Cancer Chemother Pharmacol (2001) 48: 375–382
DOI 10.1007/s002800100367

ORIGINAL ARTICLE

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Pharmacokinetics and tissue distribution of halofuginone (NSC 713205) in CD_2F_1 mice and Fischer 344 rats

Received: 25 April 2001 / Accepted: 26 July 2001 / Published online: 18 August 2001

Abstract *Purpose*: Halofuginone (HF) inhibits synthesis of collagen type I and matrix metalloproteinase-2 and is being considered for clinical evaluation as an antineoplastic agent. Pharmacokinetic studies were performed in rodents to define the plasma pharmacokinetics, tissue distribution, and urinary excretion of HF after i.v. delivery and the bioavailability of HF after i.p. and oral delivery. *Materials and methods*: Studies were performed in CD_2F_1 mice and Fischer 344 rats. In preliminary toxicity studies in mice single HF i.v. bolus doses between 1.0 and 5.0 mg/kg were used. Pharmacokinetic studies were conducted in mice after administration of 1.5 mg/kg HF. In preliminary toxicity studies in male rats HF i.v. bolus doses between 0.75 and 4.5 mg/kg were used. In pharmacokinetic studies in rats an HF dose of 3.0 mg/kg was used. Compartmental and non-compartmental analyses were applied to the plasma concentration versus time data. Plasma, red blood cells, various organs, and urine were collected for analysis. *Results*: HF doses ≥1.5 mg/kg proved excessively toxic to mice. In mice, i.v. bolus delivery of 1.5 mg/kg HF produced "peak" plasma HF concentrations between 313 and 386 ng/ml, and an AUC of 19,874 ng/ml·min, which corresponded to a total body clearance (CLtb) of 75 ml/min per kg. Plasma HF concentration versus time data were best fit by a two-compartment open linear model. The bioavailability of HF after i.p. and oral delivery to mice was 100% and 0%, respectively. After i.v. bolus delivery to mice, HF distributed rapidly to all tissues, except brain. HF persisted in lung, liver, kidney, spleen, and skeletal muscle longer than in plasma. In the oral study, HF was undetectable in plasma and red blood cells, but was easily detectable in kidney, liver, and lung, and persisted in those tissues for 48 h. Urinary excretion of HF accounted for 7–11% of the administered dose within the first 72 h after i.v. dosing and 15–16% and 16% of the administered dose within 24 and 48 h, respectively, after oral dosing. There were no observed metabolites of HF in mouse plasma or tissues. In rats, i.v. bolus delivery of 3.0 mg/kg produced a "peak" plasma HF concentration of 348 ng/ml, and an AUC of 43,946 ng/ml·min, which corresponded to a CLtb of 68 ml/min per kg. Plasma HF concentration versus time data were best fit by a two-compartment open linear model. After i.v. bolus delivery to rats, HF distributed rapidly to all tissues, with low concentrations detectable in brain and testes. HF was detectable in some tissues for up to 48 h. HF could be detected in rat plasma after a 3 mg/kg oral dose. Peak HF concentration (34 ng/ml) occurred at 90 min, but HF concentrations were less than the lower limit of quantitation (LLQ) by 420 min. Urinary excretion of HF accounted for 8–11% of the administered dose within the first 48 h after i.v. dosing. No HF metabolites were detected in plasma, tissue, or urine. *Conclusions*: HF was rapidly and widely distributed to rodent tissues and was not converted to detectable metabolites. In mice, HF

This work was supported by contract NO1-CM07106 and Grant 2P30 CA47904 awarded by the National Cancer Institute.

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was 100% bioavailable when given i.p. but could not be detected in plasma after oral administration, suggesting limited oral bioavailability. However, substantial concentrations were present in liver, kidney, and lungs. HF was present in rat plasma after an oral dose, but the time course and low concentrations achieved precluded reliable estimation of bioavailability. These data may assist in designing and interpreting additional preclinical and clinical studies of HF.

Keywords Halofuginone · Pharmacokinetics · Collagen type I · Matrix metalloproteinase

Introduction

Halofuginone (HF, Fig. 1), a quinazolinone alkaloid derived from *Dichroa febrifuga*, is an approved veterinary drug used to prevent coccidiosis in poultry [10, 34] and to treat cryptosporidiosis and theileriosis in cattle [23, 25]. The ability of HF to inhibit collagen type I synthesis is well documented [19, 20, 26, 27, 30, 31, 32, 33], and HF has been shown to reduce the excessive amount of collagen (and resulting scar tissue) associated with graft-versus-host disease [20, 26], abdominal adhesions [30], scleroderma [20], pulmonary fibrosis [27], urethral stricture formation [31], postoperative peritendinous fibrous adhesions [32], and hepatic cirrhosis [33]. HF decreases extracellular matrix deposition [11, 12], and has documented inhibitory effects on glomerular mesangial proliferation [28] and intimal hyperplasia [8, 29]. HF also inhibits matrix metalloproteinase gene expression [11, 12].

