

Preclinical and Clinical Pharmacodynamic Assessment of L-778,123, a Dual Inhibitor of Farnesyl:Protein Transferase and Geranylgeranyl:Protein Transferase Type-I

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Abstract

Farnesyl:protein transferase (FPTase) inhibitors were developed as anti-Ras drugs, but they fail to inhibit Ki-Ras activity because Ki-Ras can be modified by geranylgeranyl:protein transferase type-I (GGPTase-I). L-778,123, an inhibitor of FPTase and GGPTase-I, was developed in part because it can completely inhibit Ki-Ras prenylation. To support the clinical development of L-778,123, we developed pharmacodynamic assays using peripheral blood mononuclear cells (PBMCs) to measure the inhibition of prenylation of HDJ2 and Rap1A, proteins that are FPTase- and GGPTase-I substrates, respectively. We validated these assays in animal models and show that inhibition of HDJ2 prenylation in mouse PBMCs correlates with the concentration of FPTase inhibitors in blood. In dogs, continuous infusion of L-778,123 inhibited both HDJ2 and Rap1A prenylation in PBMCs, but we did not detect inhibition of Ki-Ras prenylation. We reported previously results from the first L-778,123 Phase I trial that showed a dose-dependent inhibition of HDJ2 farnesylation in PBMCs. In this report, we present additional analysis of patient samples from this trial and a second Phase I trial of L-778,123, and demonstrate the inhibition of both HDJ2 and Rap1A

prenylation in PBMC samples. This study represents the first demonstration of GGPTase-I inhibition in humans. However, no inhibition of Ki-Ras prenylation by L-778,123 was detected in patient samples. These results confirm the pharmacologic profile of L-778,123 in humans as a dual inhibitor of FPTase and GGPTase-I, but indicate that the intended target of the drug, Ki-Ras, was not inhibited.

Introduction

The Ras proteins are M_r 21,000 GTP binding proteins that affect cell proliferation and survival. The Ras isoforms include the splicing variants of the *Ki-ras* gene, *Ki4A-Ras* and *Ki4B-Ras*, and the *Ha-Ras* and *N-Ras* isoforms. Ras functions at the plasma membrane, where it transduces signals from extracellular growth factor receptors to downstream effectors including the Raf and phosphatidylinositol 3' kinases. Ras is normally activated by GTP binding and deactivated by GTP hydrolysis, biochemical events that are facilitated by auxiliary protein factors. The Ras pathway is dysregulated in ~30% of all human cancers through mutations that inactivate the GTPase activity of Ras, giving rise to oncogenic proteins that constitutively send proliferation and survival signals (1, 2).

Ras is post-translationally modified by an isoprenoid lipid that serves to anchor the protein at the plasma membrane, and which is required for its biological and/or transforming functions (3–6). FPTase³ catalyzes this lipid modification by conjugating the 15-carbon farnesyl group to a cysteine residue four amino acids from the COOH terminus of Ras (7). Farnesylation occurs on the cysteine that is part of the CA₁A₂X motif, where C is cysteine, A is typically an aliphatic amino acid, and X is typically serine or methionine for substrates of FPTase. In the past decade, many FTIs have been identified, and several of these are currently under clinical evaluation as a treatment for cancer (8, 9).

Cancer cells transformed with oncogenic *Ha-ras* are growth inhibited by FTIs and are typically more responsive than cells harboring wild-type *ras* or oncogenic forms of *Ki-ras* or *N-ras* (10–12). Similarly, mammary tumors formed in transgenic mice because of expression of oncogenic *Ha-ras* undergo dramatic regression when animals are treated with an FTI (13, 14). In contrast, mammary tumor growth in transgenic mice expressing oncogenic *Ki-ras* or wild-type

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³ The abbreviations used are: FPTase, farnesyl:protein transferase; FTI, farnesyl:protein transferase inhibitor; GGPTase-I, geranylgeranyl:protein transferase type-I; GGTI, geranylgeranyl:protein transferase type-I inhibitor; PBMC, peripheral blood mononuclear cell; PD, pharmacodynamic; DPI, dual prenyltransferase inhibitor; MTD, maximum tolerated dose; MDCK, Madin-Darby canine kidney cell.

N-ras is inhibited by FTI treatment, but the tumors do not dramatically regress (15, 16). The greater responsiveness of tumors containing oncogenic Ha-Ras can be explained by the finding that FTI treatment inhibits the prenylation of oncogenic Ha-Ras (17), but the Ki- and N-Ras oncoproteins remain prenylated (18, 19). Whereas all three of the Ras isoforms are substrates for FPTase *in vitro*, Ki-Ras and N-Ras are also substrates for GGPTase-I, a prenyltransferase related to FPTase (20). Whereas Ki- and N-Ras are normally farnesylated in cells, they are subject to geranylgeranylation by GGPTase-I in FTI-treated cells (18, 19). An oncogenic form of Ki-ras with a genetically altered CAAX motif that results exclusively in its geranylgeranylation can transform rodent cells (6, 21), suggesting that FTI treatment, which leads to the accumulation of geranylgeranylated Ki-Ras, does not significantly alter the oncogenic activity of Ki-Ras.

Because Ki-Ras and N-Ras prenylation is not blocked by FTIs, the antitumor effect of FTIs against tumors harboring oncogenic N-Ras or Ki-Ras may be because of inhibition of other farnesylated proteins. RhoB, a Ras-related protein, has received considerable attention as a FPTase substrate that might be critical to the antiproliferative activity of FTIs (Refs. 22, 23; reviewed in Ref. 24), although not all of the studies support this hypothesis (25). CENP-E (26, 27), a centromere associated kinesin, and PRL-1/PTP-CAAX, a protein tyrosine phosphatase (28, 29), are other farnesylated proteins that might be important to the mechanism of FTIs. Other FPTase substrates include RhoE, RheB, the nuclear lamins, HDJ2, the mammalian homologue of the yeast chaperone protein YDJ1, and a peroxisomal protein, Pxf. Proteins that play an essential role in glycogen metabolism, including phosphorylase kinase α and β , and proteins that function in vision, including transducin, cyclic GMP phosphodiesterase and rhodopsin kinase, are also farnesylated. The antitumor activity of FTIs could be attributable to the inactivation of one or more of these farnesylated proteins.

GGTIs have also been identified (reviewed in Ref. 30) and have been shown to inhibit the growth of tumor lines in culture and in nude mouse xenograft models (31, 32). Human tumor cell lines exposed to a GGTI typically arrest in G₀/G₁ (33, 34) via a mechanism that involves the p53-independent induction of the cyclin-dependent kinase inhibitor, p21 (34–36). Combination treatment with an FTI and a GGTI has been explored as a means of inhibiting Ki-Ras. FTI-GGTI combinations can inhibit the prenylation of Ki-Ras in cultured cells (31, 37), and in normal and tumor tissue in animal models (38). Furthermore, combination FTI-GGTI treatment inhibits mitogen-activated protein kinase signaling in adrenocortical cells that overexpress Ki-Ras (39) and causes the apoptosis of tumor cells in culture to a greater extent than is seen with either agent alone (38).

