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In vitro and in vivo antimalarial activity of peptidomimetic protein farnesyltransferase inhibitors with improved membrane permeability

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Abstract—A series of protein farnesyltransferase inhibitor ester prodrugs of FTI-2148 (**17**) were synthesized in order to evaluate the effects of ester structure modification on antimalarial activity and for further development of a farnesyltransferase inhibitor with in vivo activity. Evaluation against *P. falciparum* in red blood cells showed that all the investigated esters exhibited significant antimalarial activity, with the benzyl ester **16** showing the best inhibition (ED_{50} = 150 nM). Additionally, compound **16** displayed in vivo activity and was found to suppress parasitemia by 46.1% at a dose of 50 mg kg^{-1} day^{-1} against *Plasmodium berghei* in mice. The enhanced inhibition potency of the esters is consistent with improved cell membrane permeability compared to that of the free acid. The results of this study suggest that protein farnesyltransferase is a valid antimalarial drug target and that the antimalarial activity of these compounds derives from a balance between the hydrophobic character and the size and conformation of the ester moiety.

1. Introduction

Malaria is a parasitic disease commonly found in many tropical and subtropical regions of the world. Present resurgence of this disease and lack of proper treatment cause several million deaths per year and present a huge burden to the economy of these countries. Malaria causes 0.5–2.5 million deaths per year worldwide and an average of 300–500 million people become infected annually.[1] The disease is caused by protozoan parasites of the genus *Plasmodium*. The four *Plasmodium* species known to infect humans are *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. Of these, *P. falciparum* is responsible for the most severe form of malaria infections. Existing treatments for malaria include a limited number of clinically effective drugs, however the emergence of drug-resistant parasite strains indicates an urgent need for discovering new and effective antimalarial therapeutics.

New strategies for antimalarial chemotherapy include inhibition of merozoite surface protein-1 (MSP-1) processing protease as well as inhibition of *P. falciparum* fatty acid biosynthesis, phospholipid metabolism, and

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malarial lactate dehydrogenase.[2] Recent studies have suggested that protein farnesyltransferase (PFT) is a promising target for the development of novel antiparasitic agents. We have previously demonstrated that protein prenylation occurs in protozoan parasites *T. brucei*, *T. cruzi*, and *Leishmania mexicana*, and have successfully isolated, cloned, and expressed PFT from *T. brucei*.[3,4] More recently, inhibition activity against *P. falciparum* by a PFT inhibitor was also observed.[5]

PFT belongs to a family of proteins known as protein prenyltransferases that are responsible for protein prenylation. Three distinct protein prenyltransferases, PFT, and protein geranylgeranyltransferase-I and II (PGGT-I and II), have been found in higher eukaryotes.[6] The prenyltransferases catalyze the covalent transfer of a farnesyl group (15 carbon by PFT) or of a geranylgeranyl group (20 carbon by PGGT-I or -II) to cysteine residues near the C-terminus of protein substrates. Many prenylated proteins are known to play crucial roles in cellular signaling and other regulatory events that take place near the cytoplasmic surfaces of cellular membranes.[6]

In past years there has been considerable interest in protein prenyltransferases because malignant Ras activity, present in approximately 30% of human cancers, was found to be dependent on post-translational modification by PFT. Therefore, PFT has been considered as a potential target for anticancer therapeutics. PFT is known to modify proteins that contain a C-terminal CAAX tetrapeptide, where C is cysteine, AA are aliphatic amino acids and X is usually serine or methionine. On the basis of the CAAX motif we have successfully developed potent PFT inhibitors, where the aliphatic dipeptide AA portion was substituted by rigid hydrophobic spacers (Fig. 1).

Based on previous observations supporting PFT as a potential target for the development of antiparasitic agents,[3,7] we decided to investigate the antiparasitic activity of our peptidomimetic inhibitors. We initially showed that peptidomimetics FTI-276 and its methyl ester FTI-277 (Fig. 1), inhibited *T. brucei* PFT and the growth of *T. brucei* bloodstream form at low nanomolar concentrations.[3] These compounds also potently inhibited the growth of *T. cruzi* amastigote intracellular form.[8] Additionally, we have recently shown that several of our PFT inhibitors also exhibit potent antimalarial activity against *P. falciparum* in vitro.[9] In this study FTI-2148, one of our most potent mammalian PFT inhibitors (IC_{50}=1 nM), displayed no inhibition against *P. falciparum* (ED_{50}>30 μg/mL). However, the methyl ester FTI-2153 (Fig. 1) potently inhibited *P. falciparum* growth with an ED_{50} = 2 μg/mL. It is likely that this enhanced inhibition is due to the higher membrane permeability of the ester compared to that of the free acid. Guided by these findings, we decided to prepare a series of ester prodrugs of FTI-2148 to investigate the effect of ester modification on antimalarial activity. The ester prodrug strategy is a well-precedented technique commonly used to deliver drugs across cellular membranes leading to higher drug efficacy.[10] Based on this approach, a series of FTI-2148 esters were designed. This paper describes our efforts to develop PFT inhibitor ester derivatives with increased lipophilicity that exhibit in vivo antimalarial activity.

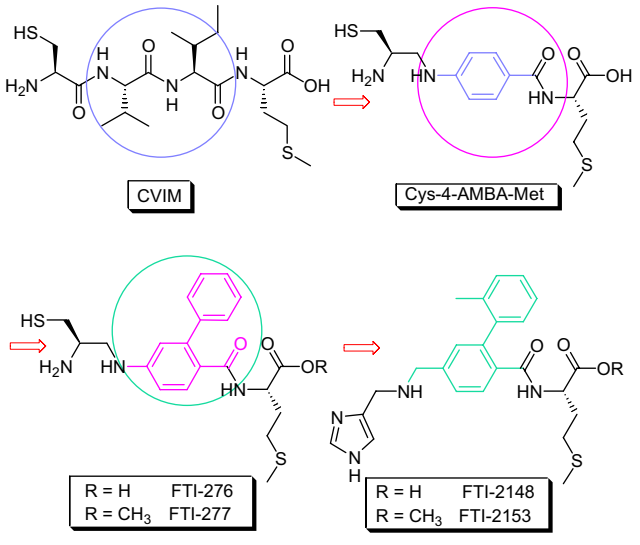


Figure 1. Evolution of CAAX peptidomimetic PFT inhibitors.