The ability of HF to inhibit both collagen type I synthesis and matrix metalloproteinase gene expression is potentially useful in reducing tumor growth, because angiogenesis in a growing tumor utilizes collagen type I [1, 11, 14, 15, 16, 17], and matrix metalloproteinases are associated with the ability of a tumor to metastasize [11, 12, 21, 39]. As a result, HF is undergoing preclinical evaluation in anticipation of subsequent clinical trials in patients with cancer. As part of this preclinical evaluation, we developed and validated an HPLC method for quantifying HF in plasma and tissue and then performed pharmacokinetic studies in mice and rats. Our intent was to define the pharmacokinetics, tissue distribution, urinary excretion, and bioavailability of HF.

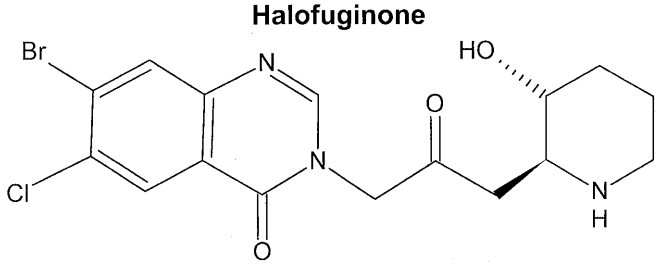

Fig. 1. Structure of halofuginone

Materials and methods

Reagents

Etoposide was obtained from Bristol-Myers Squibb (Princeton, N.J.). HPLC-grade acetonitrile and glacial acetic acid were obtained from Fisher Scientific (Fairlawn, N.J.).

Drug

HF (NSC 713205), supplied by the Toxicology and Pharmacology Branch, National Cancer Institute (Bethesda, Md.), was stored in the dark and at 4–8°C. HF dosing solutions were prepared by dissolving HF in the appropriate volume of sterile phosphate-buffered saline (1.2 m*M* KH_2PO_4, 2.9 m*M* Na_2HPO_4, 154 m*M* NaCl; GIBCO BRL Life Technologies, Rockville, Md.) adjusted to pH 5.3 with HCl. Before administration to animals, solutions were filtered through sterile, nonpyrogenic 0.45 μm syringe filters (Microstar, Costar Corporation, Cambridge, Mass.).

Mice

Specific-pathogen-free adult CD_2F_1 mice (5–6 weeks of age) were obtained from the Animal Production Program, National Cancer Institute (Frederick, Md,). Fischer 344 male rats (7–8 weeks of age) were purchased from Hilltop Lab Animals (Scottsdale, Pa.). Mice and rats without preimplanted jugular venous catheters were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility for at least 1 week before being used. Rats purchased with preimplanted jugular venous catheters were studied within 2 days of arrival. To minimize exogenous infection, mice and rats were maintained in microisolator cages in separate rooms and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Ventilation and air flow in the animal facility were set to 12 changes per hour. Room temperatures were regulated at 72 ± 2ºF, and the rooms were kept on automatic 12-h light/dark cycles. Mice and non-cannulated rats received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, Brentwood, Mo.) and water ad libitum except on the evening prior to dosing, when all food was removed and withheld until 4 h after dosing. Rats with jugular cannulae were not fasted. Sentinel animals (CD-1 mice or Sprague-Dawley rats in cages with bedding that contained 20% bedding removed from the study animal cages at cage change) were maintained in the room housing the mice and rats, respectively, and assayed at 3-month intervals for specific murine pathogens by mouse, or rat, antibody profile testing (Charles River, Boston, Mass.). Sentinel animals remained free of specific pathogens, indicating that the study mice and rats were pathogen-free.

Protein binding studies

In order to assess protein binding of HF, 100 and 300 ng/ml HF solutions were prepared in distilled water, 300 ng/ml HF solutions were prepared in mouse plasma and 100 and 300 ng/ml HF solutions were prepared in rat plasma. A 1-ml aliquot of each solution was placed into the upper chamber of an Amicon Centrifree ultrafiltration device (Amicon Company, Danvers, Mass.). After centrifugation of the ultrafiltration devices for 20 min at room temperature and 2000 *g*, HF concentrations in the resulting ultrafiltrates and in the initial solutions were determined with the HPLC method described below.

