 322.2	25 ± 5

*$P < 0.05$ vs dose-adjusted C_{max} after 100 and 300 mg/m^2

Table 3 Mean 4-MPR pharmacokinetic parameters (±SD) after

1st Administration						28th Administration								
Dose (mg/m^2)	C_{max} (μM)	t_{max} (h)	AUC_{0-24} (μM h)	AUC (μM h)	$t_{1/2}$ (h)	C_{max} (μM)	$R_{ac,C_{max}}$	t_{max} (h)	AUC_{ss} (μM h)	$R_{ac,AUC_{0-24}}$	$C_{ss,av}$ (μM)	AUC (μM h)	$t_{1/2}$ (h)	AUC_m/AUC_p
100	0.3 ± 0.1	10 ± 3	3.6 ± 1.6	6.2 ± 3.0	18 ± 9	0.6 ± 0.4	2.2	8 ± 2	9.5 ± 5.4	2.7	0.4 ± 0.2	22.9 ± 18.2	29 ± 9	0.5
300	0.4 ± 0.1	6 ± 0	5.4 ± 0.7	7.6 ± 1.5	12 ± 3	1.2 ± 1.2	2.6	11 ± 2	20.1 ± 19.9	3.5	0.8 ± 0.8	39.2 ± 33.5	23 ± 5	0.7
400	0.5 ± 0.1	6 ± 0	7.9 ± 3.1	8.7 ± 1.9	11 ± 1	2.1 ± 0.5	4.6	6 ± 0	46.2 ± 13.3	5.0	1.4 ± 0.6	85.1 ± 47.2	30 ± 9	1.1
600	0.9 ± 0.1	9 ± 4	14.2 ± 2.9	26.6 ± 9.9	16 ± 3	3.6 ± 0.8	4.2	7 ± 2	66.1 ± 16.7	4.7	2.8 ± 0.7	134.5 ± 47.8	27 ± 17	1.0
1,700	1.7 ± 0.4	10 ± 2	25.0 ± 6.1	44.0 ± 16.0	17 ± 2	7.2 ± 2.2	4.2	10 ± 5	146.7 ± 45.6	5.9	6.1 ± 1.9	497.0 ± 99.7	49 ± 36	1.3
4,000	2.5 ± 1.5*	12 ± 3	41.6 ± 28.9	95.8 ± 90.5	18 ± 2	13.5 ± 8.1	5.7	6 ± 2	247.7 ± 155.1	7.2	10.3 ± 6.5	705.6 ± 424.9	46 ± 35	1.5

*$P < 0.01$ vs C_{max} after 100 mg/m^2

Table 4 Mean 4-OXO-4-HPR pharmacokinetic parameters (±SD) after

	1st Administration					28th Administration								
Dose (mg/m^2)	C_{max} (μM)	t_{max} (h)	AUC_{0-24} (μM h)	AUC (μM h)	$t_{1/2}$ (h)	C_{max} (μM)	$R_{ac,C_{max}}$	t_{max} (h)	AUC_{ss} (μM h)	$R_{ac,AUC_{0-24h}}$	$C_{ss,av}$ (μM)	AUC (μM h)	$t_{1/2}$ (h)	AUC_m/AUC_p
100	0.6 ± 0.2	11 ± 10	7.1 ± 2.7	[a]	[a]	0.7 ± 0.3*	1.2	5 ± 4	11.4 ± 5.1	1.2	0.5 ± 0.2	[a]	[a]	*
300	0.7 ± 0.2	5 ± 1	7.9 ± 0.8	[a]	[a]	1.2 ± 0.9	1.0	8 ± 0	20.9 ± 14.8	1.6	0.9 ± 0.6	44.2 ± 24.3	32 ± 3	*
400	0.7 ± 0.2	6± 3	11.4 ± 2.1	[a]	[a]	1.4 ± 0.8	1.1	6 ± 3	26.6 ± 17.1	2.6	1.1 ± 0.7	74.4 ± 36.0	37 ± 10	*
600	1.3 ± 0.7	9 ± 3	20.1 ± 11.2	32.6 ± 16.5	15 ± 7	1.4 ± 0.5	1.2	2 ± 2	28.2 ± 9.9	1.5	1.2 ± 0.4	89.6 ± 43.2	40 ± 10	0.7
1,700	1.4 ± 0.3	11 ± 2	23.2 ± 5.5	39.7 ± 10.1	18 ± 4	2.5 ± 1.1	1.8	9 ± 4	45.8 ± 15.9	1.9	1.9 ± 0.7	142.4 ± 80.1	40 ± 14	0.4
4,000	2.0 ± 0.7*	9 ± 7	35.4 ± 15.0	63.4 ± 3.3	24 ± 5	4.5 ± 2.6	2.3	4 ± 3	81.7 ± 42.9	2.5	3.4 ± 1.8	302.4 ± 194.2	34 ± 15	0.6