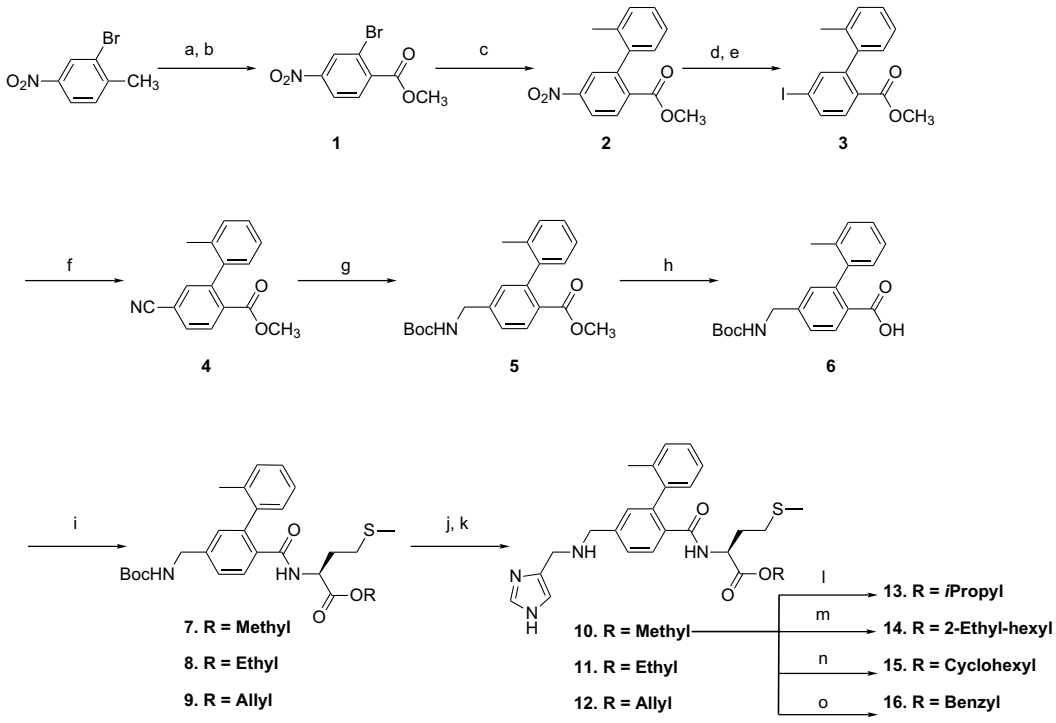
2. Results and discussion

2.1. Chemistry

The FTI-2148 esters were prepared as outlined in Scheme 1. Oxidation of 2-bromo-4-nitrotoluene with potassium permanganate followed by esterification with thionyl chloride in methanol gave **1**. The resulting 2-(bromo)-4-nitrobenzoic acid methyl ester (**1**) was then coupled to *o*-tolylboronic acid via a Suzuki coupling protocol to afford biaryl **2**. Reduction of the nitro group with tin(II) chloride dihydrate, diazotization, and Sandmeyer reaction with potassium iodide produced the iodo ester derivative **3**. Copper-mediated iodo-cyano exchange afforded nitrile **4**. The cyano intermediate **4** was then hydrogenated with palladium over activated charcoal and the resulting amine was Boc-protected in the same pot to afford intermediate **5**. Hydrolysis of **5** with LiOH in THF gave the free acid **6**.

The resulting benzoic acid derivative **6** was then coupled with L-methionine methyl, ethyl, or allyl ester using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI)/1-hydroxybenzotriazole (HOBt) to afford the respective amide precursors **7**, **8**, and **9**, respectively. Deprotection under acidic conditions followed by reductive amination of the resulting free amine with 4(5)-imidazolecarboxaldehyde and $NaBH_4$ gave the methyl (**10**), ethyl (**11**), and allyl (**12**) ester prodrugs of FTI-2148.

Since only two of the desired esters, allyl, and ethyl, could be prepared from commercially available methionine ester precursors, we turned our attention toward using a transesterification route to synthesize the



Scheme 1. Reagents and conditions: (a) $KMnO_4$, 62%; (b) $SOCl_2$, MeOH, 82%; (c) *o*-tolylboronic acid, $Pd(PPh_3)_4$, K_2CO_3, 92%; (d) $SnCl_2{\cdot}2H_2O$, AcOEt, 93%; (e) $NaNO_2$, HCl, KI, 83%; (f) CuCN, *N*-methyl-2-pyrrolidinone, 68%; (g) H_2/10% Pd–C, Boc_2O, THF, 92%; (h) LiOH, THF, 99%; (i) L-methionine ester, EDCI, HOBt, Et_3N, CH_2Cl_2, 69–88%; (j) TFA, 81–100%; (k) 4(5)-imidazolecarboxaldehyde, $NaBH_4$, 32–54%; (l) $Ti(OiPr)_4$, *i*PrOH, THF, 4 Å mol sieves, 70 °C, 99%; (m) titanium (IV)-2-ethylhexoxide, 10:1 THF/DMF, 4 Å mol sieves, 60 °C, 99%; (n) tetracyclohexyl titanate, 10:1 THF/DMF, 4 Å mol sieves, 60 °C, 44%; (o) $Ti(OiPr)_4$, benzyl alcohol, THF, 4 Å mol sieves, 85 °C, 98%.

remaining derivatives. We anticipated that this latter approach would also circumvent the low yields observed in the reductive amination step with the allyl and ethyl ester intermediates (32% and 36%, respectively). Extensive investigations into the application of transesterification reaction conditions using various model systems, revealed that a titanium-mediated transesterification could be used to efficiently prepare the desired esters from the methyl ester FTI-2153 (**10**) in a single step in very good yields (Scheme 1).

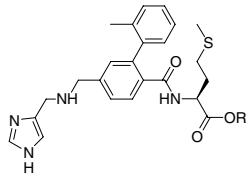
2.2. Biological activity

The activity of these PFT inhibitor ester derivatives was first evaluated against mammalian PFT both in vitro and in whole cell assays. The in vitro activity and selectivity of the FTI-2148 ester prodrugs are reported in Table 1. As expected, all the ester derivatives of FTI-2148 (**17**) exhibited higher IC_{50} values compared to the free acid. This results from the requirement of a C-terminal carboxylate for PFT substrates. In general, all compounds were more selective at inhibiting mammalian PFT over PGGT-I in vitro. The ability of these compounds to inhibit farnesylation and geranylgeranylation in whole cells was evaluated by determining the level of inhibition of H-Ras and Rap1A processing, respectively. Oncogenic H-Ras-transformed NIH 3T3 cells were treated with various concentrations of inhibitors, cells were harvested and subjected to Western blot analysis. Inhibition of farnesylation and geranylgeranylation was demonstrated by a band shift from processed (P) protein to unprocessed (U) protein. The results of the western blot are shown in Figure 2. All ester prodrugs were more effective at inhibiting H-Ras processing than the free carboxylate derivative **17** consistent with a higher cell permeability of the ester derivatives. In this series, the most potent compounds disrupted H-Ras farnesylation with an IC_{50} value of 4 nM. In addition, all compounds selectively inhibited H-Ras processing over Rap1A processing.

The inhibitory activity of these PFT inhibitor agents was then evaluated against *P. falciparum*. Evaluation against *P. falciparum* PFT showed that, as expected, all ester derivatives of FTI-2148 (**17**) were less effective at inhibiting the parasitic enzyme than the free acid, due to the inability of PFT to modify substrates that lack a C-terminal carboxylate. The antimalarial activity against *P. falciparum* growth displayed by these compounds was expected to be related to their hydrophobicity and consequently to their log *P* values. The octanol/water partition coefficient (log *P*) values of these compounds were predicted computationally using the program QikProp.[11] The results of this study are shown in Table 1.