L-778,123 was selected for clinical evaluation in part because it is a potent FTI and contains GGTI activity sufficient to inhibit the prenylation of Rap1A, a GGPTase-I substrate, as well as Ki-Ras (8, 40–42). To support the clinical development of L-778,123, we developed PD assays that use immunoblotting to measure the inhibition of protein prenylation in PBMC lysates. In this report we show that administration of L-778,123 to animals by continuous infusion inhib-

its the prenylation of the HDJ2 and Rap1A proteins, which are substrates exclusively for FPTase and GGPTase-I, respectively. Furthermore, we show that unprenylated HDJ2 and Rap1A are measurable in patient PBMC samples from a Phase I trial with L-778,123, confirming that the compound hits its intended enzyme targets in humans. However, at the administered dose in both dog and humans, L-778,123 fails to inhibit the prenylation of Ki-Ras.

Materials and Methods

Prenylation Analysis in Cell Culture. Inhibition of HDJ2, Rap1A, and Ki-Ras prenylation in PSN-1 cells was analyzed by immunoblotting as described previously (38).

Compound Dosing and Tissue Isolation in Animal Studies and from Human Subjects. Animal studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals. The Merck-West Point Animal Care and Use Committee reviewed experimental protocols. For mouse studies, compounds were solubilized in 50% DMSO and delivered using Alzet Model 2ML2 osmotic pumps (Alza Corporation, Palo Alto, CA). Pumps were implanted in the subcutis of the right flank of 8–12-week-old female *nu/nu* mice (Charles River Laboratories, Wilmington, MA). Mice were euthanized by CO₂ inhalation, blood was obtained by cardiac puncture using heparinized 20G needles, and spleen was immediately excised and quick-frozen in liquid nitrogen. Dogs were catheterized in the jugular vein and administered compound at 4 ml/h via a Pharmacia Deltec CADD-Plus ambulatory infusion pump. Dogs were fasted overnight before blood draw. Blood was drawn before dosing and at indicated times during the infusion by peripheral venipuncture. Samples from human subjects were prepared as described (43).

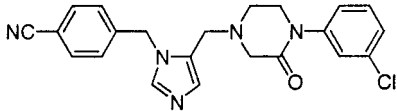
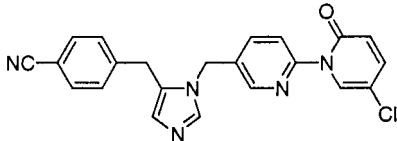
Determination of Drug Concentrations in Plasma. Concentrations of FTI-1 in mouse plasma were determined by liquid chromatography-mass spectrometry essentially as described (38), and concentrations of FTI-1 and L-778,123 in dog plasma were determined by similar methods involving reverse-phase liquid chromatography. Concentrations of L-778,123 in human samples were determined by liquid chromatography-mass spectrometry as described previously (43).

Isolation of WBCs and PBMCs. Mouse WBCs were isolated from heparinized blood by centrifugation for 15 min at 2000 \times *g* at 4°C. Buffy coat WBCs were removed from the plasma-RBC interface, and washed in calcium and magnesium-free PBS by centrifugation. Contaminating RBCs were removed by hypotonic lysis by resuspension of WBC pellets in 6 ml 0.2% w/v NaCl for 30 s, followed by the addition of 6 ml 1.6% w/v NaCl. WBCs were recovered by centrifugation and then frozen at –70°C before analysis.

PBMCs from dogs and humans were isolated over Ficoll-Hypaque density fluid using 4 ml (for dogs) or 8 ml (for humans) Vacutainer-CPT Cell Preparation Tubes (Becton Dickinson) containing sodium citrate following the manufacturers protocol. After purification, cells were washed in PBS and stored at –70°C before analysis. Total protein yields in the human PBMC samples analyzed from the clinical trial

Table 1 Prenylation inhibitors

Enzyme inhibition was determined as described previously (41, 49). Percentage of inhibition of HDJ2, Rap1A, and Ki-Ras prenylation in PSN-1 human pancreatic tumor cells was determined by scanning immunoblots of whole cell extracts as described previously in 38. The dose of compound that caused half-maximal inhibition (EC_{50}) of prenylation was determined from a plot of the percentage inhibition versus compound concentration. IC_{50} and EC_{50} values are averages of 2 or more independent determinations.

Inhibitor	Enzyme inhibition <i>in vitro</i> (IC_{50} , nM)		Prenylation inhibition in cells (EC_{50} , nM)		
	FPTase	GGPTase-I	HDJ2	Rap1A	Ki-Ras
L-778,123 	2	98	92	6,760	6,300
FTI-1 	0.26	7,340	14	>100,000	ND ^a

^a ND, not determined.

were variable; from 8 ml of blood, $720 \pm 500 \mu\text{g}$ total PBMC protein (mean \pm SD; $n = 117$) was obtained.

Immunoblot Analysis of HDJ2, Rap1A, and Ki-ras in Spleen, WBCs, and PBMCs. HDJ2, Rap1A, and Ki-Ras were analyzed from cell and tissue lysates as described previously (38). Percentage of unprenylated HDJ2 in mouse and dog samples represent single determinations; data from the clinical trials of L-778,123 are represented as the mean percentage of nonfarnesylated HDJ2 in human PBMCs, determined from four replicate analyses of each sample. Prenylated and unprenylated forms of Rap1A were detected with antibody sc-65 (Santa Cruz Biotechnology, Santa Cruz, CA). Alternatively, we used sc-1482 (Santa Cruz Biotechnology), an antibody specific for the unprenylated form of Rap1A. When using sc-1482, an antibody specific for Rab6 (Santa Cruz Biotechnology; sc-310) was added to control for protein loading. Ki-ras was analyzed by immunoprecipitation followed by Western blotting as described previously (38). Prenylated and unprenylated Ki-Ras were detected by blotting with c-K-ras Ab-1 clone 234-4.2 (Oncogene Research Products, Boston, MA). Alternatively, a polyclonal rabbit antibody specific for unprenylated Ki-Ras was used. This antibody was raised against a peptide matching the COOH-terminal sequence of Ki-Ras, GKKKKKSKTKCVIM, conjugated to keyhole limpet hemocyanin.

Results

Pharmacological profile of L-778,123 *in vitro*. L-778,123 {4-[[5-[[4-(3-chlorophenyl)-3-oxo-1-piperazinyl]methyl]-1H-imidazol-1-yl]methyl]-benzonitrile} is an inhibitor of both FPTase and GGPTase-I (Ref. 40; see Table 1). Using immunoblotting methods that distinguish between prenylated and unprenylated forms of a protein based on differences in their electrophoretic mobility (38), we confirmed the activity of L-778,123 as a DPI in PSN-1 human pancreatic tumor cells

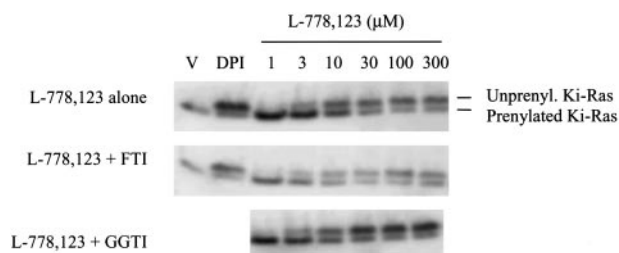


Fig. 1. Inhibition of Ki-Ras prenylation in PSN-1 tumor cells by L-778,123, either alone, or in the presence of added FTI or GGTI. Ki-Ras was detected by immunoblotting as described in "Materials and Methods" and in Ref. 38. Cells were treated for 24 h with compound before harvest. Cells were treated with the indicated dose of L-778,123, either alone or in combination with additional FTI or GGTI. In this experiment, FTI and GGTI refer to compounds described in Ref. 38; 300 nM FTI-1 and 100 nM GGTI-1. As controls, cells were also treated with vehicle (V) or DPI (10 μM DPI-1, described in Ref. 38). The IC_{50} with L-778,123 alone and in combination with added FTI-1 or GGTI-1 was determined to be 6300 nM, 4200 nM, and 1900 nM, respectively.