* $P < 0.05$ vs dose-adjusted C_{max} after 100, 300, 400 and 600 mg/m^2

[a] The value could not be calculated as plasma concentrations below the limits of quantification did not allow to correctly estimate the elimination phase

Table 5 Summary of 4-HPR pharmacokinetic parameters after 100–4,000 mg/m^2/day in children with neuroblastoma

	Single dose		Multiple doses	
	C_{max} (μM)	$t^a_{1/2}$ (h)	C_{ss} (μM)	$t^a_{1/2}$ (h)
4-HPR	0.9–6.6	12 ± 2	0.9–9.9	22 ± 8
4-MPR	0.3–2.5	15 ± 4	0.4–10.0	34 ± 22
4-oxo-4-HPR	0.6–2.0	19 ± 5	0.5–3.4	37 ± 10

[a] Mean ± SD

No statistically significant differences were seen across doses with regard to t_{max} and the dose-adjusted AUC values whereas the mean adjusted C_{max} values after 4,000 mg/m^2 resulted significantly lower ($P \leq 0.05$) than those observed after 100, 300, 400 and 600 mg/m^2. The apparent mean elimination half-life of 4-oxo-4-HPR ranged from 15 to 24 h (mean = 19 ± 5 h, see Table 5), and it was similar to that of 4-HPR. The mean C_{max} values, which ranged from 0.6 to 2.0 μM, were lower than those of the parent drug and similar to those of 4-MPR.

Repeated administration

The statistical analysis of the trough concentrations of 4-HPR, 4-MPR and 4-oxo-4-HPR indicated that the pharmacokinetics of the parent drug and of its metabolites were at steady state. Similar conclusions can be made based on the observed 4-HPR half-life of 12 h and the duration of the repeated dosing period (28 days) [24]. Therefore, the present study investigated the pharmacokinetics of 4-HPR and of its metabolites at steady state. At all doses, following repeated administrations, the AUC_{ss} were significantly higher than the values of AUC after a single administration ($P < 0.001$) indicating that, according to the superimposition principle [24], the pharmacokinetics of 4-HPR were time-dependent in the dose range investigated. The C_{max} and AUC_{ss} were significantly higher than those found after a single treatment ($P \leq 0.05$) with an accumulation ratio ($R_{ac,C_{max}}$ and $R_{ac,AUC}$) of approximately 2 (Fig. 1b and Table 2) which was higher than expected [24]. 4-HPR C_{max} occurred again within 4–10 h postdosing and ranged from 1.6 to 14.5 μM; the AUC_{ss} ranged from 21.8 to 238.0 μM h, whereas the AUC ranged from 43.7 to 504.8 μM h. 4-HPR $t_{1/2}$ was significantly longer ($P \leq 0.001$) than that observed after a single administration and ranged from 18 to 27 h (mean = 22 ± 8 h, see Table 5). The average drug concentrations during a dosing interval ($C_{ss,av}$) ranged from 0.9 to 9.9 μM. A visual inspection of the drug levels in Fig. 1b indicated that pharmacologically active concentrations (i.e., equal to or above 0.7–10 μM) [6], covering the entire 24 h dosing interval, were achieved starting from the dose of 300 mg/m^2.

The mean 4-MPR C_{max}, AUC_{ss} and $t_{1/2}$ (Fig. 1d and Table 3) were significantly higher compared with those calculated after the first administration ($P \leq 0.05$). C_{max}, occurred from 6 to 11 h postdosing, as for the parent drug, and its accumulation ratio appeared to increase with the dose (from 2.2 after 100 mg/m^2 to 5.7 after 4,000 mg/m^2). A similar trend was observed for the AUC_{ss} whose accumulation ratios increased from 2.7 after 100 mg/m^2 to 7.2 after 4,000 mg/m^2. The apparent terminal half-life ranged from 27 to 49 h (mean value = 34 ± 22 h, see Table 5). Relative to the parent drug, the plasma concentrations of 4-MPR were lower at low doses (100 and 300 mg/m^2), comparable at intermediate doses (400 and 600 mg/m^2) and higher at high doses (1,700 and 4,000 mg/m^2).