Range-finding studies

Groups of five female and five male, CD_2F_1 mice were dosed once i.v. with 5.0, 3.3, 2.2, 1.5, or 1.0 mg/kg HF (0.01 ml/g body weight)

or vehicle (0.01 ml/g body weight). Five male Fischer 344 rats were dosed i.v. with 0.75 mg/kg HF (0.005 ml/g body weight). One rat received 4.5 mg/kg body weight (0.0075 ml/g body weight), while HF doses of 3.0 and 1.5 mg/kg or vehicle were delivered in a volume of 0.005 ml/g to two, two, and five rats each. Mice and rats were observed for 14 days after dosing. Clinical observations were made twice daily. Body weights were measured twice weekly beginning 24 h after dosing. Necropsy body and tissue weights from mice were compared by both parametric and non-parametric methods using Minitab (Minitab, State College, Pa.). If one-way analysis of variance was significant, pair-wise comparisons were made using Dunnett's *t*-test. Non-parametric analyses used the Kruskal-Wallace test followed by pair-wise comparisons using the Mann-Whitney test.

Pharmacokinetic studies

Dosing

Mice were treated with a 1.5 mg/kg dose of HF as a bolus in a volume of 0.01 ml/g fasted body weight, the i.v. dose delivered through a 27-gauge needle placed into a lateral tail vein, the i.p. dose delivered through a 27-gauge needle placed into the right lower abdominal quadrant, and the oral dose delivered through a 1.5-inch 20-gauge curved gavage needle. Rats were treated with a 3.0 mg/kg dose of HF as a bolus in a volume of 0.005 ml/g body weight, the i.v. dose delivered through a 26-gauge needle placed into a lateral tail vein, and the oral dose delivered through a 3-inch 16-gauge curved gavage needle.

Sampling

In all mouse studies, three mice were sampled at each time indicated. In the 1.5 mg/kg i.v. study, blood was sampled at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 420, 960, 1440, 2880, and 4320 min after dosing. In the 1.5 mg/kg i.p. and oral studies, sampling ended at 420 and 2880 min, respectively. In the i.v. study, brain, heart, lungs, liver, kidneys, spleen, fat, red blood cells and skeletal muscle were collected from each mouse at the same times noted for blood sampling. In the oral study, lungs, liver, and kidneys were collected from each mouse. In each study, plasma and tissues from mice killed 5 min after delivery of vehicle served as controls. Blood was collected from mice by cardiac puncture into heparinized syringes, transferred to Eppendorf microcentrifuge tubes and stored on ice until centrifuged at 13,000 *g* for 4 min to obtain plasma. Red blood cells were stored at –70°C. Tissues were rapidly dissected, placed on ice until weighed, and then snap-frozen in liquid nitrogen. Sets of mice to be sampled at 1440, 2880 or 4320 min after dosing were gang-housed in metabolism cages, and urine was collected on ice until animals were killed for blood and tissue sampling.

In the rat i.v. study, groups of three rats were sampled at staggered times so that blood was obtained before drug delivery and at the following times after drug administration: 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 420, 960, 1440, and 2880 min. At the time of the final blood sample, rats were exsanguinated by cardiac puncture. Terminal sampling was carried out at 15, 180, 240, 360, 420, 960, 1440, 2880 min after drug delivery, and brain, heart, lungs, liver, kidneys, spleen, fat, red blood cells, testes and skeletal muscle were collected from each rat. Rats to be sampled at 1440 and 2880 min were housed separately in metabolism cages, and urine was collected until rats were killed. Plasma and tissues from rats injected with only vehicle served as controls. In the rat oral study, three rats were killed at each time-point (5, 10, 15, 30, 45, 60, 90, 120, 180, 360, and 420 min), at which time blood was obtained by cardiac puncture, and the livers, kidneys and lungs were removed. For both rat studies, plasma was prepared from blood, and tissues were dissected and handled as described above for the mouse samples. Plasma, tissues, urine, and dosing solutions for all studies were stored at –70°C until analysis.

Analysis of in vivo samples

Plasma, tissue, and urinary concentrations of HF were determined with HPLC. Plasma samples and red blood cells were extracted directly. Before extraction, tissue samples were thawed and immediately homogenized using a Polytron (Brinkman Instruments, Westbury, N.Y.) in phosphate-buffered saline, pH 7.2, at a ratio of 1:4 (w/v).

Extraction procedure

To a 200 µl sample of plasma, red blood cells or tissue homogenate, were added 10 µl of 20 µg/ml etoposide (internal standard) in acetonitrile and 1 ml acetonitrile. After mixing for 1 min on a Vortex Genie 2 (Model G-560, Scientific Industries, Bohemia, N.Y.) set at 8, samples were centrifuged at 14,000 *g* for 7 min. The resulting supernatants were removed, transferred to 12×75 mm glass culture tubes and evaporated to dryness under a stream of nitrogen (medical grade; Praxair, Pittsburgh, Pa.). The dried residues were resuspended in 130 µl of the mobile phase described below and vortexed for 10 s each on a Vortex Genie 2, set at 8. The resuspended samples were sonicated in a water-bath (Model FS30, Fisher Scientific) for 5 min, vortexed again, transferred to Spin-X centrifuge filter devices (Costar, Corning, N.Y.) and centrifuged at 14,000 *g* for 5 min. The resulting filtrates were placed into amber glass 0.5-ml mini-vials (Agilent Technologies, Wilmington, Del.), and 100 µl was injected by autosampler into the HPLC system.