In general, the estimated log *P* values follow an expected pattern where the least lipophilic compound is predicted to be FTI-2148 methyl ester and the most lipophilic is the 2-ethyl-hexyl ester of FTI-2148. A comparison of the computationally obtained log *P* values and the *P. falciparum* ED_{50} values shows a correlation between lipophilicity and antiparasitic activity. This is clearly

Table 1. Inhibition activity of **10**–**17** against *P. falciparum* PFT, *P. falciparum* infected RBC, mammalian PFT, and mammalian PGGT-I



Compound	R	Log *P*	*P. falciparum* PFT IC_{50} (nM)	*P. falciparum* infected RBC[a] ED_{50} (nM)	Mammalian PFT IC_{50} (nM)	Mammalian PGGT-I IC_{50} (nM)	Murine 3T3 fibroblasts ED_{50} (nM)
10	CH_3	4.44	>1000	3300	34	69,000	25,000
11	C_2H_5	4.88	750	1900	360	>10,000	10,000
12	H_2C	5.19	>1000	1800	150	>10,000	1000–10,000
13	HC	5.19	>1000	800	4000	>10,000	10,000
14	H_2C	7.09	>1000	700	3900	>10,000	1000–10,000
15	HC	4.94	>1000	350	3500	>10,000	1000–10,000
16	H_2C	6.09	1000	150	530	>10,000	1000–10,000
17	H	1.77	15	>66,000[b]	0.82	1700	>25,000

[a] RBC = red blood cell.
[b] Ref. 9.

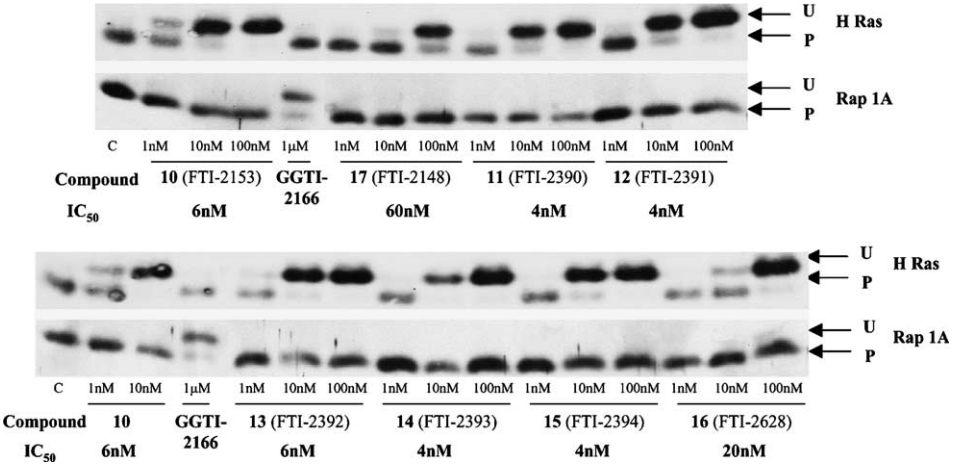


Figure 2. Mammalian H-Ras and Rap1A processing inhibition by **10**–**17** in whole cell assays.

seen in the two most lipophilic compounds **16** and **14**, that show much higher inhibition activities compared to less lipophilic **10** and **11**.

Evaluation of the seven different esters for in vitro inhibition of *P. falciparum* growth in red blood cells (Table 1) showed that all other esters had improved antiplasmodial activity compared to the methyl ester. These results indicate that the higher potency exhibited by the more hydrophobic esters appears to be due to their increased ability to enter parasite cells. In this series, branched esters **13** and **14** displayed a higher potency than linear ethyl **11** and allyl **12** derivatives. A surprising result was obtained with the cyclohexyl derivative **15** which, while not being the most hydrophobic compound, showed the second highest antiplasmodial activity in this series. Even though both **15** and **11** were predicted to be similarly hydrophobic the cyclohexyl ester derivative **15** was fivefold more active than **11**. These results indicate that, though a higher hydrophobicity leads to higher membrane permeation, other factors such as size and conformation of the ester side chain may also affect delivery of the compound into the cell and thus the inhibition activity. Thus, the activity of these compounds likely reflects a balance between the ease of membrane permeability, stability against extra-

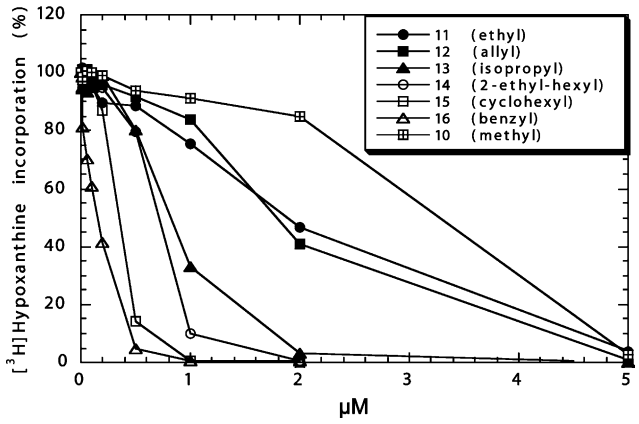


Figure 3. Inhibition of *P. falciparum* growth in red blood cells by compounds **10**–**16**. Seven different esters of compound **17** (FTI-2148) were tested at the indicated concentrations for in vitro inhibition of *P. falciparum* 3D7 growth in red blood cells. The malaria growth was measured by the [^{3}H]hypoxanthine-incorporation assay[14] and expressed as % incorporation of the no-drug control level.

Table 2. In vivo study results of **16** against *P. berghei*

Group	Dose (mg/kg)	% Parasitemia	% Inhibition
Control	0	12.42	0.0
Chloroquine	50	0.00	100
16	50	6.70	46.1

cellular hydrolytic activity (in serum and red blood cell), and hydrolysis rate by esterases in the parasite cells. Optimization of these factors would be expected to improve antiparasitic inhibition by increasing inhibitor concentration inside the parasites.

In this series, the benzyl ester **16** showed the best inhibition activity against *P. falciparum* in red blood cells with an ED_{50} value of 150 nM (Fig. 3). This promising in vitro result encouraged us to further evaluate the antimalarial activity of benzyl ester **16** in murine malarial models infected with *Plasmodium berghei*. Using Peters' four-day suppressive test,[12] compound **16** was administered to *P. berghei*-infected mice by ip injection at a daily dose of 50 mg kg^{-1} and chloroquine was used as a positive control (Table 2). In this study, the benzyl ester prodrug was found to suppress parasitemia by 46.1% as compared to untreated infected controls and promoted no apparent toxicity in the mice.

3. Conclusions

In conclusion, we have shown that ester derivatives of **17** with increased lipophilicity show enhanced inhibition activity against *Plasmodium falciparum* growth in red blood cells. Additionally, **16** showed in vivo activity using murine malaria models infected with *Plasmodium berghei*. Further investigations exploring other prodrug mechanisms in order to generate new antimalarial agents with potent and long duration of action are ongoing.

4. Experimental

4.1. General methods

Melting points were determined with an electrothermal capillary melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-500 and 400 spectrometer. Chemical shifts were reported in δ (ppm) relative to tetramethylsilane. All coupling constants were described in Hz. Elemental analyses were performed by Atlantic Microlab, Inc., GA. Flash column chromatography was performed on silica gel (40–63 μm) under a pressure of about 4 psi. Solvents were obtained from commercial suppliers and purified as follows: tetrahydrofuran and ether were distilled from sodium benzophenone ketyl; dichloromethane was distilled over calcium hydride. Synthesized final compounds were checked for purity by analytical HPLC, which was performed using a Rainin HP controller and a Rainin UV-C detector with a Rainin 250 mm × 4.6 mm, 5 μm Microsorb C-18 column, eluted with gradient 10–90% of CH_3CN in 0.1% TFA in H_2O in 30 min. High-resolution mass spectra (HRMS) and low-resolution mass spectra (LRMS) were performed on a Varian MAT-CH-5 (HRMS) or VG 707 (LRMS) mass spectrometer.

