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The plasma pharmacokinetics and cerebrospinal fluid penetration of the thymidylate synthase inhibitor raltitrexed (Tomudex™) in a nonhuman primate model

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Abstract *Purpose*: Raltitrexed (Tomudex™, ZD1694) is a novel quinazoline folate analog that selectively inhibits thymidylate synthase. Intracellularly, raltitrexed is polyglutamated to its active form which can be retained in cells for prolonged periods. The pharmacokinetics of raltitrexed in plasma and cerebrospinal fluid (CSF) were studied in a nonhuman primate model. *Methods*: Animals received 3 mg/m^2 ($n = 1$), 6 mg/m^2 ($n = 3$), or 10 mg/m^2 ($n = 3$) i.v. over 15 min, and frequent plasma samples were obtained over 48 h. CSF samples were drawn from an indwelling 4th ventricular Ommaya reservoir over 48 h. Plasma and CSF raltitrexed concentrations were measured with a novel, sensitive enzyme inhibition assay with a lower limit of quantification of 0.005 μM. A three-compartment pharmacokinetic model was fitted to the raltitrexed plasma concentration-time data. *Results*: The plasma concentration-time profile of raltitrexed was triexponential with a rapid initial decline and a prolonged terminal elimination phase ($t_{1/2} > 24$ h), which was related to retention of raltitrexed in a deep tissue compartment. At the peak approximately 30% of the administered dose was in the deep tissue compartment, and 24 h after the dosing >20% of the administered dose remained in the body with >99% in the deep tissue compartment. The mean peak (end of infusion) plasma concentrations after the 3, 6, and 10 mg/m^2 doses were 1.5, 2.4 and 4.8 μM, respectively. The clearance of raltitrexed ranged from 110 to 165 ml/min per m^2, and the steady-state volume of distribution exceeded 200 l/m^2. The CSF penetration of raltitrexed was limited (0.6 to 2.0%) and drug could only be detected in the CSF following a 10 mg/m^2 dose. *Conclusions*: The elimination of raltitrexed is triexponential with a prolonged terminal elimination phase. The pharmacokinetic profile is consistent with extensive polyglutamation and intracellular retention of ralitrexed. The three-compartment model presented here may be useful for the analysis of the pharmacokinetics of raltitrexed in humans.

Key words Antifols · Thymidylate synthase inhibitor · Pharmacokinetic model · Enzyme inhibition assay

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Introduction

Folates are cofactors in the biosynthetic pathways for purines and thymidine, and antifolates that block these pathways at various sites have been used in the treatment of a variety of human cancers. The classical antifol, methotrexate (MTX), blocks *de novo* nucleotide synthesis primarily by depleting cells of chemically reduced tetrahydrofolate cofactors through inhibition of dihydrofolate reductase (DHFR). In addition, MTX polyglutamates, which are synthesized by folylpolyglutamate synthetase (FPGS) and retained in cells, and dihydrofolates, which accumulate as a result of DHFR inhibition, may inhibit thymidylate synthase (TS) and other enzymes involved in the purine biosynthetic pathway [4]. Other antifolates that inhibit DHFR, such as trimetrexate and 10-ethyl-10-deazaaminopterin, have been clinically evaluated, and antifolates that are directed against other targets, such as TS and glycinamide ribonucleotide (GAR) transformylase, are being developed.

The quinazoline-based antifol, raltitrexed (Tomudex™, ZD1694), is a specific inhibitor of TS, the enzyme that converts deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and is the first of a series of such compounds to reach advanced clinical development [6, 12]. Similar to MTX, raltitrexed is transported into cells via a folate carrier in cell membranes and is polyglutamated by FPGS. Polyglutamation is essential for TS inhibition and for prolonged

intracellular retention [13]. Raltitrexed has demonstrated activity against a variety of tumors in preclinical studies and clinical trials, including partial and complete responses in colorectal and breast cancer [7, 8, 14, 19].