Also for 4-oxo-4-HPR the plasma concentrations increased compared with the first administration ($P \leq 0.05$), even if to a lower extent compared to 4-MPR (Fig. 1f and Table 4): the accumulation ratio ranged from 1.0 to 2.3 for C_{max} and from 1.2 to 2.5 for AUC_{ss}. At steady state, the terminal half-life of 4-oxo-4-HPR was longer ($P \leq 0.05$) compared with the first administration (mean value = 37 ± 10 h, see Table 5). The average 4-oxo-4-HPR concentrations during a dosing interval ($C_{ss,av}$) ranged from 0.5 to 3.4 μM (Tables 4 and 5) and from visual inspection of Fig. 1f, these concentrations were maintained during the whole dosing interval. Contrary to what we observed for 4-MPR, the levels of 4-oxo-4-HPR were lower (AUC_m/AUC_p: 0.4–0.7) than those of the parent drug after all the tested doses.

Table 5 summarizes the most relevant pharmacokinetic parameters of 4-HPR and its metabolites. At steady state, after multiple doses, the plasma concentrations of 4-HPR (range 0.9–9.9 μM) and its active metabolite 4-oxo-4-HPR (range 0.5–3.4 μM) were both pharmacologically relevant taking into consideration that, in vitro, the inhibiting concentrations 50% (IC_{50}) against human neuroblastoma cell lines ranged from 0.7 to 6.9 μM for 4-HPR and from 0.4 to 3.5 μM for 4-oxo-4-HPR [6].The elimination half-lives of the parent drug and its metabolites are the average values calculated at all doses, as no apparent differences were observed across doses. The average elimination half-life of 4-HPR, being in the range of 22 h, supports a once daily administration schedule.

Inhibition of neuroblastoma cells proliferation after continuous and intermittent treatment schedules

It is known that the in vitro growth inhibitory effects of 4-HPR and 4-oxo-4-HPR are dose-proportional [6], but it is not known whether the two compounds must be present continuously in order to exhibit the optimal antiproliferative effect. We investigated the growth inhibitory effects of 4-HPR and 4-oxo-4-HPR in two neuroblastoma cell lines by adopting two treatment schedules: one by exposing the cells to the drug for a short time (3 days) followed by a washout period of 3 days during which no drug was added to the culture medium, the other one by exposing the cells to the drug for 3 days followed by additional 3 days drug exposure (total 6 days continuous exposure). 4-MPR, which has no growth inhibitory effects in these cell lines [6], was not tested. 4-HPR and 4-oxo-4-HPR were tested at IC_{50} concentrations, i.e., 5 and 1 μM for 4-HPR and 1 and 0.4 μM for 4-oxo-4-HPR in SK-N-BE and SK-N-MC cells, respectively [6]. In both cell lines, the removal of 4-HPR (Fig. 2a, b) and of 4-oxo-4-HPR (Fig. 2c, d) following a 3-day exposure, was associated with a significantly lower growth inhibition compared with continuous exposure.

Discussion

The objectives of the present study were to assess the pharmacokinetics of 4-HPR and its metabolites after oral administration of single and repeated administrations of the drug in children with neuroblastoma and to investigate the optimal schedule to achieve optimal tumor growth inhibitory effects in vitro. Pharmacokinetic data of 4-HPR and its metabolites in man in studies specifically designed with this aim are scant and therefore, until now, doses and administration schedules have been selected empirically in