4.1.1. 2-Bromo-4-nitrobenzoic acid. A solution of 2-bromo-4-nitrotoluene (25.2 g, 116 mmol) in pyridine (100 mL) and water (200 mL) was warmed to 97 °C and $KMnO_4$ (101 g, 642 mmol) was added in portions. The reaction was refluxed for 5 h and an additional amount of $KMnO_4$ (27.5 g, 174 mmol), H_2O (40 mL) and pyridine (80 mL) were added. The reaction mixture was allowed to reflux overnight and the hot solution was then filtered over Celite. The resulting filtrate was acidified with 12 M HCl (110 mL) and extracted with AcOEt (600 mL). The organic layer was dried with Na_2SO_4 and the solvent was evaporated under vacuum to give the free acid as white crystals (17.6 g, 62%). Mp 153–155 °C; ^{1}H NMR (CD_3OD) δ 8.43 (d, J = 2.2 Hz, 1H), 8.18 (dd, J = 2.2 and 8.5 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H); ^{13}C NMR (CD_3OD) δ 168.47, 150.97, 141.29, 132.98, 130.21, 123.81, 122.46.

4.1.2. 2-Bromo-4-nitrobenzoic acid methyl ester (1). Absolute MeOH (50 mL) was cooled to 0 °C and $SOCl_2$ (6 mL, 82 mmol) was added dropwise. A solution of the free acid (7.5 g, 30.5 mmol) in MeOH (25 mL) was added and the reaction mixture was then refluxed overnight. The white solid was filtered, and the filtrate was concentrated under reduced pressure. The white precipitates were combined and recrystallized from hexanes and ether to afford the product as white crystals (6.53 g, 82%). Mp 82–85 °C; ^{1}H NMR ($CDCl_3$) δ 4.02 (s, 3H), 7.96 (d, J = 8.4 Hz, 1H), 8.24 (dd, J = 2.2 and 8.6 Hz, 1H), 8.55 (d, J = 2.2 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 165.61, 149.65, 138.35, 132.17, 129.57, 122.55, 122.40, 53.50.

4.1.3. 2′-Methyl-5-nitro-biphenyl-2-carboxylic acid methyl ester (2). A solution of **1** (0.259 g, 1 mmol) in DMF (1.5 mL) was flushed with N_2 for 30 min.

$Pd(PPh_3)_4$ (0.038 g, 33 μM) was added and the reaction was stirred for 10 min. To the resulting red reaction mixture, *o*-tolylboronic acid (0.149 g, 1.1 mmol) and K_2CO_3 (0.415 g, 3 mmol) were added. The mixture was stirred at 80 °C for 1 h and at 120 °C overnight. H_2O (1.5 mL) was added and the mixture was extracted with Et_2O (4 × 3 mL). The organic layer was washed with 1 M HCl (2 × 1.5 mL) and dried over anhydrous $MgSO_4$. Solvent evaporation followed by SiO_2 column chromatography with hexanes/AcOEt = 10:1 afforded the product as a pale yellow oil (0.25 g, 92%). ^1H NMR ($CDCl_3$) δ 8.26 (dd, J = 2.4 and 8.6 Hz, 1H), 8.13 (d, J = 2.2 Hz, 1H), 8.07 (d, J = 8.6 Hz, 1H), 7.34–7.21 (m, 3H), 7.08 (d, J = 7.4 Hz, 1H), 3.65 (s, 3H), 2.09 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 166.79, 149.55, 144.68, 139.30, 136.72, 135.54, 131.39, 130.30, 128.78, 128.71, 126.20, 126.03, 122.40, 52.89, 20.35; LRMS (CI) *m/z* calcd for $C_{15}H_{13}NO_4H^+$ 272, found 272; HRMS (CI) *m/z* calcd for $C_{15}H_{13}NO_4H^+$ 272.0923, found 272.0922.

4.1.4. 5-Iodo-2′-methyl-biphenyl-2-carboxylic acid methyl ester (3). To a solution of **2** (4.46 g, 16.4 mmol) in AcOEt (120 mL) was added $SnCl_2{\cdot}2H_2O$ (39.6 g, 175 mmol). The reaction was refluxed for 4 h and cooled to room temperature. To the mixture AcOEt (370 mL) was added and the resulting mixture was poured into saturated $NaHCO_3$ (500 mL). The organic layer was washed with brine (300 mL) and dried over anhydrous $MgSO_4$. Solvent evaporation afforded the free amine desired product (3.68 g, 93%). ^1H NMR ($CDCl_3$) δ 1.95 (s, 3H), 3.44 (s, 3H), 3.94 (br s, 2H), 6.26 (d, J = 2.4 Hz, 1H), 6.45 (dd, J = 2.5 and 8.5 Hz, 1H), 6.93 (d, J = 7.4 Hz, 1H), 7.04–7.13 (m, 3H), 7.74 (d, J = 8.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 167.65, 150.52, 146.09, 142.80, 135.66, 133.06, 129.68, 128.49, 127.37, 125.58, 119.17, 114.20, 113.21, 51.82, 20.36; LRMS (CI) *m/z* calcd for $C_{15}H_{15}NO_2H^+$ 242, found 242; HRMS (CI) *m/z* calcd for $C_{15}H_{15}NO_2H^+$ 242.1181, found 242.1178.

A solution of the free amine (1.85 g, 7.64 mmol) in HCl (2.8 mL), H_2O (2.4 mL), AcOH (0.8 mL) and MeOH (2.0 mL) was cooled to 0 °C. To the mixture $NaNO_2$ (0.604 g, 8.75 mmol) in H_2O (1.8 mL) was added and the mixture was stirred at 0 °C. A mixture of KI (1.90 g, 11.4 mmol) in HCl (5 mL) was added and the reaction was stirred at room temperature for 5 min and then at 60 °C for 15 min. The mixture was extracted with AcOEt (300 mL) and the organic layer was washed with 5% Na_2SO_3 (200 mL). The organic layer was dried over $MgSO_4$ and the solvent was evaporated. Purification by SiO_2 column chromatography with hexanes/AcOEt = 8:1 afforded the product as a yellow oil (2.23 g, 83%). ^1H NMR ($CDCl_3$) δ 2.06 (s, 3H), 3.60 (s, 3H), 7.04 (d, J = 7.3 Hz, 1H), 7.16–7.30 (m, 3H), 7.63 (d, J = 1.8 Hz, 1H), 7.67 (d, J = 8.4 Hz, 1H), 7.78 (dd, J = 1.8 and 8.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 166.16, 143.62, 138.80, 135.32, 134.15, 131.10, 130.37, 128.50, 127.54, 127.44, 127.26, 126.63, 124.31, 50.97, 18.94; LRMS (CI) *m/z* calcd for $C_{15}H_{13}IO_2H^+$ 353, found 353; HRMS (CI) *m/z* calcd for $C_{15}H_{13}IO_2H^+$ 353.0039, found 353.0037.

