Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 2027–2030

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

The Synthesis and Biological Evaluation of a Series of Potent Dual Inhibitors of Farnesyl and Geranyl-Geranyl Protein Transferases

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Received 16 January 2002; accepted 12 March 2002

Abstract—We have prepared a series of potent, dual inhibitors of the prenyl transferases farnesyl protein transferase (FPTase) and geranyl-geranyl protein transferase I (GGPTase). The compounds were shown to possess potent activity against both enzymes in cell culture. Mechanistic analysis has shown that the compounds are CAAX competitive for FPTase inhibition but geranyl-geranyl pyrophosphate (GGPP) competitive for GGPTase inhibiton.

Ras proteins are a family of GTPases which are processed by a series of intracellular enzymes. Ras functions primarily as a molecular on–off switch, cycling between an inactive GDP-bound form and an active GTP-bound form.[1] The biologically inactive pro-Ras peptide becomes activated by anchorage to the inner surface of cell membranes through a series of post-translational C-terminal modifications.[1] Ras proteins share a C-terminal CAAX motif, and the first and most important post-translational modification covalently adds a farnesyl group from farnesyl diphosphate onto the cysteine residue of the CAAX sequence catalyzed by farnesyl protein transferase (FPTase).[2] The fully modified and anchored Ras proteins initiate a cascade of phosphorylations, leading to a nuclear signal for cell division. In the case of mutant Ras proteins, the signal is always activated and is believed to be responsible for the oncogenic response.[1] Inhibition of FPTase has thus been accepted as a viable approach to treating tumors in which mutant Ras proteins are involved.

Upon inhibition of FPTase, N-Ras and K-Ras act as substrates for geranyl-geranyl protein transferase I (GGPTase), resulting in geranyl-geranylated Ras proteins that are functionally equivalent to the corresponding farnesylated proteins.[3] This suggests that inhibition of FPTase in and of itself may not be completely effective as a means of blocking the signaling process, as cross prenylation by GGPTase could restore the function of the prenylated proteins involved in the signaling cascade. Therefore, when attempting to block mutant Ras protein function pharmacologically, it may be critical to inhibit both FPTase and GGPTase activity. Sebti and co-workers[4] have shown that inhibition of FPTase is more effective than inhibition of GGPTase at inhibiting tumor growth in mouse xenograft models; however, these studies were conducted with less than optimal inhibitors at extremely high exposures. A critical need remains for highly potent dual FPTase-GGPTase inhibitors that could be used experimentally to further elucidate the roles of these enzymes in the control of cellular proliferation. This paper describes our efforts toward the design and synthesis of potent, dual inhibitors of FPTase and GGPTase.

In the preclinical evaluation of the Merck FPTase inhibitor **1a** (Fig. 1), it was noted that the compound also inhibited GGPTase activity through a mechanism that was GGPP competitive rather than CAAX competitive as was seen with FPTase.[5] This suggested that the

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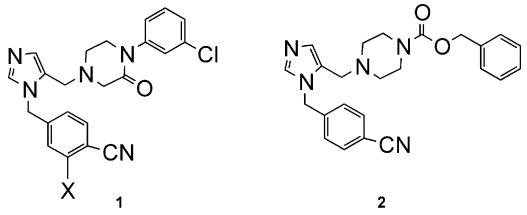
0960-894X/02/$ - see front matter
PII: S0960-894X(02)00308-6

molecule might occupy part of the binding region used by GGPP in the GGPTase active site. A previous manuscript[6] has described a similar series of compounds derived from **1a** that possess dual inhibitor activity. These compounds (Fig. 1, **1b**) used large lipophilic groups appended to the cyanobenzyl moiety of **1a** to provide a series that possessed activity against both prenylation enzymes. The data presented in this previous manuscript showed that a large arylthio or aryloxy group adjacent to the cyano group provided compounds with potent activity against both enzymes. The results obtained with this series of compounds as well as with **1a** indicate that both the large lipophilic group appended to the cyanobenzyl moiety and the *m*-chlorophenyl ring on the opposite end of the molecule play a key role in binding to GGPTase.

Based on the discussion above, as well as previous modeling studies with various compounds in this series,[7] we theorized that the lipophilic *m*-chlorophenyl moiety of **1a** and **1b** was occupying part of the lipophilic GGPP binding region in the GGPTase active site. We therefore began a synthetic program designed to investigate the placement of larger, more lipophilic groups off of the piperazinone ring rather than on the cyanobenzyl moiety as previously reported.[6] For synthetic ease, we used the simple piperazine ring system, and replaced the *m*-chlorophenyl ring with a series of aryl carbamate moieties. Since compound **2** (Fig. 1), the prototype compound for this series, exhibited reasonable potency versus both enzymes, we followed this compound with a series of substituted aryl carbamates.