The pharmacokinetics of raltitrexed were initially studied in mice, using a high-pressure liquid chromatography (HPLC) assay with a limit of quantification of 0.2 μ*M*. This method is too insensitive to characterize the terminal elimination phase of raltitrexed [16]. We developed a novel, sensitive enzyme inhibition assay (lower limit of quantification 0.005 μ*M*) to measure raltitrexed in biological fluids (Wideman et al., manuscript in preparation). To better define the disposition of raltitrexed and to determine its penetration into the cerebrospinal fluid (CSF), we studied the pharmacokinetics of raltitrexed in nonhuman primates.

Methods

Instrumentation

The raltitrexed assay was carried out in a Biotek EL 340 microplate kinetic spectrophotometer (BioTek Instruments, Winooski, Vt.) which was interfaced to a Macintosh SE 30 computer running Delta Soft II v.3.3B software (Bio-Metallics, Princeton, N.J.). A solid phase extraction vacuum manifold, VAC Elut SPS24 and 3 ml C_{18} solid phase extraction columns were obtained from Varian (Harbor City, Calif.), and 96-well flat-bottom microplates were obtained from Costar Corporation (Cambridge, Mass.). A sample evaporator was obtained from Zymark (Hopkinton, Mass.).

Reagents, drug and chemicals

Dihydrofolate (FH_2), 2-mercaptoethanol, reduced nicotinamide adenine dinucleotide (NADPH), Tris-HCl, Tris-base, tetrahydrofolate (FH_4), dUMP, EDTA and adenosine 5′-triphosphate (ATP) were obtained from Sigma Chemical Company (St. Louis, Mo.); methanol, formaldehyde, potassium chloride (KCl) from Fisher Scientific Company (Fair Lawn, N.J.); magnesium chloride ($MgCl_2$) from Mallinckrodt Specialty Chemicals Company (Paris, Ky.); DHFR and TS from *Lactobacillus casei* from Biopure Corporation (Cambridge, Mass.); L-glutamic acid from Grand Island Biological Company (Grand Island, N.Y.). FPGS from an *E. coli* mutant was kindly provided by Dr. Barry Shane, University of California (Berkeley, Calif.). Raltitrexed was kindly provided by Zeneca Pharmaceuticals (Wilmington, Del.).

Drug administration

The dose of raltitrexed was diluted in 0.9% sodium chloride to yield a final concentration of 0.1 mg/ml and sterilized by filtration through a 0.22-μm filter. No loss of drug was observed following filtration (data not shown). A single dose of 3 mg/m^2 was administered i.v. over 15 min to the first animal and a dose of 6 or 10 mg/m^2 was administered i.v. over 15 min to three animals each.

Animal experiments

Animals

Six adult male Rhesus monkeys (*Macaca mulatta*) weighing 9.5–13.6 kg were used for this study (one animal was studied twice and received 6 mg/m^2 and 10 mg/m^2 raltitrexed). The animals were fed Purina Monkey Chow twice daily and were group housed in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council, National Academy Press, Washington DC, 1996. The experiments for this study were approved by the National Cancer Institute Animal Care and Use Committee. Blood samples were drawn through a catheter placed in either the femoral or saphenous vein contralateral to the site of drug infusion. CSF samples were drawn from a subcutaneously implanted Ommaya reservoir attached to an indwelling Pudenz catheter, with its tip located in the fourth ventricle [18].

Experiments

Blood samples of 2–3 ml were collected before and at the end of the 15-min infusion, and then 5, 15, 30 and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 26, and 48 h after the end of the infusion. Plasma was immediately separated by centrifugation and frozen at −70 °C until analysis. Ventricular CSF samples of 0.3 ml were collected before and at the end of the raltitrexed infusion, and then 0.5, 1, 2, 3, 4 and 6 h after the end of the infusion in the first three animals. In subsequent experiments CSF samples (1.0 ml) were collected before the infusion and 0.5, 1, 2 and 4 h after the end of the infusion.