(Table 1). L-778,123 inhibits the prenylation of the FPTase substrate, HDJ2, the GGPTase-I substrate, Rap1A, and because it is a DPI, also blocks the prenylation of Ki-Ras (see Table 1; Fig. 1; Ref. 42).

Whereas FTIs have displayed excellent tolerability in animal models (e.g., Ref. 13), we have found that GGITs are toxic to mice when administered by continuous infusion for 2 days or more (38). Ideally, a DPI designed for clinical targeting of Ki-Ras should be balanced in its FTI and GGTI activities such that there is sufficient GGTI activity to inhibit the cross-prenylation of Ki-Ras by GGPTase-I but that avoids excessive GGTI activity, which might confer undesirable side effects. To assess whether L-778,123 has a suitable balance of inhibitory activities, we measured the inhibition of Ki-Ras prenylation in response to L-778,123 either alone or in combination with selective inhibitors of FPTase or GGPTase-I

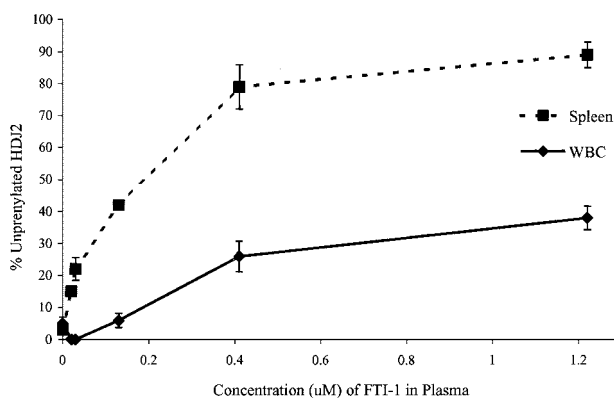


Fig. 2. Inhibition of HDJ2 prenylation in mice infused with FTI-1. Mice were implanted with 7-day osmotic pumps containing either vehicle or different concentrations of FTI-1 (in half-log increments ranging from 0.3 to 30 mg/ml), which administered a dose range of FTI-1 between 1.44 and 144 mg/kg/day. On day 7 after implantation, mice were euthanized, spleens were removed, and WBCs and plasma were isolated from whole blood as described in "Materials and Methods." Each data point represents the mean percentage of unprenylated HDJ2 from 5 animals per dose group plotted as a function of the mean plasma concentration of FTI-1 per dose group; bars, \pm SE.

(38). The added FTI or GGTI were used at concentrations that maximally inhibit their respective enzymes (38). We found that the IC_{50} for inhibition of Ki-Ras prenylation by L-778,123 was not appreciably altered by the added FTI or GGTI (Fig. 1). Therefore, L-778,123 is reasonably well optimized and balanced in its FTI and GGTI activities for inhibiting Ki-Ras.

Preclinical Evaluation of PD Assays for FPTase and GGPTase-I Inhibition. We have demonstrated previously the inhibition of HDJ2 and Rap1A prenylation *in vivo* in mouse spleen and tumor tissue by FTIs or GGITs, respectively (15, 38). To develop a PD assay suitable for clinical monitoring, we evaluated whether WBCs derived from whole blood could be used to assess the inhibition of HDJ2 and Rap1A prenylation. In our initial studies we used FTI-1 {4-[[1-[(5-chloro-2-oxo[1(2H),2'-bipyridin]-5'-yl)methyl]-1H-imidazol-5-yl]methyl]-benzotriazole},⁴ a potent FTI, which lacks inhibitory activity against GGPTase-I (Table 1). FTI-1 was administered to mice by continuous infusion for 7 days using osmotic pumps, and then animals were euthanized for analysis of HDJ2 prenylation in both spleen tissue and in WBCs obtained from whole blood. As seen in Fig. 2, FTI-1 caused a dose-dependent inhibition of HDJ2 prenylation in both spleen and WBCs. The level of unprenylated HDJ2 reached a plateau level that was higher in spleen (~90%) than in WBCs (~40%). In another experiment, FTI-1 was administered via a 14-day osmotic pump at a fixed dose (96 mg/kg/day), and HDJ2 prenylation was monitored periodically between days 2–14 by euthanizing the mice at time points during the 2-week infusion. In this experiment, the mean plasma concentrations of FTI-1 ranged between 0.28 and 0.74 μ M, and the level of unprenylated HDJ2 remained

⁴ S. Young *et al.*, Preparation of oxopyridinylmethylimidazolylmethylbenzotriazoles and related compounds as inhibitors of farnesyl-protein transferase. PCT Int. Appl. WO 9829119, 1998.

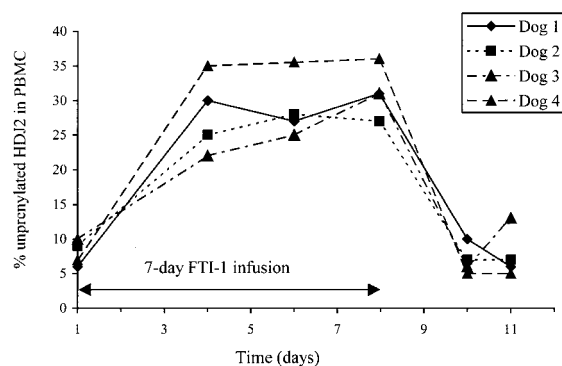


Fig. 3. Inhibition of HDJ2 prenylation in dog PBMCs from animals infused with FTI-1. Four individual dogs were administered 20 mg/kg/day FTI-1 over a 7-day period via continuous infusion delivered by infusion pumps. Blood samples (3 ml) were withdrawn on the indicated days, and PBMCs were isolated and analyzed by immunoblotting. Although drug concentrations were not determined in this study, in a similar experiment, 2 dogs administered 20 mg/kg/day FTI-1 for 5 days had average plasma concentrations during the infusion of 1.8 and 2.2 μ M, respectively.

reasonably constant throughout, ranging from 53–65% un-prenylated HDJ2 in the spleen and 30–45% un-prenylated HDJ2 in WBCs (average of 4 animals/group).⁵ Although higher plateau levels of unprenylated HDJ2 are observed in spleen, these experiments indicate that WBCs are suitable for monitoring FPTase inhibition.