HPLC

The HPLC system consisted of a Hewlett Packard 1100 autosampler (Hewlett Packard, Wilmington, Del.), a Waters model 600 automated gradient controller (Waters Corporation, Milford, Mass.), a Waters 501 pumps, a Hypersil BDS C18 guard column and a Hypersil BDS C18 column (3 µm, 4.6 mm i.d. × 100 mm) (Alltech Associates, Deerfield, Ill.). The mobile phase, consisting of acetonitrile/50 m*M* sodium phosphate, pH 4.5 (20:80 v/v), was pumped at 1 ml/min. Column eluent was monitored at 243 nm with a Waters 2487 dual-wavelength absorbance detector. Under these conditions, the retention time of HF and internal standard were approximately 12 and 17 min, respectively, and the overall run time was 27 min. The detector signal was processed with Chrom Perfect software (Justice Innovations Chromatography Data Systems, Mountain View, Calif.) so as to integrate the area under each peak. The HF concentration in each sample was calculated by determining the ratio of the HF peak area to that of the corresponding internal standard peak and comparing that ratio with concomitantly performed duplicate standard curves that were prepared using plasma or control tissue homogenates. Standard curves included HF concentrations of 30, 50, 100, 300, 500, and 1000 ng/ml.

Some control tissue samples contained endogenous material that could have potentially co-eluted with HF. Therefore, the concentration of acetonitrile in the mobile phase was adjusted to between 18% and 22% as needed to avoid such interference. Recovery of HF from spiked plasma samples containing 300 and 500 ng/ml was 73 ± 7%. The lower limit of quantitation (LLQ) in plasma, red blood cells and tissue homogenates was 30 ng/ml, and the coefficients of variation in plasma at a low mid-range concentration (50 ng/ml) and a high mid-range concentration (300 ng/ml) were 10.1% and 8.9%, respectively. The standard curve of HF in plasma was linear between 30 and 1000 ng/ml. Samples containing concentrations above the upper limits of each standard curve were reassayed after dilution in the appropriate matrix to a degree calculated to produce a concentration within the linear range.

Pharmacokinetic analysis

The time courses of plasma concentrations of HF were analyzed by both non-compartmental and compartmental methods. The area

under the curve from zero to infinity (AUC) and the terminal half-life (t1/2) were estimated by non-compartmental analysis using the LaGrange function [40] as implemented by the computer program LAGRAN [35]. Total body clearance (CLtb) was calculated from the equation:

$$CLtb = dose/AUC$$

Individual concentrations of HF detected in plasma versus time were fit to compartmental models with the program ADAPT II [9] using maximum likelihood estimation. One-, two-, and three-compartment open linear models were fit to the data. Model discrimination was based on Akaike's information criteria (AIC) [4], calculated as:

$$AIC = 2p + n(\ln WSSR)$$

where p represents the number of parameters, n is the number of observations and WSSR is the weighted sum of squares residuals.

Results

Plasma protein binding

When 100 and 300 ng/ml solutions of HF in distilled water were centrifuged in a Centrifree device, $94 \pm 7\%$ was recovered in the ultrafiltrate. When 300 ng/ml solutions of HF in mouse plasma were processed in a similar manner $15.1 \pm 0.9\%$ was ultrafilterable. When 100 and 300 ng/ml solutions of HF in rat plasma were processed in a similar manner, $16.7 \pm 1.4\%$ and $12.1 \pm 0.1\%$, respectively, were ultrafilterable. These results indicate that HF is 85–90% protein bound and that the binding was quantitatively similar in mouse and rat plasma.

Range-finding studies

In mice, i.v. HF doses of 5.0 and 3.3 mg/kg were toxic to both sexes, and all animals receiving these doses were killed between 1 and 4 days after HF administration. An i.v. HF dose of 2.2 mg/kg resulted in the death, within 4 days, of one of five male mice and three of five female mice. Mice given 1.5 mg/kg of HF i.v. had not returned to their pretreatment weight by 24 h after treatment, but suffered no other obvious consequences of treatment, and mice given 1 mg/kg of HF did not show any behavior different from that of vehicle-treated controls.

No untoward effects were observed in rats treated i.v. with 0.75 or 1.5 mg/kg HF. The one rat given 4.5 mg/kg of HF died within 48 h of dosing. The two rats given 3.0 mg/kg of HF survived the study but had not returned to their pretreatment body weight by the end of the 14-day study.