Because the administered doses of raltitrexed were close to or exceeded the maximum tolerated dose in humans based on adult phase I trials (maximum tolerated dose, 3.5 and 4.5 mg/m^2), leucovorin, (10–20 mg, i.v.) was administered three times daily for 2 days starting 26 h after administration of raltitrexed. Animals were evaluated for signs of clinical toxicity and monitored with complete blood counts, serum electrolytes, blood urea nitrogen and creatinine twice weekly for 2 weeks after each experiment.

Raltitrexed assay

Raltitrexed was quantified using a novel TS enzyme inhibition assay developed for a UV microplate reader. In brief, plasma and CSF samples were initially applied to a 3-ml C_{18} solid-phase extraction column which had been previously wetted with 3 ml methanol followed with 3 ml water and equilibrated with 3 ml sodium acetate buffer (pH 5.0, 0.1 *M*). After sample application, the extraction columns were washed with 3 ml sodium acetate buffer and the samples were eluted with 3 ml methanol. Eluted samples were evaporated to dryness under nitrogen at 50 °C and redissolved in 120 μl Tris buffer (pH 8.5, 0.16 *M* with 8.3 m*M* glutamic acid, 33 m*M* $MgCl_2$ and 167 m*M* KCl). Raltitrexed in reconstituted samples was polyglutamated by adding 20 μl 8.8 μ*M* FPGS and 10 μl 0.1 *M* ATP and incubating at 37 °C for 3 h. This converts >90% of raltitrexed-$(glutamate)_1$ to raltitrexed-$(glutamate)_3$ (Wideman et al., manuscript in preparation).

The polyglutamated sample was added to a reaction mixture containing 0.00032 U TS, 7 μg dUMP, 0.0008 U DHFR, 20 μg NADPH, 0.4 mg $MgCl_2$ and 5,10-methylene tetrahydrofolate (prepared by incubating 2.6 μg FH_4 with 28 μg formaldehyde in the presence of 156 μg 2-mercaptoethanol). In this reaction mixture, the synthesis of dTMP from dUMP by TS converts 5,10-methylene tetrahydrofolate to FH_2 and DHFR converts this FH_2 to FH_4 and oxidizes NADPH to NADP. The oxidation of NADPH to NADP was monitored at 340 nm for 65 min in a kinetic microplate reader at room temperature. The reaction rate was linear over 65 min and the change in absorption at 340 nm was proportional to the amount of raltitrexed in the reaction mixture. The lower limit of quantification for raltitrexed in plasma and CSF was 0.005 μ*M* and 0.01 μ*M*, respectively. The within-day coefficients of variation (CV) for 0.007, 0.01, and 0.07 μ*M* standards were 19%, 9% and 9%, respectively, and the between-day CVs were 25%, 10% and 7% at these concentrations.

Pharmacokinetic analysis

A three-compartment pharmacokinetic model (Fig. 1) was fitted to the plasma raltitrexed concentration-time data using MLAB, a mathematical modeling program [17]. As the pharmacokinetics

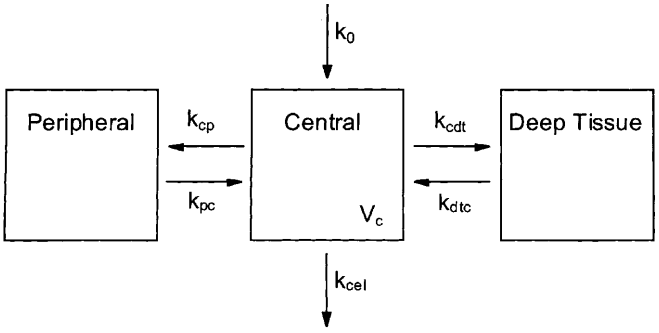

Fig. 1 Three-compartment pharmacokinetic model with elimination of drug from the central compartment used to describe raltitrexed distribution and elimination (k_0 drug infusion rate; V_c volume of the central compartment; k_{cel} elimination rate constant; k_{cp}, k_{pc}, k_{cdt}, and k_{dtc} rate constants describing the exchange of drug between compartments)