To additionally simplify the PD assay we used commercially available cell preparation tubes (Vacutainer CPT) containing Ficoll-Hypaque density fluid to isolate PBMCs from whole blood. To confirm that PBMCs are a subpopulation of WBCs suitable for monitoring HDJ2 prenylation, we performed FTI-1-infusion studies in dogs. In these experiments, we followed both the onset of accumulation of unprenylated HDJ2 on initiation of the treatment and the rate of recovery of prenylated HDJ2 on termination of the infusion. In one study, dogs were continuously infused for 7 days with 20 mg/kg/day FTI-1, and blood was sampled by venipuncture at times before, during, and after the infusion. As shown in Fig. 3, a constant level of ~30% un-prenylated HDJ2 was observed in immunoblots of PBMCs isolated between days 3 and 7 of the infusion; by 2 days after the infusion, the levels of un-prenylated HDJ2 returned to the baseline predose level.

Having established that PBMCs were suitable for monitoring FPTase inhibition, we turned our studies to the characterization of L-778,123 in dogs. Two dogs (#1 and #2) were administered 35 mg/kg/day L-778,123 for 7 days by continuous infusion, whereas two other dogs (#3 and #4) were administered a slightly higher dose (50 mg/kg/day). A dose dependency was not observed with these two doses of L-778,123, with the two dogs dosed at 35 mg/kg/day and the two dogs dosed at 50 mg/kg/day showing mean plasma concentrations of 8.5, 6.2, 3.5, and 6.1 μ M L-778,123, respectively, (average of concentrations at days 1, 2, 4, and 7). Immunoblot analysis of PBMCs isolated from these dogs

⁵ D. Liu and R. Lobell, unpublished observations.

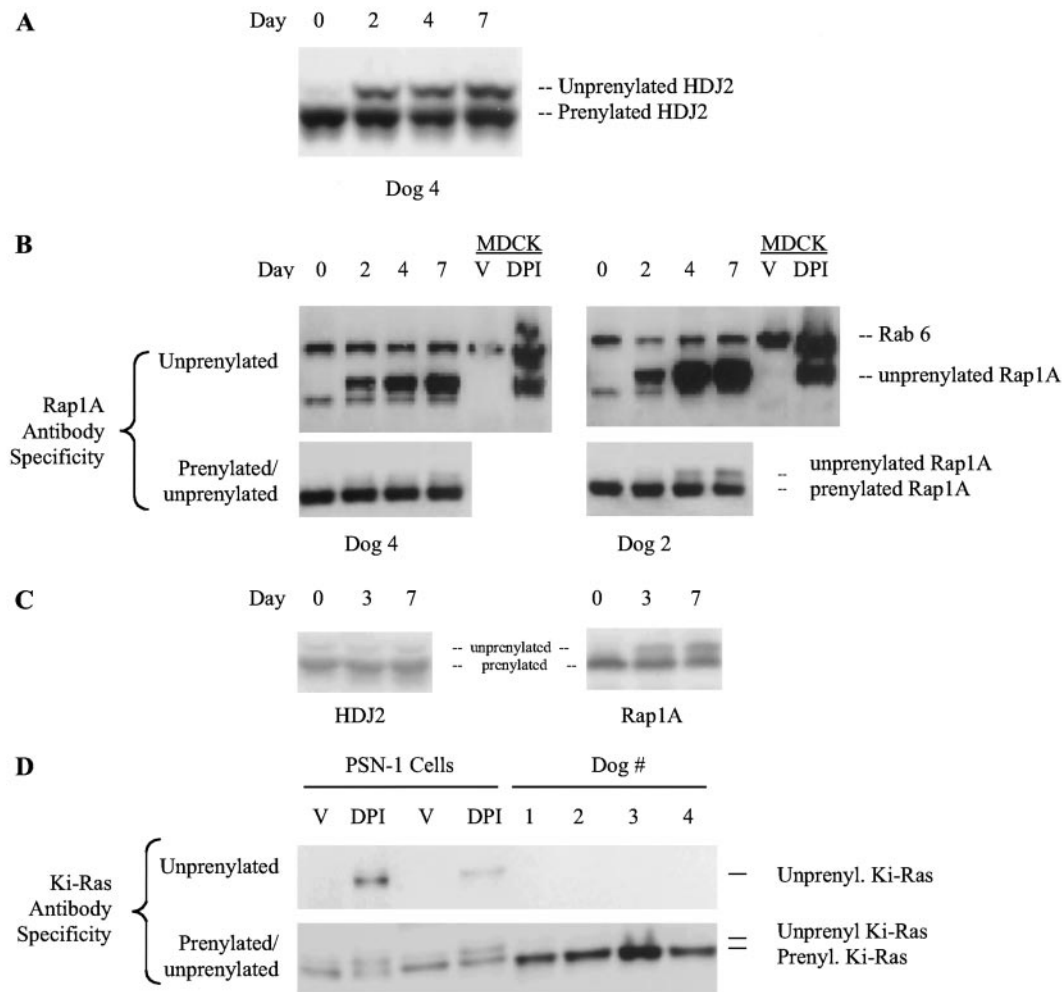


Fig. 4. Inhibition of FPTase and GGPTase-I in dogs. Dogs were treated with either 35 (dogs 1 and 2) or 50 (dogs 3 and 4) mg/kg/day L-778,123 for 7 days. Blood was drawn on the indicated day, and PBMCs were isolated and analyzed by immunoblotting for HDJ2 (A), Rap1A (B), or Ki-Ras (D) as described in "Materials and Methods." Immunoblots from dogs 2 and 4 are shown in A and B; comparable results were seen in the other 2 dogs. In A and B, 50 μ g PBMC lysate, derived from ~0.25 ml whole blood, was analyzed. In B, lysate from MDCK cells treated with vehicle or DPI (30 μ M L-778,123) was used as a control to show that reactivity of the unprenylated-specific Rap1A antibody depends on DPI treatment. In D, 20 μ g of lysates from PSN-1 cells treated with 10 μ M L-778,123 was either loaded directly (Lanes 1 and 2) or immunoprecipitated with Pan-Ras antibody (Lanes 3 and 4). For analysis of Ki-Ras in dog PBMCs, PBMCs were isolated from 8 ml of whole blood obtained on day 7 of the infusion, and PBMC lysate was immunoprecipitated with Pan-Ras antibody. Blots were probed first with the unprenylated-specific Ki-Ras-specific antibody and detected by chemiluminescence with enhanced chemiluminescence reagent, and then the blots were re-probed with the antibody specific for both prenylated and unprenylated Ki-Ras followed by detection with ECF fluorescent alkaline phosphatase substrate (Amersham Pharmacia Biotech, Piscataway, NJ; "Materials and Methods"). C, HDJ2 and Rap1A prenylation inhibition in PBMCs isolated on the indicated day from a dog treated by continuous infusion for 7 days with 0.2 mg/kg/day GGTI (GGTI-2, reported in Ref. 38).

showed that HDJ2 prenylation was inhibited to a comparable extent in all four of the animals, with 25%, 31%, and 40% unprenylated HDJ2 at days 2, 4, and 7 during the infusion, respectively (average percentage of unprenylated HDJ2 – predose levels; $n = 4$ dogs); a representative immunoblot of PBMC lysates from dog #4 is shown in Fig. 4A.