4.1.5. 5-Cyano-2′-methyl-biphenyl-2-carboxylic acid methyl ester (4). A solution of **3** (0.33 g, 0.93 mmol) and CuCN (0.187 g, 2.1 mmol) in *N*-methyl-2-pyrrolidinone (4.0 mL) was stirred at 200 °C for 4 h. To the mixture was added NaCN solution (0.391 g in 8.0 mL of H_2O) and the mixture was extracted with AcOEt (250 mL). The organic layer was dried with $MgSO_4$ and the solvent was evaporated. Purification of the crude product by SiO_2 column chromatography with hexanes/AcOEt = 4:1 afforded the product as a white solid (0.16 g, 68%). Mp 65–68 °C; ^1H NMR ($CDCl_3$) δ 2.07 (s, 3H), 3.63 (s, 3H), 7.03 (d, J = 6.5 Hz, 1H), 7.20–7.33 (m, 3H), 7.56 (d, J = 1.5 Hz, 1H), 7.72 (dd, J = 1.7 and 8.1 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 166.51, 143.68, 138.91, 135.13, 134.66, 134.48, 130.69, 130.55, 129.83, 128.34, 128.20, 125.60, 117.86, 115.15, 52.45, 19.97; LRMS (CI) *m/z* calcd for $C_{16}H_{13}NO_2H^+$ 252, found 252; HRMS (CI) *m/z* calcd for $C_{16}H_{13}NO_2H^+$ 252.1025, found 252.1029; Anal. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21; N, 5.57. Found: C, 76.06; H, 5.12; N, 5.53.

4.1.6. 5-(*tert*-Butoxycarbonylamino-methyl)-2′-methyl-biphenyl-2-carboxylic acid methyl ester (5). A solution of 10% Pd–C (0.116 g) in THF (10 mL) was preactivated for 30 min under 40 psi H_2 atmosphere. To the mixture a solution of **4** (0.551 g, 2.20 mmol) and Boc_2O (0.972 g, 4.45 mmol) in THF (12 mL) was added and the solution was hydrogenated under 60 psi H_2 atmosphere overnight. The mixture was filtered over Celite and the filtrate was concentrated under reduced pressure. Purification with SiO_2 column chromatography with hexanes/AcOEt = 4:1 afforded the desired product as a white solid (0.719 g, 92%). Mp 68–71 °C; ^1H NMR ($CDCl_3$) δ 1.46 (s, 9H), 2.05 (s, 3H), 3.60 (s, 3H), 4.39 (d, J = 6.0 Hz, 2H), 4.91 (br s, 1H), 7.05 (d, J = 7.8 Hz, 1H), 7.14 (d, J = 1.3 Hz, 1H), 7.17–7.25 (m, 3H), 7.34 (dd, J = 1.7 and 8.1 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 167.49, 155.84, 143.40, 142.89, 141.34, 135.26, 130.52, 129.56, 129.45, 129.17, 128.41, 127.31, 125.87, 125.25, 51.84, 44.22, 28.37, 20.01; LRMS (ESI) *m/z* calcd for $C_{21}H_{25}NO_4H^+$ 356, found 356; HRMS (ESI) *m/z* calcd for $C_{21}H_{25}NO_4H^+$ 356.1862, found 356.1872.

4.1.7. 5-(*tert*-Butoxycarbonylamino-methyl)-2′-methyl-biphenyl-2-carboxylic acid (6). To a solution of **5** (1.2 g, 3.38 mmol) in THF (12 mL) and MeOH (3 mL), was added LiOH (0.487 g, 11.6 mmol) in H_2O (9.5 mL) and the mixture was refluxed overnight. The reaction mixture was neutralized with 10% citric acid (2.5 mL) and the solvent was evaporated. The residue was then acidified with 10% citric acid (23 mL) and extracted with AcOEt. The organic layer was dried over anhydrous $MgSO_4$ and concentrated under vacuum to afford the desired product as a colorless foam (1.14 g, 99%). ^1H NMR ($CDCl_3$) δ 1.45 (s, 9H), 2.06 (s, 3H), 4.40 (br s, 2H), 4.93 (br s, 1H), 7.07 (d, J = 7.3 Hz, 1H), 7.13 (s, 1H), 7.18–7.25 (m, 3H), 7.35 (d, J = 8.4 Hz, 1H), 8.02 (d, J = 8.1 Hz, 1H); ^{13}C NMR (CD_3OD) δ 170.05, 157.48, 143.58, 143.14, 142.01, 135.41, 130.45, 130.08, 129.53, 129.36, 128.57, 127.02, 125.57, 125.11, 79.24, 43.53, 27.64, 19.19; LRMS (ESI) *m/z* calcd for

$C_{20}H_{23}NO_4H^+$ 342, found 342; HRMS (ESI) *m/z* calcd for $C_{20}H_{23}NO_4H^+$ 342.1705, found 342.1712.

4.1.8. 2-{[5-(*tert*-Butoxycarbonylamino-methyl)-2′-methyl-biphenyl-2-carbonyl]-amino}-4-methylsulfanyl-butyric acid methyl ester (7). A solution of benzoic acid **6** (1.03 g, 3.01 mmol), methionine methyl ester hydrochloride (0.667 g, 3.33 mmol), HOBt (0.455 g, 3.33 mmol) and Et_3N (0.46 mL, 3.33 mmol) in CH_2Cl_2 (15 mL) was cooled to −10 °C. To the cooled solution was added EDCI (0.590 g, 3.01 mmol) and the mixture was stirred for 36 h at room temperature. The reaction mixture was extracted with CH_2Cl_2 (130 mL) and satd. $NaHCO_3$ (55 mL). The organic layer was then extracted with 10% citric acid (50 mL) and brine. Anhydrous $MgSO_4$ was added and the dried organic layer was concentrated under vacuum. The crude product was purified by SiO_2 column chromatography with 2:1 = hexanes/ethyl acetate to afford a white foamy product (1.00 g, 69%). ^{1}H NMR ($CDCl_3$) δ 1.45 (s, 9H), 1.52–1.65 (m, 1H), 1.81–1.91 (m, 1H), 1.96–2.20 (m, 8H), 3.66 (s, 3H), 4.39 (d, J = 5.2 Hz, 2H), 4.57–4.67 (m, 1H), 4.96 (br s, 1H), 5.90 (d, J = 7.5 Hz, 1H), 7.11 (s, 1H), 7.15–7.41 (m, 5H), 7.94 (dd, J = 8.0 Hz (each), 1H); ^{13}C NMR ($CDCl_3$) δ 172.15, 167.91, 156.23, 140.33, 136.71, 131.05, 130.95, 130.55, 130.29, 129.63, 129.33, 128.83, 126.88, 126.73, 126.64, 80.15, 52.75, 52.10, 44.58, 32.11, 29.68, 28.76, 20.44, 15.61; LRMS (ESI) *m/z* calcd for $C_{26}H_{34}N_2O_5SH^+$ 487, found 487; HRMS (ESI) *m/z* calcd for $C_{26}H_{34}N_2O_5SH^+$ 487.2267, found 487.2270.

4.1.9. 2-{[5-(*tert*-Butoxycarbonylamino-methyl)-2′-methyl-biphenyl-2-carbonyl]-amino}-4-methylsulfanyl-butyric acid ethyl ester (8). This compound was prepared in a similar manner to that described for compound **7** with **6** and methionine ethyl ester hydrochloride to yield a colorless foam. This compound was immediately used for the next reaction without further purification.