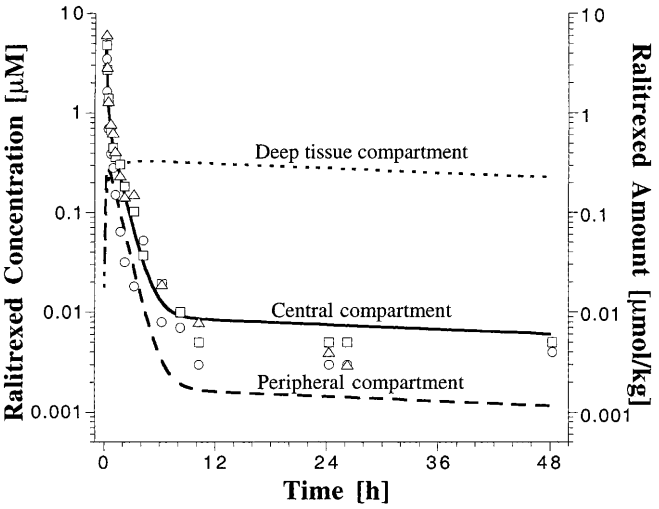

Fig. 2 Plasma concentration-time curve for three animals that received 10 mg/m^2 raltitrexed (*open symbols* measured raltitrexed plasma concentrations, *solid line* model-predicted central compartment concentrations of raltitrexed). The amount (μmol/kg) of raltitrexed in the peripheral compartment (*dashed line*) and the deep tissue compartment (*dotted line*) were simulated from the model-derived parameters

appeared linear, data from the animals studied at the 6 and 10 mg/m^2 dose levels were combined and fitted simultaneously (i.e. combined into one data file). Plasma concentrations were weighted using the MLAB EWT function which computes a weight vector from estimates of reciprocal variance values [17]. Clearance (Cl) was calculated from $\mathrm{Cl} = V_c \cdot k_{cel}$, and the area under the concentration-time curve (AUC) was determined by dividing the dose by Cl. The maximum fraction of the dose of raltitrexed in the deep tissue compartment was calculated by dividing the peak amount of raltitrexed in the deep tissue compartment (simulated from the model) by the dose administered. The triexponential function $C(t) = \sum_{n=1}^{3} A_n \cdot e^{-\lambda_n \cdot t}$ was fitted to the model-predicted central compartment raltitrexed concentration-time data for the 6 and 10 mg/m^2 dose levels using MLAB. Terminal half-lives were then calculated by dividing 0.693 by the terminal rate constant (λ_3). Volume of distribution at steady state was calculated from $V_{ss} = \mathrm{Dose} \cdot \mathrm{AUMC}/\mathrm{AUC}^2 - T \cdot \mathrm{Dose}/2 \cdot \mathrm{AUC}'$ where AUMC is the area under the moment curve and T is the duration of the infusion. The CSF AUC was determined using the trapezoidal method.

Results

The plasma concentration-time profile of raltitrexed was triexponential with a rapid initial decline and a prolonged terminal elimination phase (Fig. 2). This profile was well described by the three-compartment model, which includes central, peripheral and deep tissue compartments and elimination of raltitrexed from the central compartment. The model parameters for the three dose levels are listed in Table 1. The movement of drug out of the deep tissue compartment occurred at a rate (k_{dtc}) that was only 1% of the influx rate (k_{cdt}) and was also considerably slower than the drug elimination rate (k_{cel}). This retention of raltitrexed in the deep tissue compartment accounts for the prolonged terminal elimination phase of the drug.

Using the fitted model parameters, the amount of drug in the deep tissue compartment was simulated (Fig. 2). The amount of raltitrexed in the deep tissue compartment was predicted to peak at 105, 150, and 270 min after raltitrexed administration for the 3, 6, and 10 mg/m^2 dose levels, and at the peak the percent of the administered dose in this deep tissue compartment at the 3, 6, and 10 mg/m^2 dose levels was 27%, 28%, and 30%, respectively. For the 6 and 10 mg/m^2 doses, the model simulations predicted that 21% and 26% of the administered dose would remain in the body at 24 h after administration of raltitrexed and that >99% of the drug would be in the deep tissue compartment at this later time-point.