Because L-778,123 is also an inhibitor of GGPTase-I (Table 1), we monitored the inhibition of Rap1A prenylation in PBMCs as a PD marker of GGPTase-I inhibition. In some of our immunoblotting studies, we used a commercially available Rap1A antibody that we found to be highly specific and sensitive for unprenylated Rap1A. The specificity of this antibody for unprenylated Rap1A is demonstrated by the finding that Rap1A immunoreactivity is only seen when cells are

treated with either GGITs⁶ or with DPIs such as L-778,123 (Fig. 4B). As a loading control, samples were blotted concurrently with an antibody specific for the Rab6 protein, a protein geranylgeranylated by GGPTase-II of which the prenylation is unaffected by inhibitors of GGPTase-I. Immunoblotting with the unprenylated-specific Rap1A antibody indicated that L-778,123 infusion caused the accumulation of unprenylated Rap1A in dog PBMCs (Fig. 4B, top panel). However, immunoblotting with an antibody that recognizes both prenylated and unprenylated forms of Rap1A showed that only a small percentage of unprenylated Rap1A ($\leq 5\%$) is

⁶ M. Abrams and R. Lobell, unpublished observations.

produced by treatment with 35 or 50 mg/kg L-778,123 (Fig. 4B, bottom panel). To determine whether more dramatic levels of unprenylated Rap1A could be observed, we performed infusions with a GGTI (GGTI-2 described in Ref. 38) that has 1000-fold greater potency against GGPTase-I than L-778,123. A 7-day infusion of this more potent GGTI failed to inhibit HDJ2 prenylation but significantly inhibited GGPTase-I, with 31% unprenylated Rap1A observed at day 7 of the infusion (Fig. 4C). This result suggests that higher levels of unprenylated Rap1A in dog PBMCs might be achieved by treatment with doses of L-778,123 higher than 35–50 mg/kg/day.

We also monitored Ki-Ras prenylation in PBMCs from these L-778,123-treated dogs. In these experiments, we first immunoprecipitated Ras proteins with an antibody reactive with all of the Ras isoforms, and then immunoblotted with either an antibody specific to the prenylated and unprenylated forms of Ki-Ras, or with an antibody we developed that is specific for the unprenylated form of Ki-Ras. This unprenylated Ki-Ras-specific antibody was raised against a peptide corresponding to the unprenylated COOH terminus of Ki-Ras and shows reactivity to Ki-Ras in cells treated with DPI but not in vehicle-treated cells (Fig. 4D, top panel) or FTI-treated cells.⁵ Whereas prenylated Ki-Ras was detectable in dog PBMCs (Fig. 4D, bottom panel), no unprenylated Ki-Ras was observed using either the antibody that recognizes both prenylated and unprenylated Ki-Ras or with the unprenylated Ki-Ras-specific antibody (Fig. 4D).

Assessment of Prenylation Inhibition by L-778,123 in Clinical Samples. We reported recently on results from a Phase I continuous infusion/dose-escalation trial of L-778,123 in which the HDJ2-PBMC PD assay described here was used to monitor the inhibition of farnesylation in patient samples (43). In this trial, patients were dosed with a 7-day continuous infusion of L-778,123, and in many cases, patients were given multiple cycles of treatment (C1, C2, and so on) every 3 weeks. A representative HDJ2 immunoblot of PBMCs from patients dosed at 280, 560, and 1120 mg/m² L-778,123 from the 7-day infusion trial is shown in Fig. 5A. In this trial, a dose-dependent inhibition of HDJ2 prenylation by L-778,123 was observed, and we noted an apparent plateau level of ~35% unprenylated HDJ2 at a dose-level of ~560 mg/m² L-778,123, the MTD of the compound (43). L-778,123 has also been evaluated in a Phase I trial involving prolonged (2- or 4-week) infusion (44). The majority of patients in this trial were dosed at the MTD established in the 7-day infusion study, 560 mg/m² L-778,123. A comprehensive analysis of HDJ2 prenylation in PBMC samples from patients in this trial is shown in Fig. 5B. Notably, a constant level of unprenylated HDJ2 is observed in PBMCs in patients treated for up to 4 weeks with 560 mg/m² L-778,123 (Fig. 5B); the level of unprenylated HDJ2 at this dose was quite comparable with that observed in the prior 7-day L-778,123 infusion trial (43).

In Fig. 6, we represent data from the 7-day and 14-/28-day infusion trial where both HDJ2 prenylation status and plasma concentrations of L-778,123 was available. Although there is a fair amount of scatter in the data, there is a trend toward higher levels of unprenylated HDJ2 with increasing plasma concentrations of L-778,123 (Fig. 6). Whereas our initial anal-

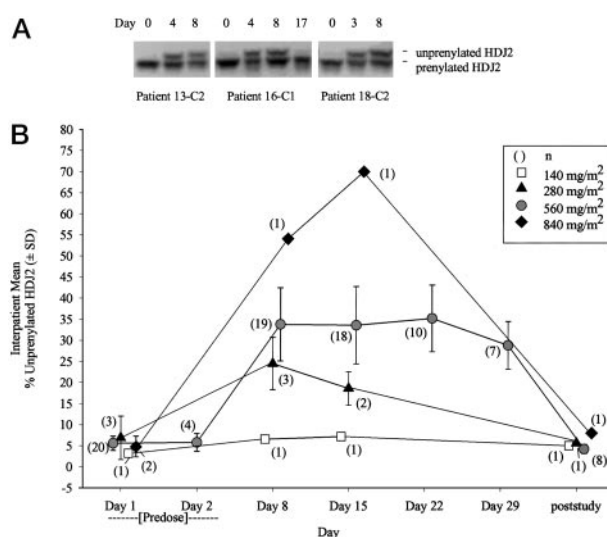


Fig. 5. Inhibition of FPTase in patients treated with L-778,123. A, HDJ2 immunoblots of PBMCs from 3 patients treated with 280, 560, or 1120 mg/kg/day L-778,123 for 7 days (patients 13, 16, and 18, respectively). B, HDJ2-PBMC analysis from the 2-/4-week L-778,123 infusion study. The interpatient mean percentage of unprenylated HDJ2 for the four L-778,123 dose groups is shown. The number of different patients measured at each time point is indicated (n); bars, \pm SD.