Pharmacokinetic studies

Mice

Based on the results of the range-finding studies, a 1.5 mg/kg dose was used in the pharmacokinetic studies. An i.v. dose of 1.5 mg/kg, produced "peak" plasma HF concentrations of 313–386 ng/ml at 5 min after injection (Fig. 2). Thereafter, plasma HF concentrations declined in a manner best fit by a two-compartment open linear model described by a volume of the central compartment (Vc) of 3097 ml/kg, an elimination constant (ke) of 0.023 min^{-1}, a transfer constant from central to peripheral compartments (kcp) of 0.051 min^{-1}, and a transfer constant from peripheral to central compartments (kpc) of 0.050 min^{-1}. After 180 min, plasma concentrations of HF were below the LLQ. When calculated with non-compartmental methods, the AUC of HF produced by a 1.5 mg/kg i.v. dose was 19,874 ng/ml·min, corresponding to a CLtb of 75 ml/min per kg.

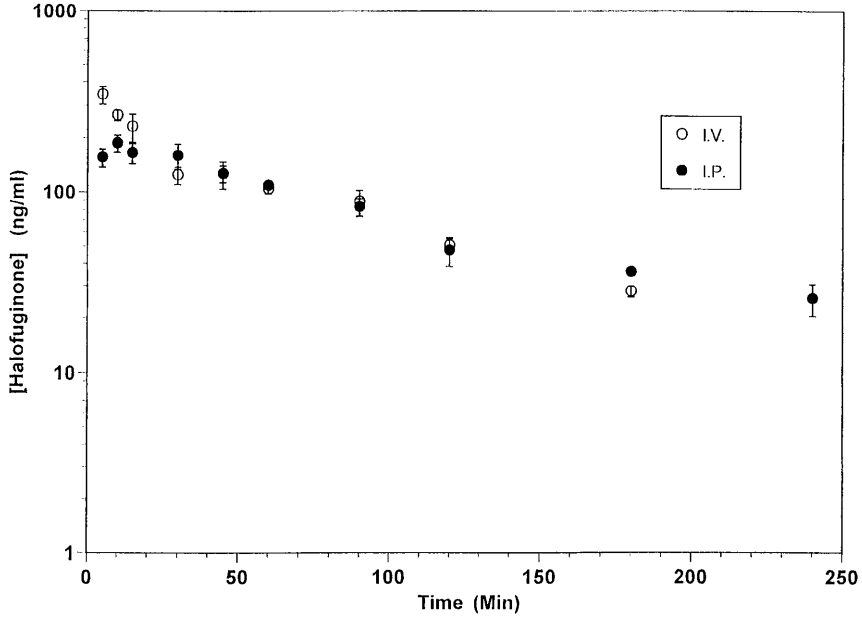

Fig. 2 Concentrations of halofuginone detected in plasma of female CD_2F_1 mice given HF i.v. (*open circles*) or i.p. (*closed circles*) at a dose of 1.5 mg/kg. The data points represent the means from three mice at each time-point (*error bars* ±SD)

There was no evidence of any metabolite of HF. Specifically, there was no material in the plasma of HF-treated mice that was not present in plasma of vehicle-treated control mice or in the dosing solution. Within the first 24, 48, and 72 h after i.v. HF administration, urinary excretion of HF accounted for 4.4–8.5%, 0.9%, and 1.6%, respectively, of the delivered dose.

After i.v. delivery of a 1.5 mg/kg dose, HF was widely distributed to tissues, but did not achieve detectable concentrations in brain (Table 1). The highest tissue concentrations of HF were observed in kidney and lung, with progressively lower concentrations found in heart, liver, spleen, skeletal muscle, fat, and red blood cells. Although HF concentrations in all tissues declined with time, HF was detected in all tissues for at least 90 min after drug delivery and persisted in lung and liver for 72 h (Table 1). When expressed as AUC, the exposure of most tissues to HF was substantially greater than that of plasma (Table 1). As with plasma and urine, no organ contained evidence of putative HF metabolites.

After completion of the i.v. pharmacokinetic study, an effort was made to define the bioavailability of HF after i.p. and oral delivery. After i.p. delivery of a 1.5 mg/kg dose of HF, plasma HF concentrations increased rapidly, with peak concentrations of 173–209 ng/ml observed at 10 min. They then declined to less than the LLQ by 240 min (Fig. 2). Modeled in a non-compartmental fashion, the AUC of HF produced by a 1.5 mg/kg i.p. dose was 21,626 ng/ml·min, indicating a bioavailability of 100%. These plasma concentration versus time data were also modeled compartmentally and were best fit by a two-compartment open linear model with first-order absorption from the peritoneum. In this model, values for Vc, absorption constant (ka), kcp, kpc, and ke were 333 ml/kg, 0.0136 min^{-1}, 0.289 min^{-1}, 0.0011 min^{-1}, and 0.0004 min^{-1}, respectively.