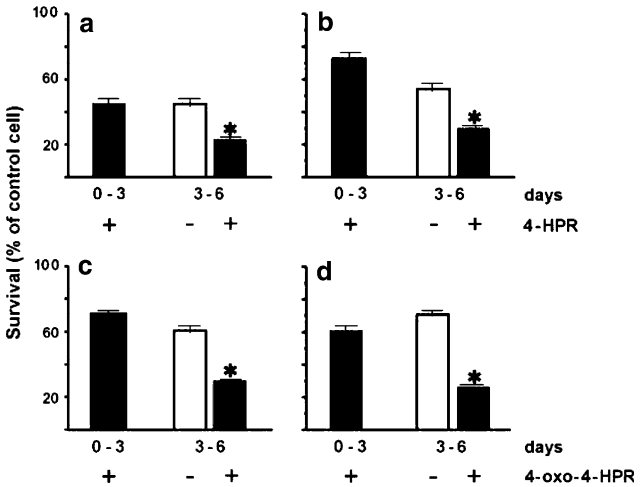

Fig. 2 4-HPR and 4-oxo-4-HPR growth inhibitory effects on SK-N-BE and SK-N-MC neuroblastoma cell lines. Cells were treated 24 h after seeding with the IC_{50} of 4-HPR and 4-oxo-4-HPR: SK-N-BE cells were treated with 5 μM 4-HPR (**a**) or 1 μM 4-oxo-4-HPR (**c**); SK-N-MC cells were treated with 1 μM 4-HPR (**b**) or 0.4 μM 4-oxo-4-HPR (**d**). Surviving cell number was evaluated 3 days after treatment (0–3, *black columns*); cells were then divided and incubated for additional 3 days (3–6) with the drugs (*black columns*) or without the drug (*white columns*). Mean values ± SD; representative experiment of three experiments with similar results. * $P \leq 0.01$ Student's *t*-test

phase I–II clinical trials [5, 12–15]. As a result of this, there is currently a lack of consensus on how future clinical studies with 4-HPR should be designed in terms of both dose and administration schedule. In addition, the safety and efficacy results obtained in different studies in which 4-HPR has been administered at different doses and with different schemes of treatment [14, 15] are difficult to compare. We therefore decided to investigate the pharmacokinetics of 4-HPR and its metabolites in a study that was specifically designed for this aim in children with neuroblastoma.

The results indicated that repeated oral administrations of 4-HPR at doses ranging from 100 to 4,000 mg/m^2 once daily for 28 days affords steady state plasma concentrations of the drug that are pharmacologically effective, i.e., in the 0.7–10 μM range (6), and that these concentrations are maintained for the dosing interval of 24 h, after administrations of doses ranging from 300 to 4,000 mg/m^2. We have previously shown that in adults treated with 4-HPR at 200 mg/day, equivalent to 122 mg/m^2, 1 μM 4-HPR concentrations were achieved at steady state and that the elimination half-life from plasma averaged 27 h [8]. These results are in reasonable agreement with the results obtained in children in the present study in which the average steady state 4-HPR concentrations after 100 mg/mg^2 were 0.9 μM and the average elimination half-life from plasma was 22 h.

The present study allowed an accurate calculation of 4-HPR elimination half life at steady state using two methods, i.e., by calculating the slope of the terminal elimination phase of the plasma concentration vs time plots and by calculating the accumulation ratios compared to the single dose administration [24]. In children with neuroblastoma the elimination half life of 4-HPR, calculated at steady state using the two methods described above, was 22 h and this value remains approximately constant in the dose range 100–4,000 mg/m^2. The agreement of the results obtained with the two methods is remarkable in light of the high inter-subject variability and the limited number of patients enrolled in the present study.

The elimination half-life of 22 h at steady state supports a once daily administration schedule for 4-HPR. Administered with this schedule, i.e., daily for 28 days followed by a 7-day interruption, 4-HPR at doses ranging from 100 to 4,000 mg/m^2/day resulted in manageable toxicity [15]. On the contrary, a less favorable tolerability profile was found in children with solid tumors in which similar daily doses (from 350 to 3,300 mg/m^2/day) were divided into two or three daily doses and were administered in cycles of 1 week followed by periods of 2 weeks during which no drug was administered [14]. A further support for the once daily continuous drug administration, instead of an intermittent schedule of higher doses split during the day, comes from the results of the in vitro experiments. The results obtained in human neuroblatoma cell lines indicated that a treatment schedule that foresaw a longer exposure to 4-HPR and to the active metabolite 4-oxo-4-HPR, produced a greater growth inhibitory activity compared to a shorter treatment schedule followed by a washout period. These findings suggest that administration schemes that foresee weekly cycles of treatment with split daily doses separated by long washout periods, i.e., 2 weeks [12–14], might not yield optimal exposure and thus tumor growth inhibitory activity and might be associated with a worse safety profile [14]. In addition, the difference between the elimination half-lives determined after a single dose (12 ± 2 h) and those determined at steady state (22 ± 8 h) indicates that the pharmacokinetics of 4-HPR are time-dependent and suggest that administration schemes that foresee long (2 weeks) periods of treatment discontinuation might not be optimal as, at each cycle of therapy, drug pharmacokinetics have to return to steady state conditions.