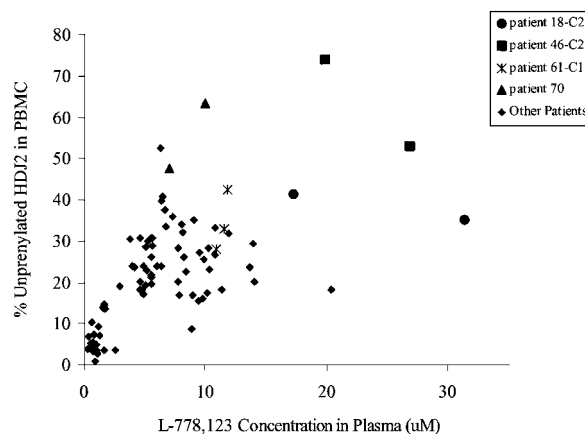


Fig. 6. HDJ2 prenylation inhibition in human PBMCs as a function of L-778,123 concentration in plasma. The data sets for the two L-778,123 clinical trials involving either 7-day or 14-/28-day infusions were pooled for this analysis, representing 34 different patients, and in some cases, multiple treatment cycles per patient. Each data point represents the percentage of unprenylated HDJ2 and L-778,123 concentration on day 4 or day 8 for patients treated for 7 days, day 8 or 15 from patients treated for 14 days, and from day 8, 15, 22, and 28 from patients treated for 28 days. The 4 patients who became myelosuppressed during therapy are indicated by distinct symbols relative to the other patients.

ysis with L-778,123 from the 7-day infusion indicated a plateau level of ~35% unprenylated HDJ2, several patients in the 2-/4-week infusion trial, including one treated with 840 mg/m² L-778,123 (Fig. 5B), displayed considerably higher levels of unprenylated HDJ2 (Fig. 6; see also Tables 2 and 3). Another patient with exceptionally high levels of unprenylated HDJ2, patient 46, was dosed for two separate 14-day infusion cycles at the 560 mg/m² dose. Unexpectedly, the

Table 2 Inhibition of the FPTase substrate HDJ2 and the GGPTase-I substrate Rap1A in humans by L-778,123

PBMC samples from patients in the 7-day infusion study (43), or in the 14- or 28-day infusion study (44) were analyzed as described in "Materials and Methods" and in Figure 7. The values of percentage of unprenylated HDJ2 are reported as percentage of unprenylated HDJ2 at the indicated time of PBMC isolation minus the predose unprenylated value (typically ~5%).

Patient #	Dose (mg/m ²)	Length of infusion (days)	Cycle #	Time of PBMC isolation (day of infusion)	% unprenylated HDJ2	% unprenylated Rap1A	Plasma Concentration (μM)
13	280	7	2	4	29	0.6	5.1
				8	31	0	5.6
16	560	7	1	4	28	0	7.8
				8	23	5.0	5.2
				17	2.7	0	nd ^a
18	1120	7	2	3	35	1.8	31
				8	41	25	17
43	560	14	1	8	26	0	5.5
				15	31	2.2	4.7
44	560	14	1	8	31	0	5.5
				15	30	0	3.8
46	560	14	1	8	38	4.1	6.6
				15	36	0	7.3
			2	8	53	32	27
61	560	28	1	12	74	46	20
				8	28	16	11
				15	33	19	12
				22	42	nd	nd
70	840	14	1	8	48	nd	7.0
				15	64	nd	10

^a nd, not determined.

Table 3 Hematologic data for myelosuppressed patients in the clinical trials with 7- and 14-/28-day infusions of L-778,123

Patient #/dose/ length of infusion	Hematologic toxicity (grade)		Hematologic data [WBC, platelet counts (× 10 ⁹ /liter)] % unprenylated HDJ2 L-778,123 plasma concentration (μM)												
	Neutropenia	Thrombocytopenia	Day	1	2	3	8	10	14	14	14	14	14	14	14
#18-C2 1120 mg/m ² 7 days	3	3	Day	1	2	3	8	10	14						
			WBC	5.8	6	5.9			1.9	5.9					
			Plts ^a	490	590	470			280	87					
			HDJ2	2.8		38	44								
			778,123			31	17								
#46-C2 560 mg/m ² 12 days	4	3	Day	1	8	10	12	15	19	22	24	25	28		
			WBC	8.8	9.8	5.3	1.4	0.1	0.1	2.6	10	16	39		
			Plts	250	150	78	48	5	19	8	23	24	120		
			HDJ2	3.7	57		78								
			778,123		27		20								
#61-C1 560 mg/m ² 22 days	3	3	Day	pre	1	8	15	22	24	25	26	27	29	33	34
			Plts	270	250	250	110	77	51	40	26	18	16	82	99
			HDJ2		6.4	34	39	49							
			778,123			11	12								
#70-C1 840 mg/m ² 14 days	4	4	Day	pre	1	8	15	16	17	18	19	21			
			Plts	nd	nd		27	25	7	42	30	20			
			WBC	3.6	4.6		1.7	1.2	0.8	0.5	0.4	0.3			
			HDJ2		6.5	54	70								
			778,123			7	10								

^a Plts, platelets; Pre, pre-study sample; nd, not determined.

second cycle of infusion resulted in plasma concentrations of up to 27 μM L-778,123, a level ~3-fold higher than concentrations achieved in the first drug cycle (Table 2). Interestingly, levels of unprenylated HDJ2 in PBMCs in the second cycle approached 75% unprenylated HDJ2, significantly higher than the typical level of ~35% unprenylated HDJ2 seen in the 560 mg/m² L-778,123 dose group. These excep-

tionally high levels of unprenylated HDJ2 may be related to myelosuppression (see Table 3), a possibility we discuss additionally in the "Discussion."

We have also analyzed selected patient samples for inhibition of GGPTase-I in PBMCs. Initially, we focused on samples from three different individuals from the 7-day infusion trial representative of the 280, 560, and 1120 mg/m²

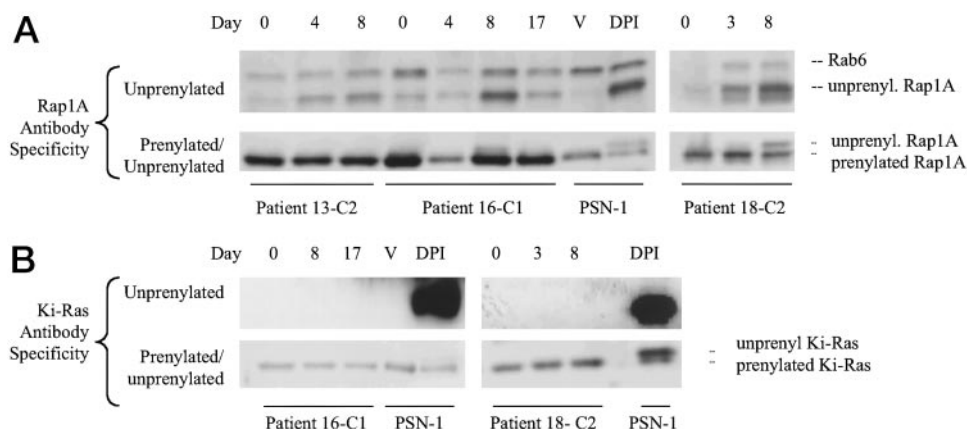


Fig. 7. Inhibition of GGPTase-I in patients treated with L-778,123. Samples were analyzed for Rap1A (A) and Ki-Ras (B) prenylation as described in Fig. 4. For controls, PSN-1 cells were treated for 24 h with either vehicle or 10 μ M L-778,123 (DPI; 30 μ M L-778,123 was used in the Ki-Ras gel for Patient 18-C2). In B, Ki-Ras prenylation was examined as described in Fig. 4; for each time point, either 1 mg (patient 16-C1) or 200 μ g (Patient 18-C2) PBMC lysate was immunoprecipitated with Pan-Ras antibody before immunoblotting.