A final mouse pharmacokinetic study was undertaken in which a 1.5 mg/kg dose of HF was administered by oral gavage. At no time after oral dosing were plasma concentrations of HF above the LLQ of the assay employed. However, HF was detectable in tissues (Table 2), with highest concentrations in kidney (1648 ng/g), liver (1023 ng/g), and lung (474 ng/g) occurring 2–3 h after dosing. Detectable concentrations of HF persisted in these tissues for the full 48 h of the study. Within the first 7, 24, and 48 h after oral HF administration, urinary excretion of HF accounted for 3.2–7.1%, 9.1–11.4%, and 15.9%, respectively, of the delivered dose.

Rats

Based on the results of the range-finding studies, a 3.0 mg/kg dose was used in the pharmacokinetic studies.

Table 2 Concentrations of halofuginone in plasma and tissues of mice given a 1.5 mg/kg oral dose of halofuginone. Values are the means from three samples at each time-point

Time (min)	Plasma (ng/ml)	Lung (ng/g)	Liver (ng/g)	Kidney (ng/g)
5	0	86	276	239
10	0	255	376	258
15	0	196	626	459
30	0	304	791	1059
45	0	255	591	759
60	0	289	818	1088
90	0	355	731	1205
120	0	474	1002	1648
180	0	249	1023	1257
240	0	260	949	989
360	0	237	426	661
420	0	161	241	270
960	0	137	238	336
1440	0	132	652	452
2880	0	160	401	619

Table 1 Concentrations and AUCs of halofuginone in plasma and tissues of mice injected i.v. with a 1.5 mg/kg dose of halofuginone. Values are the means from three samples at each time-point

Time (min)	Plasma (ng/ml)	Brain (ng/g)	Heart (ng/g)	Lung (ng/g)	Liver (ng/g)	Kidney (ng/g)	Spleen (ng/g)	Skeletal muscle (ng/g)	Fat (ng/g)	RBCs (ng/ml)
5	342	0	3442	5840	3380	17,534	2081	1212	518	538
10	265	0	2693	5213	3424	17,149	2557	1235	325	325
15	229	0	2318	5527	3429	18,368	3504	1328	412	412
30	125	0	1605	5009	1787	13,040	2455	943	479	479
45	127	0	1179	3883	1666	9,016	2463	889	326	326
60	104	0	1012	3515	1265	11,215	1931	947	219	219
90	89	0	653	2626	1408	6,792	2154	722	138	138
120	51	0	423	2039	1107	5,308	2048	698	0	0
180	28	0	278	1072	1037	2,949	1443	524	0	0
240	18	0	135	916	700	1,925	1160	380	0	0
360	0	0	38	529	442	1,156	639	253	0	0
420	0	0	0	449	380	937	673	204	0	0
960	0	0	0	433	224	402	281	100	0	0
1440	0	0	0	178	164	243	0	0	0	0
2880	0	0	0	215	156	0	0	0	0	0
4320	0	0	0	98	90	0	0	0	0	0
AUC (μg/ml·min)	20	0	181	1752	1305	2,526	1007	372	35	

After an i.v. dose of 3.0 mg/kg, the "peak" plasma HF concentration of 348 ng/ml occurred at 10 min after injection (Fig. 3). Although HF could be detected in plasma until 960 min after injection, concentrations of HF were below the LLQ after 420 min. When calculated with non-compartmental methods, the AUC of HF produced by a 3.0 mg/kg i.v. dose was 43,946 ng/ml·min, corresponding to a CLtb of 68 ml/min per kg. The decline in plasma HF concentrations was best fit by a two-compartment open linear model with values of Vc, ke, kcp, and kpc of 7457 ml/kg, 0.0085 min^{-1}, 0.031 min^{-1}, and 0.025 min^{-1}, respectively. At no time did plasma contain any evidence of an HF metabolite.

Due to the structure of the sampling schedule employed, the distribution of HF to rat tissues (Table 3) could not be defined as thoroughly as was the distribution to mouse tissues (Table 1). At 15 min, concentrations of HF in most tissues were greater than those in plasma (Table 3), with the highest concentrations of HF measured in kidney, lung, and liver. Tissue concentrations of HF decreased with time, but even at 48 h, remained detectable in liver, kidney, and lung (Table 3). HF had limited access to testes and brain (Table 3). At no time did any tissue contain any evidence of an HF metabolite.