The rate (C_{max}) and extent (AUC) of 4-HPR bioavailability after a single treatment was found to be linear, i.e., dose-proportional, in the dose interval from 100 to 1,700 mg/m^2, whereas after administration of the 4,000 mg/m^2 dose the bioavailability of 4-HPR deviated from linearity being lower than expected. Increased drug metabolism and thus clearance at higher doses can probably be excluded because the pharmacokinetic parameters of 4-MPR and 4-oxo-4-HPR followed the same trend indicating that the biotransformation of 4-HPR was not induced. These results could be more likely due to a saturation of the absorption process at this dose level. In support of this hypothesis is the finding that two out of three children receiving the 4,000 mg/m^2 dose reported diarrhea [15] and this might have affected the absorption of 4-HPR. In fact, 4-HPR is poorly soluble in water and its solubility might be even lower in the presence of watery stools. However, the low number of patients and the high inter-patients variability of 4-HPR C_{max} and AUC at this dose do not allow any further speculation. Specific studies should be conducted to investigate 4-HPR pharmacokinetic linearity at doses higher than 1,700 mg/m^2 by investigating different rising single doses and a higher number of subjects at each dose.

After multiple daily administration, the pharmacokinetics of 4-HPR appeared to be time-dependent, that is the pharmacokinetics of the drug after repeated doses cannot be extrapolated from those after a single administration. At steady state, accumulation of 4-HPR was observed. Both C_{max} and AUC_{ss} increased twofold to threefold relative to the first administration and the estimated half life was found to be longer (22 vs 12 h). Therefore, the efficacy of 4-HPR is expected to be maintained after repeated doses whereas any safety concerns observed after a single dose,

that are related to the plasma concentrations of 4-HPR, are likely to be maintained and possibly worsen after treatment periods of long duration. The accumulation observed for 4-HPR after repeated administration was likely due to the saturation of an elimination process, likely the excretion. Impairment in 4-HPR metabolism seems to be excluded, as the 4-HPR metabolites accumulated at steady state and autoinduction of 4-HPR metabolism after repeated doses was reasonably excluded (see above).

The present study investigated also the pharmacokinetics of two 4-HPR metabolites, namely 4-MPR and 4-oxo-4-HPR. 4-MPR was the main metabolite in plasma. Similarly to 4-HPR, the pharmacokinetics of 4-MPR were linear up to 1,700 mg/m^2 and time-dependent. After repeated treatments, the rate of 4-MPR accumulation increased with the dose increase and, at higher doses, the concentrations of 4-MPR were higher than those of 4-HPR. The apparent elimination half life of 4-MPR was found to be longer than that of 4-HPR (34 h). Nothing is known about the importance of 4-HPR, 4-MPR and 4-oxo-4-HPR in determining the toxicities associated with 4-HPR treatment. However, as 4-MPR is pharmacologically ineffective, any adverse event due to its circulating levels is expected to be, in the dose range investigated, worse at higher doses and after chronic treatment and this is not expected to be accompanied by any improvement in the therapeutic effects.

The results obtained for the other 4-HPR metabolite, 4-oxo-4-HPR, allow few speculations as the plasma concentrations of this metabolite were in some cases below the limit of quantitation of the bioanalytical method employed. However, the steady state plasma concentrations of 4-oxo-4-HPR could be evaluated and they ranged from 0.5 to 3.4 μM. 4-oxo-4-HPR is in vitro more potent than the parent drug and, in neuroblastoma cell lines, 4-oxo-4-HPR IC_{50} ranged from 0.4 to 3.5 μM [6]. Therefore, the present study indicates that the plasma concentrations of 4-oxo-4-HPR might be pharmacologically relevant. Moreover, any pharmacological and toxicological effect produced by 4-oxo-4-HPR is expected to be maintained after repeated doses.

A possible limitation of the present study was the limited number of patients evaluated in light of the observed high inter-subject variability. Future studies in larger pediatric patients cohorts should allow a confirmation of the results presented here. In addition, evaluation of the influence of age and BMI on 4-HPR pharmacokinetic profile should be also assessed.

In conclusion, this is to our knowledge the first study in which the pharmacokinetics of 4-HPR and its major metabolites have been investigated in a rigorously designed trial. The information gathered should be useful for the selection of the appropriate dose and dosing schedule in studies aimed at exploring the therapeutic potential of 4-HPR after oral administration in this patient population and indicate that a continuous rather than intermittent dosing schedule should be selected for optimal therapeutic effects. Finally, the data presented here should provide a reference for future studies aimed at investigating the performance of new formulations of 4-HPR designed to improve its bioavailability.

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