L-778,123 dose groups. Immunoblotting with the antibody specific for unprenylated Rap1A suggested inhibition of GGPTase-I in these samples, particularly in the patient dosed at 1120 mg/m² L-778,123 (Fig. 7A, Patient 18-C2). Densitometric quantitation of the unprenylated Rap1A and Rab6 bands in the blot from these patient PBMC samples suggests that L-778,123 infusion measurably inhibited GGPTase-I in all three of the patients. The densitometric ratio of unprenylated Rap1A to Rab6 in each sample was determined to be as follows: patient 13-C2, 0.9, 2.1, and 1.8 (day 0, 4, and 8, respectively); patient 16-C1, 0.4, 0.7, 2.3, and 1.1 (day 0, 4, 8, and 17, respectively); and patient 18-C2, 5.1 and 11.6 (day 3 and day 8, respectively). Blotting with the Rap1A antibody that detects both prenylated and unprenylated Rap1A allowed a more quantitative assessment of Rap1A prenylation inhibition in these samples. The samples from the patient administered 280 mg/m² L-778,123 (patient 13) showed no detectable unprenylated Rap1A with this antibody, but patient 16 (dosed at 560 mg/m²) and patient 18 (dosed at 1120 mg/m²) both displayed unprenylated Rap1A by the last day (day 8) of the infusion (Fig. 7A). Densitometric quantitation revealed 5% and 25% unprenylated Rap1A in the day 8 patient samples from patients 16 and 18, respectively (Table 2). Analysis of PBMC lysates from an additional patient in the 560 mg/m² dose group with the unprenylated-specific Rap1A antibody clearly demonstrated the presence of unprenylated Rap1A in this patient (#15-C1); blotting with the other Rap1A antibody indicated that \leq 5% of the Rap1A in this sample was unprenylated.⁵ This limited data set indicates that 7-day L-778,123 infusion inhibited GGPTase-I in patients in this Phase I study.

Rap1A PD analysis from five patients in the 2-/4-week infusion study is reported in Table 2. Two patients in this study dosed at 560 mg/m² showed no inhibition of Rap1A prenylation, whereas two other patients receiving this dose showed significant inhibition of Rap1A (Table 2). Of note, in drug cycle 2, PBMCs from patient #46 not only displayed exceptionally high levels of unprenylated HDJ2 but also the highest levels of unprenylated Rap1A (46%) we have observed in any of the patients.

We have also analyzed PBMC samples from several patients (#16-C1, #18-C2, #46-C2, and #61-C1) for inhibition of

Ki-Ras prenylation. These patient samples were chosen for analysis because their PBMCs all displayed appreciable inhibition of GGPTase-I, with levels of unprenylated Rap1A ranging from 5% to 46% (Table 2). Whereas prenylated Ki-Ras was detected in all of these samples, no unprenylated Ki-Ras was detected in any of these samples (see Fig. 7B; data not shown).⁵

Discussion

We have developed and validated PD assays that quantitatively measure FPTase and GGPTase-I inhibition in PBMCs. These PD assays use immunoblotting methods to assess the inhibition of HDJ2 and Rap1A, substrates specific for FPTase and GGPTase-I, respectively. Using the FPTase-specific inhibitor FTI-1 (Table 1), we showed that continuous infusion of FTI-1 in mice caused the accumulation of unprenylated HDJ2 in both spleen and WBCs in a dose-dependent manner (Fig. 2). Similarly, FTI-1 infusion caused the accumulation of unprenylated HDJ2 in dog PBMCs, which resolved back to predose levels 48 h after infusion (Fig. 3). In other studies, we observed unprenylated HDJ2 in dog PBMCs 24 h after the start of FTI infusion.⁵ Thus, HDJ2 is a dynamic reporter of FPTase inhibition *in vivo*.

We also performed validation studies with L-778,123, an investigational compound with inhibitory activity against both FPTase and GGPTase-I (Ref. 41; Table 1). In the dog model, L-778,123 inhibited HDJ2 prenylation (Fig. 4A) and produced measurable, albeit slight (\sim 5%), levels of unprenylated Rap1A in PBMCs (Fig. 4B). By administering a more potent GGTI to dogs, we showed that higher levels of unprenylated Rap1A (31%) could be produced in PBMCs (Fig. 4C). We conclude that PBMCs are suitable for quantitative, PD measurement of both FPTase and GGPTase-I inhibition.

Using these PD assays, we showed that L-778,123 inhibited both FPTase and GGPTase-I in patients treated with this compound (Figs. 5–7; Table 2). In a prior study, we used the PBMC-HDJ2 assay to monitor the inhibition of FPTase in patients infused for 7 days with L-778,123 (43, 45). Our results suggested a dose-dependent inhibition of FPTase with an apparent plateau of \sim 35% unprenylated HDJ2 at the MTD of L-778,123 (560 mg/m²; Ref. 43). Similarly, in our

current study, we found that patients dosed with 560 mg/m² for up to 28 days exhibited a mean level of unprenylated HDJ2 of ~35% (Fig. 5B). Additional analysis of patient samples demonstrated that L-778,123 can inhibit GGPTase-I in humans at the 560 mg/m²/day dose level (Fig. 7; Table 2). Of the six different 560 mg/m² L-778,123 patient treatment cycles analyzed, two showed fairly high levels of unprenylated Rap1A in PBMCs, ranging from 16% to 46% (Table 2). The patient dosed with 1120 mg/m²/day L-778,123 also displayed a high level of unprenylated Rap1A (25%). The presence of higher levels (≥5%) of unprenylated Rap1A in PBMCs correlates with plasma concentrations of L-778,123 ranging from 11 to 31 μM (Table 2). The four other patients dosed with 560 mg/m² L-778,123 that showed measurable, albeit lower levels of unprenylated Rap1A (≤5%) had plasma concentrations of L-778,123 ranging from 3.8 to 7.8 μM (Table 2). Similarly, in the dog study, L-778,123 concentrations ranging from 3.5 to 8.5 μM resulted in low levels (≤5%) of unprenylated Rap1A (Fig. 4B). These results indicate that the *in vivo* IC₅₀ for Rap1A prenylation inhibition is several fold higher than the *in vitro* IC₅₀ measured in cultured cells (6.8 μM; see Table 1). To our knowledge, these results represent the first demonstration of GGPTase-I inhibition in humans.

In the two L-778,123 monotherapy trials, no objective tumor responses were observed (43, 44). Whereas the HDJ2-PBMC assay results demonstrate that significant inhibition of FPTase was achieved in these trials, it is possible that a more potent FTI that can achieve a higher level of FPTase inhibition might prove to be efficacious. Whereas HDJ2 is a convenient FPTase marker for PD assays, we note that a single substrate cannot provide a complete profile of FPTase inhibition. FPTase has a variety of substrates, and presumably, each one will have a different affinity for the enzyme. For example, the affinity of Ki-Ras for FPTase is 10–20-fold higher than the affinity of the other Ras isoforms, and consequently, 10–20-fold higher concentrations of a CAAX-competitive FTI is required to inhibit the farnesylation of Ki-Ras *in vitro* (20). Furthermore, in cells treated with a GGTI to prevent cross-prenylation of Ki-Ras, 20–50-fold higher concentrations of FTI are required to inhibit the farnesylation of Ki-Ras compared with the concentration required to inhibit HDJ2 farnesylation (see Ref. 38 and Table 1). The kinetic constants (K_m and K_{cat}) have been characterized for only a limited number of FPTase substrates, and furthermore, the FPTase substrate(s) critical for the antiproliferative effects of FTIs are not well defined. Therefore, it is unclear whether HDJ2 is a sufficient PD marker for clinical monitoring of FTIs. In clinical studies with another FTI, R115777, HDJ2 and prelamin A were used as PD markers of FPTase (46, 47). However, in cell culture, the accumulation of prelamin A occurs over an FTI dose-range similar to the doses required for inhibition of HDJ2 prenylation (48), suggesting that measurement of both HDJ2 and prelamin A may be redundant.