After oral delivery to rats at 3.0 mg/kg, HF was detectable in plasma (Fig. 3). HF concentrations in plasma "peaked" at 90 min after oral delivery, but were never much greater than 30 ng/ml, the LLQ of the assay (Fig. 3, Table 4). Concentrations of HF in liver, kidney, and lung were much greater than concomitant concentrations in plasma. Peak HF concentrations in liver (1848 ng/g), kidney (1960 ng/g), and lungs (2934 ng/g) occurred at 15, 120, and 240 min, respectively (Table 4). HF concentrations in all three tissues remained well above 30 ng/g at 420 min. At no time after oral HF

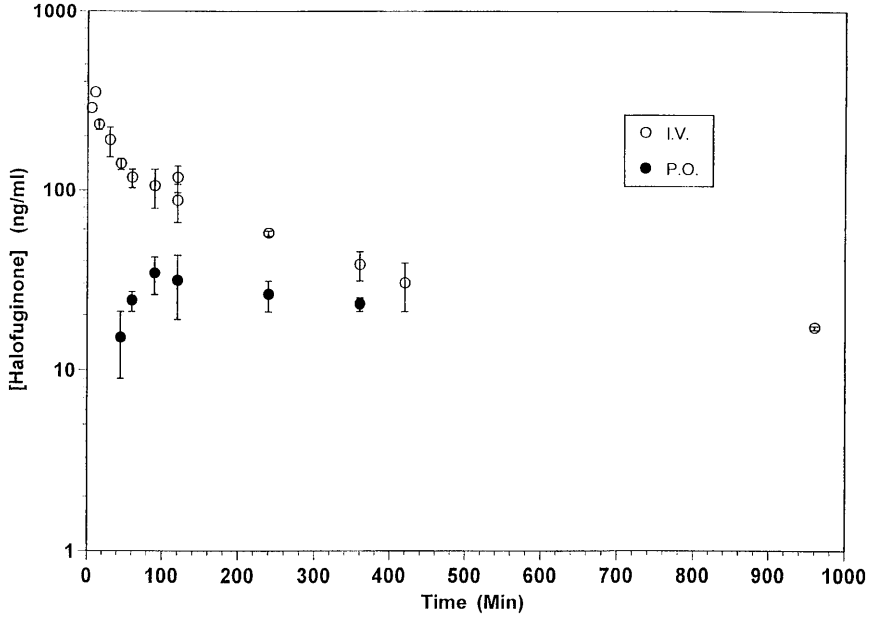

Fig. 3 Concentrations of HF detected in plasma of male Fischer 344 rats given HF i.v. (*open circles*) or orally (*closed circles*) at a dose of 3.0 mg/kg. The data points represent the means from three samples at each time-point (*error bars* ±SD)

Table 3 Concentrations of halofuginone in plasma and tissues of rats injected i.v. with a 3.0 mg/kg dose of halofuginone. Values are the means from three samples at each time-point, except for one sample of plasma at 5 and 10 min, and two samples of all tissues at 420 min

Time (min)	Plasma (ng/ml)	RBCs (ng/ml)	Brain (ng/g)	Heart (ng/g)	Lung (ng/g)	Liver (ng/g)	Kidney (ng/g)	Spleen (ng/g)	Skeletal muscle (ng/g)	Fat (ng/g)	Testes (ng/g)
5	285	88									
10	348	211									
15	231	637	53	4079	7129	4841	11,616	4674	2325	222	52
30	189	489									
45	140	407									
60	117	430									
90	105	318									
120	87	263									
180	117	189	26	1110	6259	1864	4,812	5040	1492	218	92
240	57	138	32	685	6415	1982	4,291	4214	1447	168	97
360	38	103		482	5719	1719	2,931	2710	884	85	94
420	30			439	4974	1729	2,789	2211	835	88	72
960	17			194	2490	957	1,185	1758	271	30	78
1440	0			67	686	474	425	521	93		46
2880	0				106	133	67	21			

Table 4 Concentrations of halofuginone in plasma and tissues of rats given a 3.0 mg/kg oral dose of halofuginone. Values are the means from three samples at each time-point, except for one sample of plasma at 5 and 10 min, and two samples of all tissues at 420 min

Time (min)	Plasma (ng/ml)	Lung (ng/g)	Liver (ng/g)	Kidney (ng/g)
5	0	229	476	0
10	0	234	988	0
15	0	350	1848	357
30	0	411	1409	356
45	15	667	1767	472
60	24	1743	1551	1232
90	34	1926	822	1716
120	31	2864	899	1960
180	31	2632	635	1398
240	27	2934	497	1169
360	23	2153	1719	801
420	0	1931	1729	508

delivery did plasma, liver, kidney, or lung contain a material that might have represented an HF metabolite.