Table 2 lists the plasma and CSF pharmacokinetic parameters for raltitrexed in nonhuman primates. The mean peak (end of infusion) plasma concentrations and AUCs increased in proportion to the dose (Table 2). For the animal studied at the 6.0 mg/m^2 and 10 mg/m^2 dose level the clearance was 250 ml/min per m^2 at both dose levels, and the AUC was 0.87 $\mu M \cdot$ h and 1.47 $\mu M \cdot$ h, respectively. Plasma raltitrexed concentrations had declined to ≤0.015 μM by a median of 6 h (range 3–8 h), but still could be measured in six of seven animals 24 h after drug administration and in four of seven animals 48 h after drug administration. The concentrations at these late time-points approached the limit of quantification for the assay, and thus an accurate characterization of the terminal elimination phase was difficult. The estimates of the terminal half-lives for the 6 and 10 mg/m^2 dose levels were considerably longer than 24 h.

Raltitrexed could only be detected in CSF following administration of 10 mg/m^2. Detectable concentrations (0.01 to 0.015 μM) occurred 30 min after the dose in two animals and at 30 and 60 min in one animal. The assumption was made that time-points immediately prior to (i.e. preinfusion of raltitrexed in three animals) and following the last detectable CSF raltitrexed concentrations (60 min after the end of the raltitrexed infusion in two animals and 120 min after the raltitrexed infusion in one animal) were at the limit of detection for the assay,

Table 1 Model parameters from fitting a three-compartment model with elimination from the central compartment (see Fig. 1) to the plasma concentration-time data for raltitrexed studied in seven animals at three dose levels. Values represent the fitted parameter ± standard deviation

Dose level (mg/m^2)	n	V_c (l/m^2)	k_{cel} (h^{-1})	k_{cp} (h^{-1})	k_{pc} (h^{-1})	k_{cdt} (h^{-1})	k_{dtc} (h^{-1})
3	1	2.4 ± 0.6	2.9 ± 1.5	2.4 ± 2.9	2.7 ± 1.6	1.1 ± 1.8	0.11 ± 0.37
6	3	2.5 ± 0.2	4.0 ± 3.0	2.1 ± 1.3	2.1 ± 1.2	1.7 ± 1.8	0.021 ± 0.055
10	3	2.2 ± 0.3	3.1 ± 2.0	2.8 ± 1.9	1.6 ± 0.6	1.4 ± 1.2	0.013 ± 0.030

Table 2 Pharmacokinetics parameters derived as described in the Methods section in six animals at three dose levels (*UD* undetectable, < 0.01 μM)

Dose level (mg/m^2)	n	C_{max}		Clearance ($ml/min/m^2$)	AUC ($\mu M \cdot h$)	V_{ss} (l/m^2)	$t_{1/2}$ (h)
		Plasma (μM)	CSF (μM)				
3	1	1.5	UD	116	0.94	–	–
6	3	2.4 (2.2–2.6)[a]	UD	165	1.3	200	46
10	3	4.8 (3.5–6.1)[a]	0.01 (0.01–0.015)[a]	110	3.3	250	78

[a] Mean (range)

which results in a potential overestimation of the raltitrexed CSF penetration. Using this approach the penetration of raltitrexed in the CSF based on the ratio AUC_{CSF}:AUC_{plasma}, was estimated to range from 0.6 to 2.0%.

The animals tolerated the administration of raltitrexed, followed by leucovorin rescue, well. There was no evidence of clinical toxicity, myelosuppression, or other laboratory abnormalities.