Whereas PBMCs are readily obtained in the clinical setting, we note that there may be pitfalls in using PBMCs for PD analysis of FPTase inhibition. Because L-778,123 (43) and other FTI candidates in development can cause myelo-

suppression (9), the composition of cells in PBMCs might change significantly during the course of an FTI-induced myelosuppressive event. In our mouse studies, we observed that the maximum percentage of unprenylated HDJ2 at each dose of FTI-1 was less in WBCs than in spleen (Fig. 2). By analogy, it is possible that the different cell types in PBMCs (lymphocytes and monocytes) might exhibit different maximal levels of unprenylated HDJ2 at a given dose of FTI. Therefore, if the relative numbers of lymphocytes and monocytes in PBMCs changed during myelosuppressive FTI therapy, the observed level of unprenylated HDJ2 might be a function not only of the dose of FTI but also of the level of myelosuppression. As discussed below, our analysis of clinical samples suggests that myelosuppression can indeed alter the dose-response relationship between FTI dose and the level of unprenylated HDJ2 in PBMCs.

Whereas the majority of patients treated at the MTD of L-778,123 (560 mg/m²) showed maximal levels of ~35% unprenylated HDJ2 (Fig. 5B), several patients who became myelosuppressed during the course of L-778,123 treatment had much higher levels of unprenylated HDJ2 (Tables 2 and 3; Fig. 5B; Fig. 6). For example, in the second cycle of treatment with 560 mg/m² L-778,123, patient #46 displayed 53% and 74% unprenylated HDJ2 at days 8 and 12 during the infusion, respectively. This patient experienced clinical grade 4 leukopenia and grade 3 thrombocytopenia with an 84% drop in WBCs and an 81% drop in platelets by infusion-day 12 (Table 3), at which point the infusion was terminated. During treatment, patient #46 had plasma concentrations of 27 and 20 μM L-778,123 at days 8 and 12 during the infusion, respectively (Table 2), approximately 2.2–3-fold higher than the mean steady state concentration of L-778,123 in patients receiving the 560 mg/m² dose (43). Another patient (#70), who was dosed with 840 mg/m² L-778,123 for 2 weeks displayed a 53% drop in WBCs by day 14, with WBC levels that continued to drop after infusion (Table 3). Whereas the plasma concentration of L-778,123 in patient #70 was unremarkable (7–10 μM), this patient had 48% and 64% unprenylated HDJ2 in PBMCs at days 8 and 15, respectively. Another patient (#61) became myelosuppressed by 22 days of infusion, at which point treatment was stopped. This patient displayed a rising level of unprenylated HDJ2 between days 8 and 22 of the infusion, reaching almost 50% unprenylated HDJ2 by day 22 (Table 3). In the 7-day infusion trial, myelosuppression was one of the dose-limiting toxicities of L-778,123, but we were unable to obtain PBMC-HDJ2 data from 3 of 4 individuals that showed significant (clinical grade 3) neutropenia (43) because of very low PBMC yields from these patients. Patient 18-C2 was the one neutropenic individual from this trial where HDJ2 was analyzed; this patient displayed no change in WBC levels at day 3 of infusion but had WBC levels approximately one-third that of the predose level by day 10 (Table 3). At the end of the infusion (day 8), the PBMCs from this patient displayed 44% unprenylated HDJ2, a level somewhat higher than the 35% unprenylated HDJ2 plateau. Although our data set is somewhat limited, our analysis suggests that myelosuppression can contrib-

ute to atypically high levels of unprenylated HDJ2 in PBMCs.

It should also be noted that the 3 patients (#18, 46, and 61) who became myelosuppressed during the course of L-778,123 treatment (Table 3) showed the highest levels of unprenylated Rap1A (Table 2); insufficient sample precluded an analysis of Rap1A in the other myelosuppressed patient (#70). In mouse studies, we have found that GGTTs can cause myelosuppression (38). We suggest that in addition to FPTase inhibition, inhibition of GGPTase-I could have contributed to the myelosuppression in humans caused by L-778,123 (Table 3; Ref. 43).

Whereas L-778,123 was selected for clinical evaluation in part because it can inhibit the prenylation of Ki-Ras, we were unable to demonstrate inhibition of Ki-Ras prenylation in PBMCs with L-778,123 in either the dog model (Fig. 4D) or in 4 different patients from the clinical trials (Fig. 7B).⁵ We also did not detect unprenylated Ki-Ras in mice infused for 24 h with 2300 mg/kg L-778,123, a dose that caused only modest inhibition of GGPTase-I (approximately 5–10% unprenylated Rap1A).⁷ In other studies in mice with DPIs more potent than L-778,123, we have found that inhibition of Ki-Ras prenylation required concentrations of DPI higher than is required to inhibit Rap1A prenylation; this is surprising because the DPI inhibited Rap1A and Ki-Ras prenylation with similar potency in cultured cells (38). Because L-778,123 infusion resulted in only partial inhibition of Rap1A prenylation in both dogs (Fig. 4B) and humans (Fig. 7A; Table 2), the lack of inhibition of Ki-Ras prenylation might be explained by the incomplete inhibition of GGPTase-I. The lack of unprenylated Ki-Ras in PBMCs might also be explained by the fact that this is a nonproliferating tissue. In experiments with dog MDCK cells in culture, we have observed that accumulation of unprenylated Ki-Ras is highly dependent on the rate of cell proliferation, whereas the accumulation of unprenylated HDJ2 is not affected.⁵ Whereas treatment of MDCK cells plated at low density (35% in S phase) with L-778,123 resulted in a dose-dependent increase in unprenylated Ki-Ras, no unprenylated Ki-Ras was detected in L-778,123-treated cells plated at high density (12% in S phase). Therefore, a tissue with more actively proliferating cells might be more suitable than PBMCs for PD monitoring of Ki-Ras prenylation.

Our studies suggest that whereas L-778,123 inhibited FPTase and partially inhibited GGPTase-I in humans, the compound may have lacked the potency required for inhibition of Ki-Ras prenylation *in vivo*. Therefore, the clinical trials with L-778,123 may not have adequately tested the hypothesis of whether inhibition of Ki-Ras prenylation by a DPI can cause significant tumor responses. Subsequent to the selection of L-778,123 as a clinical candidate, we found that DPIs were lethal in mice when administered at a dose that inhibited Ki-Ras prenylation (38). In addition, continuous infusion with a GGTTI for 48–72 h is lethal to mice when administered at a dose that inhibits GGPTase-I at a level sufficient to inhibit Ki-Ras prenylation in the presence of an FTI (38). These results do not make a compelling argument in

favor of clinical targeting of Ki-Ras with DPIs more potent than L-778,123.

Acknowledgments

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