During the first 48 h after i.v. delivery of HF, urinary excretion accounted for $9.6 \pm 1.6\%$ of the dose administered to rats, which agreed closely with the value reported for urinary excretion of HF by mice.

Discussion

Ideally, rational use of any drug should reflect consideration of the pharmacology of that drug. This philosophy may receive even more emphasis in antineoplastic chemotherapy as increased effort is being devoted to developing target-directed agents [2, 3, 5, 6, 7, 18, 22, 24, 36, 37, 38]. Among the targets being actively pursued are matrix metalloproteinases [21, 39] and angiogenesis [14, 15, 16, 17, 22]. HF, a compound used widely in the poultry and meat industries [10, 23, 25, 34], has documented ability to inhibit the former of these targets [11, 12, 13, 28] and to inhibit synthesis of type I collagen [1, 8, 11, 13, 19, 20, 26, 27, 28, 29, 30, 31, 32, 33], which is essential for angiogenesis. Therefore, it is not surprising that there is active interest in evaluating HF as a potential antitumor agent [11, 12]. In anticipation of subsequent clinical trials of HF, effort is being invested in a more extensive preclinical evaluation of HF. The results reported here represent part of that effort. In that regard, discussion of a number of aspects of the current studies is warranted.

The studies in mice in which HF was given by i.p. and oral routes showed the bioavailability by the former route to be 100%, and implied that the bioavailability of the latter route was essentially zero. The excellent bioavailability of HF after i.p. delivery means that antitumor efficacy studies should be able to utilize that route instead of the logistically much more difficult i.v. route. The low bioavailability of HF after oral administration may not be surprising. In previous studies either much less of a pharmacodynamic effect of HF administered orally has been found when compared with i.v. administration [38], or higher oral doses have been used than could be administered i.v. [1, 11, 13, 30, 33]. Our failure to detect HF in the plasma of mice given a dose of 1.5 mg/kg obviously reflects a relative lack of sensitivity of the HPLC assay used. The fact that HF was easily detected in organs of mice given a 1.5 mg/kg oral dose of HF clearly demonstrates that some of the dose was absorbed. Furthermore, the time course of tissue concentrations of HF after oral administration is compatible with an initial hepatic sequestration of this material, with subsequent redistribution to lung and kidney, reflective of the order of capillary beds encountered by an orally administered agent.

The ability of HF to be absorbed after oral administration was also demonstrated directly in rats, which were given a larger dose of HF orally than were mice. Still, the bioavailability of HF in rats was low, with the concentrations of HF detected in rat plasma remaining very close to the LLQ of the HPLC assay. The sensitivity of the assay was a minor problem in terms of the i.v. studies performed in mice and rats and the i.p. studies performed in mice because 85%, 75%, and 81%, respectively, of the plasma $AUC_{0\text{-inf}}$ occurred before plasma HF concentrations fell below the LLQ of the assay. However, it would be desirable to extrapolate $< 10\%$ of the $AUC_{0\text{-inf}}$. This problem of assay sensitivity should be overcome by using LC/MS instrumentation instead of the HPLC with an absorbance detection system used in the current studies, and we are currently actively developing such a method.

The tissue distribution data presented demonstrate the degree of and relative differences in exposure to HF as well as the duration of time that HF persists in various tissues. Furthermore, these data are being used to construct a physiological flow model of HF pharmacokinetics, which should be useful in estimating the pharmacokinetics of this material in humans if it does undergo clinical evaluation.

The failure to demonstrate metabolites of HF in plasma or tissue is consistent with previous reports (www.fda.gov/cvm/efoi/section2/140824s041992.html), although those previous reports are not particularly detailed. It is still possible that there could be metabolites of HF without the chromophore and which would therefore not be detectable in our assay. Finally, our data indicate that HF is more toxic to mice than rats and argue against these differences in toxic doses being due to interspecies differences in metabolism, HF protein binding, or clearance. Based on our results and more extensive toxicology studies that they engendered, the mouse has been defined as being exceedingly sensitive to the toxic effects of HF. Although the cause for this sensitivity remains unknown, this information has prompted the NCI to shift its antitumor activity testing of HF from mouse models to rat models.

In summary, we performed a series of pharmacokinetic studies characterizing the plasma pharmacokinetics and tissue concentrations associated with several routes of administration of HF. The results should be useful in

the design of additional preclinical efficacy and toxicology studies of HF as well as clinical trials. Furthermore, it should be possible to enhance the sensitivity of the HPLC method by interfacing it with mass spectrometric detection.

Acknowledgements We thank Diane Mazzei and her colleagues in the University of Pittsburgh Animal Facility without whose expert assistance, these studies would not have been possible. We also thank Mr. Ezekiel Woods for excellent secretarial assistance in preparation of the manuscript and Collgard Biopharmaceuticals for their insightful comments and suggestions regarding this paper.

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