Discussion

Raltitrexed is a classical quinazoline folate analog and a specific inhibitor of TS. Raltitrexed enters cells via a folate carrier and undergoes extensive polyglutamation by FPGS which markedly enhances the intracellular retention of raltitrexed and the ability to inhibit TS [2, 11, 13, 15]. The plasma concentration-time profile of raltitrexed was characterized by a rapid initial decline and a prolonged terminal elimination phase, and was best described by a three-compartment model consisting of a central, peripheral and deep tissue compartment. Rapid uptake and prolonged retention of up to 30% of the administered raltitrexed dose into the deep tissue compartment accounted for the long terminal half-lives of 46 and 78 h for the 6 and 10 mg/m^2 dose levels, respectively.

Triexponential elimination of raltitrexed and a prolonged terminal elimination phase has also been found in mice and dogs [3], but no terminal half-life was calculated. The pharmacokinetic parameters obtained in nonhuman primates in this study are comparable to those reported in adult patients with colorectal cancer who received raltitrexed as a 15-min bolus infusion. The maximum raltitrexed plasma concentration after a dose of 3.0 and 3.5 mg/m^2 was 1.5 μM followed by a rapid decline in raltitrexed concentrations and prolonged elimination with terminal half lives ranging from 36.8 to 43.1 and 105 to 172.3 h for the 3.0 and 3.5 mg/m^2 dose levels respectively [7]. A sensitive radioimmunoassay (lower limit of detection 0.2 ng/ml) was used to analyze plasma raltitrexed concentrations in this phase I trial and samples were obtained for up to 96 h, which may have allowed for a more accurate determination of the terminal elimination half-life of raltitrexed.

Even though intracellular drug exposure and polyglutamation were not measured, the pharmacokinetic profile presented here is consistent with extensive polyglutamation and intracellular retention of raltitrexed. More than 30% of the administered dose was present in the deep tissue compartment at the peak and was retained for a prolonged time period, 25% of the dose still being present in this compartment 24 h after administration of raltitrexed.

Extensive polyglutamation and prolonged retention of raltitrexed has been found in mice that received 5 mg/kg [5-^{3}H]raltitrexed. Raltitrexed polyglutamates were detected in liver, kidney and gut tissue and accounted for at least 75% of the total drug concentration in these tissues. Tissue to plasma ratios of raltitrexed 24 h after raltitrexed administration raltitrexed were approximately 70:1, 50:1, 30:1 and 3:1 for liver, kidney, small gut mucosal scrapes and gastrocnemius muscle, respectively [11]. Similar results have been obtained in mice and dogs when raltitrexed and raltitrexed-polyglutamates were measured with a radioimmunoassay with 100% cross-reactivity for raltitrexed polyglutamates [3]. Retention of raltitrexed has also been demonstrated in a mouse tumor model [10] and in humans with colorectal cancer [9] with tumor to plasma raltitrexed ratios of 50:1 and 90:1 at 24 h after raltitrexed administration, respectively.

Our pharmacokinetic model predicted a deep tissue to plasma raltitrexed ratio of 38:1 at 24 h after raltitrexed administration. Even though the amount of raltitrexed retained in the deep tissue compartment of our pharmacokinetic model may represent more than intracellular polyglutamate exposure, this ratio is com-

patible with extensive polyglutamation and intracellular retention of raltitrexed in nonhuman primates, and is in agreement with the findings of the tissue studies described above.

Our model also predicts that plasma raltitrexed concentrations at later time-points significantly underestimate the amount of raltitrexed retained intracellularly, and this is also supported by the actual tissue to plasma ratios measured in other species.

The CSF penetration of raltitrexed is poor. CSF drug concentrations could only be quantified at single time-points after the administration of 10 mg/m^2 raltitrexed, which significantly exceeds the recommended adult dose of 3.0 mg/m^2. The CSF penetration was estimated to range from 0.6 to 2.0%, which is similar to that of methotrexate, a classical antifolate with a CSF penetration of 3% [4].

As with other anticancer drugs, this nonhuman primate model may be predictive of human pharmacokinetics [1, 5]. It appears that the pharmacokinetics of raltitrexed are significantly influenced by polyglutamation and intracellular retention of raltitrexed. The three-compartment model presented here may be useful for the analysis of the pharmacokinetics of raltitrexed in humans.

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