4.1.10. 2-{[5-(*tert*-Butoxycarbonylamino-methyl)-2′-methyl-biphenyl-2-carbonyl]-amino}-4-methylsulfanyl-butyric acid allyl ester (9). This compound was prepared in a similar manner to that described for compound **7** with **6** and methionine allyl ester sulfonate to afford a white foamy residue. This compound was used for the next reaction without further purification.

4.1.11. 2-[(5-{[(1H-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid methyl ester (FTI-2153) (10). A solution of **7** (0.92 g, 1.90 mmol) in CH_2Cl_2 (9.2 mL) was cooled to 0 °C. To the cooled solution was added TFA (10 mL) and the reaction was stirred at room temperature for 30 min. Evaporation of the solvent was followed by extraction with CH_2Cl_2 (150 mL) and 5% NH_4OH (100 mL). The organic layer was washed with brine and dried over $MgSO_4$. Evaporation of the solvent afforded the free amine (0.74 g). This material was used for the next reaction without further purifications.

To a solution of the free amine (0.74 g, 1.91 mmol) in MeOH (7.2 mL) was added 4(5)-imidazolecarboxaldehyde (0.184 g, 1.91 mmol) and the reaction mixture was stirred for 30 min at room temperature. To the mixture was added $NaBH_4$ (0.072 g, 1.9 mmol) and the reaction was stirred for 36 h. Evaporation of the solvent afforded a residue which was extracted with 10% MeOH in CH_2Cl_2 (350 mL) and saturated $NaHCO_3$ (80 mL). The organic layer was washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent followed by purification by SiO_2 column chromatography with 9:1:0.1 = CH_2Cl_2/MeOH/NH_4OH afforded the product as a white foam (0.48 g, 54%). TLC R_f 0.23 (CH_2Cl_2/MeOH/NH_4OH 9:1:0.1); HPLC t_R 11.93; ^{1}H NMR ($CDCl_3$) δ 1.58–1.63 (m, 1H), 1.85–1.87 (m, 1H), 2.01–2.19 (m, 8H), 3.66 (s, 3H), 3.80 (s, 2H), 3.86 (s, 2H), 4.43–4.52 (m, 1H), 6.03 (d, J = 7.4 Hz, 1H), 6.88 (s, 1H), 7.16–7.42 (m, 6H), 7.48 (s, 1H), 7.78 (dd, J = 7.9 and 26.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 172.32, 168.57, 140.56, 140.29, 139.97, 136.58, 135.64, 130.97, 130.87, 130.55, 130.04, 129.79, 129.63, 129.36, 128.75, 127.80, 126.56, 52.85, 52.78, 52.24, 45.61, 31.83, 29.71, 20.42, 15.56; LRMS (ESI) *m/z* calcd for $C_{25}H_{30}N_4O_3SH^+$ 467, found 467; HRMS (ESI) *m/z* calcd for $C_{25}H_{30}N_4O_3SH^+$ 467.2117, found 467.2112.

4.1.12. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid ethyl ester (FTI-2390) (11). This compound was prepared in a manner similar to that described for FTI-2153 (**10**) with **8** to give the product as a white foam (42 mg, 36%). TLC R_f 0.31 ($CHCl_3$/MeOH/NH_4OH 9:1:0.1); HPLC t_R 12.56; ^{1}H NMR ($CDCl_3$) δ 1.23 (t, J = 7.2 Hz, 3H), 1.56–1.65 (m, 1H), 1.80–1.91 (m, 1H), 1.97–2.22 (m, 8H), 3.82 (s, 2H), 3.87 (s, 2H), 4.06–4.17 (m, 2H), 4.53–4.65 (m, 1H), 5.95 (d, J = 7.0 Hz, 1H), 6.91 (s, 1H), 7.15–7.44 (m, 6H), 7.56 (s, 1H), 7.90 (dd, J = 8.0 and 20.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 171.86, 168.40, 140.69, 139.93, 136.68, 135.54, 131.02, 130.92, 130.60, 130.51, 130.17, 129.90, 129.70, 129.34, 128.80, 127.86, 126.72, 61.92, 52.97, 52.36, 45.65, 32.12, 29.65, 20.53, 15.67, 14.53; LRMS (FAB) *m/z* calcd for $C_{26}H_{32}N_4O_3SH^+$ 481, found 481; HRMS (FAB) *m/z* calcd for $C_{26}H_{32}N_4O_3SH^+$ 481.2273, found 481.2275.

4.1.13. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid allyl ester (FTI-2391) (12). This compound was prepared in a manner similar to that described for FTI-2153 (**10**) with **9** to afford the product as a colorless foam (60 mg, 32%). TLC R_f 0.30 ($CHCl_3$/MeOH/NH_4OH 9:1:0.1); HPLC t_R 13.44; ^{1}H NMR ($CDCl_3$) δ 1.54–1.67 (m, 1H), 1.79–1.92 (m, 1H), 1.95–2.22 (m, 8H), 3.82 (s, 2H), 3.88 (s, 2H), 4.55 (d, J = 5.7 Hz, 2H), 4.57–4.69 (m, 1H), 5.20–5.32 (m, 2H), 5.79–5.91 (m, 1H), 5.94 (d, J = 7.6 Hz, 1H), 6.91 (s, 1H), 7.13–7.46 (m, 6H), 7.56 (s, 1H), 7.91 (dd, J = 8.2 and 21.7 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 171.32, 168.28, 140.87, 139.27, 136.31, 136.06, 135.80, 135.43, 135.15, 133.09, 131.69, 131.09, 130.21, 129.54, 129.09, 126.75, 124.57, 121.56, 119.39, 66.48, 52.40, 51.39, 40.78, 31.56, 29.74, 20.14, 15.50; LRMS (FAB) *m/z* calcd for $C_{27}H_{32}N_4O_3SH^+$ 493, found 493; HRMS (FAB) *m/z* calcd for $C_{27}H_{32}N_4O_3SH^+$ 493.2273, found 493.2272.

4.1.14. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid isopropyl ester (FTI-2392) (13). To a solution of FTI-2153 (**10**) (23.6 mg, 0.051 mmol) in *i*PrOH (1 mL) was added two small 4 Å molecular sieves. The mixture was flushed with N_2 and stirred at room temperature for 15 min. To the mixture was added four drops of $Ti(OiPr)_4$ and an additional amount of *i*PrOH (1 mL). The mixture was stirred at 70 °C for 3 days under N_2. HPLC analysis was used to determine completion of the reaction. Evaporation of the solvent gave a residue that was dissolved in MeOH (2 mL) and H_2O (0.1 mL). The resulting mixture was concentrated and filtered over SiO_2 with 5:1:0.1 = $CHCl_3$/MeOH/NH_4OH. Evaporation of the solvent gave the desired product, which was further purified by preparatory HPLC to give a white foam (25 mg, 99%). TLC R_f 0.34 ($CHCl_3$/MeOH/NH_4OH 9:1:0.1); HPLC t_R 13.18; ^1H NMR ($CDCl_3$) δ 1.17–1.24 (m, 6H), 1.51–1.64 (m, 1H), 1.78–1.89 (m, 1H), 1.96–2.23 (m, 8H), 3.86 (s, 2H), 3.91 (s, 2H), 4.48–4.63 (m, 1H), 4.94–5.01 (m, 1H), 5.94 (d, J = 7.9 Hz, 1H), 6.93 (s, 1H), 7.14–7.63 (m, 7H), 7.89 (dd, J = 7.9 and 19.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 171.06, 168.27, 140.81, 139.31, 136.34, 136.05, 135.68, 135.15, 133.05, 131.04, 129.99, 129.55, 129.29, 129.06, 126.73, 124.62, 121.57, 69.89, 52.58, 51.34, 40.74, 31.68, 29.65, 22.00, 20.22, 15.53; LRMS (FAB) *m*/*z* calcd for $C_{27}H_{34}N_4O_3SH^+$ 495, found 495; HRMS (FAB) *m*/*z* calcd for $C_{27}H_{34}N_4O_3SH^+$ 495.2430, found 495.2430.

4.1.15. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid 2-ethyl-hexyl ester (FTI-2393) (14). This ester was prepared in a similar manner to that described for **13** with FTI-2153 (**10**), titanium (IV)-2-ethylhexoxide in 10:1 THF/DMF to yield the product as a colorless foam (40 mg, 99%). TLC R_f 0.37 ($CHCl_3$/MeOH/NH_4OH 9:1:0.1); HPLC t_R 17.73; ^1H NMR ($CDCl_3$) δ 0.73–0.86 (m, 6H), 1.12–1.30 (m, 9H), 1.42–1.57 (m, 1H), 1.71–1.85 (m, 1H), 1.88–2.16 (m, 8H), 3.76 (s, 2H), 3.82 (s, 2H), 3.90 (d, J = 5.6 Hz, 2H), 4.49–4.62 (m, 1H), 5.85 (d, J = 8.0 Hz, 1H), 6.82–7.57 (m, 8H), 7.83 (dd, J = 7.8 and 17.6 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 171.85, 168.27, 140.83, 140.79, 139.38, 136.43, 136.06, 133.30, 132.99, 131.09, 130.35, 130.17, 129.57, 129.33, 129.10, 126.75, 125.26, 68.44, 52.31, 51.33, 41.24, 39.01, 31.91, 30.64, 29.68, 29.23, 24.02, 23.30, 20.23, 15.51, 14.39, 11.29; LRMS (FAB) *m*/*z* calcd for $C_{32}H_{44}N_4O_3SH^+$ 565, found 565; HRMS (FAB) *m*/*z* calcd for $C_{32}H_{44}N_4O_3SH^+$ 565.3212, found 565.3210.

4.1.16. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid cyclohexyl ester (FTI-2394) (15). Tetracyclohexyl titanate was prepared by reacting titanium tetraisopropoxide (0.143 g, 0.5 mmol) with freshly distilled cyclohexanol (0.26 mL, 2.5 mmol) in anhydrous toluene (3.1 mL) under N_2 atmosphere and the toluene–isopropanol azeotrope was removed by distillation. Additional toluene was added periodically with continuous removal of toluene–isopropanol azeotrope in order to drive the reaction to completion. Excess unreacted cyclohexanol was removed by vacuum distillation and the obtained tetracyclohexyl titanate was immediately used in the synthesis of ester **15** without further purification. ^1H NMR ($CDCl_3$) δ 1.28 (br s, 5H), 1.55 (br s, 1H), 1.74 (br s, 2H), 1.90 (br s, 2H), 3.62 (br s, 1H).

A solution of FTI-2153 (**10**) (25.5 mg, 0.055 mmol) in 10:1 THF/DMF (2 mL) was added to a round bottom flask containing freshly prepared tetracyclohexyl titanate (0.16 mmol) with a syringe to avoid minimal exposure to atmospheric moisture. To the mixture, was added five small 4 Å molecular sieves and the reaction was stirred at 60 °C for 9 days under N_2 atmosphere. During this period, additional amounts of THF were added to keep approximately 2 mL of solvent in the reaction flask. The solvent was evaporated and to the residue was added MeOH (2 mL) and H_2O (0.1 mL). The reaction mixture was stirred for an additional 5 min at room temperature and was concentrated under vacuum. The residue was filtered over SiO_2 with 5:1:0.1 = $CHCl_3$/MeOH/NH_4OH. Evaporation of the solvent gave the crude product, which was further purified by preparatory HPLC to afford the final product as a colorless foam (13 mg, 44%). HPLC t_R 15.14; ^1H NMR ($CDCl_3$) δ 1.18–1.44 (br m, 5H), 1.46–1.56 (br m, 1H), 1.56–1.74 (br m, 3H), 1.74–1.89 (br m, 3H), 1.90–2.20 (br m, 8H), 4.22 (s, 2H), 4.33 (s, 2H), 4.47–4.61 (br m, 1H), 4.68–4.79 (br s, 1H), 6.27 (br d, J = 16.9 Hz, 1H), 6.87–7.67 (m, 7H), 7.80 (br s, 1H), 8.34 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 172.10, 169.58, 139.98, 139.90, 139.46, 137.79, 137.48, 131.77, 131.76, 131.73, 129.76, 129.60, 128.82, 128.34, 128.31, 127.75, 125.41, 73.26, 51.03, 50.36, 30.66, 30.34, 28.68, 28.12, 24.13, 22.51, 18.90, 14.18; LRMS (FAB) *m*/*z* calcd for $C_{30}H_{38}N_4O_3SH^+$ 535, found 535; HRMS (FAB) *m*/*z* calcd for $C_{30}H_{38}N_4O_3SH^+$ 535.2743, found 535.2745.

4.1.17. 2-{[(5-[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid benzyl ester (FTI-2628) (16). A solution of FTI-2153 (**10**) (146 mg, 0.313 mmol) in THF (3.1 mL) was flushed with N_2 for 25 min. To the solution was added benzyl alcohol (3.2 mL, 31 mmol), and activated 4 Å molecular sieves (0.62 g). The reaction was stirred under N_2 for an additional 15 min, and $Ti(OiPr)_4$ (21.7 μL) was added. The reaction mixture was stirred at 85 °C for 2 days under N_2 atmosphere. The solvent was evaporated and the residue was dissolved in MeOH (16 mL) and H_2O (0.8 mL) and stirred for an additional 10 min at room temperature. The resulting mixture was concentrated and filtered over SiO_2 with 5:1:0.1 = $CHCl_3$/MeOH/NH_4OH. Evaporation of the solvent gave the crude product, which was further purified by preparatory HPLC to give **16** as a white foam (164.5 mg, 98%). HPLC t_R 15.61; ^1H NMR ($CDCl_3$) δ 1.55–168 (m, 1H), 1.78–1.88 (m, 1H), 1.89–2.10 (m, 8H), 4.19 (s, 2H), 4.30 (s, 2H), 4.52–4.66 (br m, 1H), 5.07 (s, 2H), 6.27 (br d, J = 30 Hz, 1H), 6.96–7.56 (m, 12H), 7.78 (s, 1H), 8.30 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 171.32, 168.47, 140.88, 139.13, 136.28, 135.73, 135.45, 135.32, 135.17, 133.07, 130.99, 130.08, 129.60, 129.28, 129.15, 129.05, 128.76, 128.70, 124.44, 121.59, 67.73, 52.33, 51.48, 40.79, 31.46, 29.65, 20.12, 15.49; LRMS

(FAB) *m/z* calcd for $C_{31}H_{34}N_4O_3SH^+$ 543, found 543; HRMS (FAB) *m/z* calcd for $C_{31}H_{34}N_4O_3SH^+$ 543.2430, found 543.2428.

4.1.18. 2-[(5-{[(1*H*-Imidazol-4-ylmethyl)-amino]-methyl}-2′-methyl-biphenyl-2-carbonyl)-amino]-4-methylsulfanyl-butyric acid. 2TFA salt (FTI-2148.2TFA) (17). A solution of FTI-2153 (**10**) (1.41 g, 3.02 mmol) in THF (21 mL) was cooled to 0 °C and LiOH (0.251 g, 5.99 mmol) in H_2O (12.1 mL) was added dropwise. The reaction was stirred at room temperature for 30 min and neutralized with 0.5 N HCl (15 mL). Solvent evaporation gave a white residue which was further purified by gel permeation on Sephadex LH-20 with MeOH/$CHCl_3$ = 1:1 to give FTI-2148. The product was dissolved in CH_2Cl_2 (9.5 mL) and MeOH (1.1 mL) and the solution was cooled in ice-water bath. TFA (1.3 mL) was then added and the solvent was rapidly removed under vacuum to give the product as a cream yellow TFA salt (1.72 g, 84%). HPLC t_R 10.46; ^{1}H NMR (CD_3OD) δ 1.65–1.82 (m, 1H), 1.95–2.26 (m, 9H), 4.39 (s, 2H), 4.39–4.48 (m, 1H), 4.48 (s, 2H), 7.13–7.33 (m, 4H), 7.45 (d, J = 12.0 Hz, 1H), 7.61–7.73 (m, 2H), 7.74 (s, 1H), 8.81 (s, 1H); ^{13}C NMR (CD_3OD) δ 175.09, 171.77, 142.41, 141.05, 139.10, 137.76, 137.18, 134.49, 133.75, 131.75, 131.09, 130.49, 130.21, 129.74, 127.21, 126.12, 122.75, 53.27, 52.07, 41.99, 32.12, 31.25, 20.89, 15.45; LRMS (ESI) *m/z* calcd for $C_{24}H_{28}N_4O_3SH^+$ 453, found 453; HRMS (ESI) *m/z* calcd for $C_{24}H_{28}N_4O_3SH^+$ 453.1960, found 453.1952.

4.1.19. Log *P*. Theoretical values of log *P* were predicted using QikProp program established at Yale University.[13] The 3D structures were entered by using XChem-Edit and were optimized using BOSS program. The octanol/water partition coefficients were computed with the commercial software QikProp version 1.6.[11]

4.1.20. Assay for in vitro inhibition of *P. falciparum* growth.[14] *P. falciparum* 3D7 in human red blood cells (0.4% parasitemia, 2% hematocrit) was cultured with the compound (added as 1 μL DMSO solution) in a 200-μL medium using a 96-well plate. After 48 h, 0.8 μCi of [8-^{3}H]hypoxanthine (American Radiolabeled Chemicals) was added to the culture, and the incubation was continued for an additional 24 h. The malaria growth was quantified by measuring the radioactivity incorporation into the parasites and expressed as % incorporation of the no-drug control level. Murine 3T3 fibroblasts were grown in 96-well plates in the presence of serially diluted compounds for 72 h. Viability was quantified using the AlamarBlue reagent (Alamar Biosciences Inc., Sacramento, Calif.).

4.1.21. In vivo antimalarial test. Blood was taken from donor mice infected with *P. berghei* (ANKA strain) and diluted in heat inactivated fetal calf serum to a parasitemia of 1% (equivalent of 1×10^7 infected erythrocytes); 0.2 mL of the diluent was used to infect female BALB/c mice (18–20 g). The mice were randomly sorted into groups of 5 and dosing commenced 2 h post-infection. Compounds were prepared as a stock solution of 10 mg/mL in 10% DMSO/PBS. Compound **16** and the control drug, chloroquine, were administered i.p. for four consecutive days in a 0.2 mL volume at, respectively, doses of 50 mg/kg and 10 mg/kg. On day 5, blood smears were taken, fixed in methanol and Giemsa stained. The percentage of suppression was calculated from the parasitemia in untreated and treated groups.

4.1.22. In vitro mammalian PFT and PGGT-I activity assay. In vitro inhibition assays of mammalian PFT and PGGT-I were done by measuring the incorporation of [^{3}H] FPP (Amersham Biosciences, Piscataway, NJ) and [^{3}H] GGPP (Perkin–Elmer Life and Analytical Sciences, Boston, MA) into wild type H-Ras (PFT) and H-Ras-CVLL (PGGT-I), respectively, as previously described.[15] Briefly, 75 μg of 60,000*g* post-microsomal supernatant from Daudi cells was incubated in the presence of increasing concentration of compound, 10 μg H-Ras or H-Ras-CVLL substrate, and 0.5 μCi/sample of either [^{3}H] FPP or [^{3}H] GGPP. Samples were TCA-precipitated and then filtered onto glass fiber filters; unbound [^{3}H] FPP or [^{3}H] GGPP was washed through the filters. Samples were counted on a scintillation counter and activity compared to vehicle controls to obtain IC_{50} values.

4.1.23. Mammalian Ras and Rap1A processing assay. Subconfluent NIH 3T3 mouse fibroblast cells stably transfected with H-Ras61L (H-Ras/3T3) were treated for 48 h with increasing concentrations of the compounds to determine their inhibitory activity against the farnesylation of H-Ras protein and geranylgeranylation of Rap1A protein. Cells were lysed in 30 mM Hepes, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EGTA, 1% Triton-X-100, 10% glycerol, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 25 μg/mL leupeptin, and 2 mM phenylmethylsulfonylfluoride on ice for 30 min. Cleared lysates were resuspended in 2× SDS-PAGE sample buffer and run on 12.5% SDS-PAGE gels to separate proteins. Proteins were transferred to nitrocellulose and probed with 25 μg/mL Y13-289 pan-Ras antibody for detection of H-Ras or with 0.2 μg/mL Rap1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Results were visualized using peroxidase-conjugated antirat or antirabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and an enhanced chemiluminescence detection system (Perkin–Elmer).

4.1.24. Assay for in vitro inhibition of *P. falciparum* PFT. The PFT assay used to determine the IC_{50}s of the compounds is based on the glass fiber filter method.[16] Assays were carried out in 30 mM potassium phosphate pH 7.7, with 5 mM DTT, 0.5 mM $MgCl_2$, 20 μM $ZnCl_2$, 0.3 μCi (0.75 μM) [^{3}H] farnesyl pyrophosphate (15 μCi/mmol, American Radiolabeled Chemicals.), 1 μM RAS-CVIM protein substrate[3] in a total volume of 20 μL which included 1 μL of FTI solution in DMSO and 3 μL of partially purified Pf PFT.[17] Assays in the absence of FTI and Pf PFT were included as positive and negative controls, respectively. Reaction mixtures were incubated at 30 °C for 30 min, and the reaction was terminated by addition of 200 μL of 10% HCl/ethanol. After overnight

incubation at room temperature, the mixtures were filtered onto a Whatman glass fiber filter (VWR, San Francisco, CA) using a 96-well vacuum manifold. After washing with 100% ethanol, the filter was cut, and individual slices were counted in a beta-scintillation counter. IC_{50} values were calculated using linear regression analysis of the plots of the ^{3}H-incorporation into protein versus concentration of compounds.

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