Inhibitors **7**–**23** were synthesized as shown below in Scheme 1.[8] Reductive amination of the aldehyde **3**[9] with Boc-piperazine was effected utilizing titanium isopropoxide catalysis.[10] Equivalent amounts of the aldehyde, amine, and titanium isopropoxide were premixed and stirred for 1 h, with a trace of THF added to provide efficient stirring. The resulting slurry was diluted with dry ethanol, and the mixture treated with 0.7 equiv of $NaBH_3CN$ to give the desired products **4** in good yield. These reductive amination conditions provided consistently good results with this series of compounds. Compound **4** was deprotected upon treatment with TFA to provide the free amine **5**. Reaction of **5** with the appropriate *p*-nitrophenyl carbonate (prepared by reaction of the appropriate alcohol with *p*-nitrophenyl chloroformate) provided the target compounds **6**–**23** in good yield.[8]



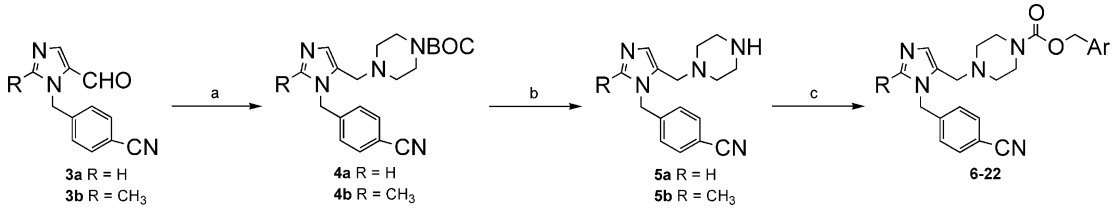
1a X = H
1b X = various lipophilic groups

	IC 50 (nM)	
	FPTase	GGPTase
1a	2	99
2	63	61

Figure 1. Structure and inhibitory potency for **1** and **2**.

Compounds **6**–**23** were evaluated for their ability to inhibit both FPTase and GGPTase in vitro. Compounds were tested as FPTase inhibitors using purified recombinant enzyme to catalyze the reaction between [1-^{3}H]FPP and a recombinant protein substrate containing the K-ras C-terminus.[11] The IC_{50} value reported is the concentration of inhibitor required to reduce radiolabel incorporation by 50%, as compared to uninhibited controls.[11] Inhibition of GGPTase was evaluated in a scintillation proximity assay, by carrying out the enzymatic reaction between [1-^{3}H]GGPP and a biotinylated peptide that represents the C-terminus of the K4B-Ras protein in the presence of 5 mM ATP and varying concentrations of inhibitor.[12] IC_{50} values were reported as described above for FPTase. Enzyme inhibition data for the compounds are detailed in Table 1; all reported values are the average of at least two determinations.

In general, most of the compounds evaluated in this study were found to be potent inhibitors of both enzymes. Addition of a small to moderate-sized lipophilic 2-substituent to the aryl ring of the carbamate moiety provided compounds that demonstrated a marked enhancement in both FPTase and GGPTase inhibitory potency (**6** and **8**–**13**). Introduction of a more polar methanesulfonyl moiety to the 2-position of the aryl ring (**14**) tempered the activity against both enzymes, although **14** remained a more potent inhibitor of GGPTase than the unsubstituted aryl compound **2**. Compound **12** was an extremely potent, sub-nanomolar inhibitor of both enzymes. Movement of the lipophilic 2-substituent to the 3-position (**7** and **18**) gave compounds that exhibited a loss in inhibitory potency towards both enzymes as compared to the corresponding 2-substituted analogues. The 2,3-disubstituted



3a R = H
3b R = CH_3
4a R = H
4b R = CH_3
5a R = H
5b R = CH_3
6-22

Scheme 1. Reagents and conditions: (a) $Ti(OPr)_4$, $NaBH_3CN$, Boc-piperazine/THF–EtOH; (b) TFA/CH_2Cl_2; (c) appropriate benzyl-(*p*-nitrophenyl) carbonate, DIEA/DMF, 80 °C.

analogues **15**–**17** retained good inhibitory potency versus both enzymes; however, the activity was somewhat less than that observed for the 2-monosubstituted derivatives. The 3,4-methylenedioxy derivative **19** demonstrated a marked loss in potency versus both enzymes when compared to the 2,3-disubstuted **17**, suggesting that a 4-substituent is detrimental to activity versus both enzymes.

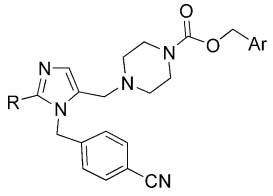
Addition of a methyl group to the 2-position of the imidazole ring (Table 1, **20**–**23**) led to profound activity changes compared to the previous compounds. While these compounds remained potent inhibitors of FPTase, a marked decrease in GGPTase inhibitory potency was observed for all of these analogues. The structural basis for the decreased GGPTase inhibitory activity exhibited by these 2-methyl imidazole analogues is unclear at present.

All of the compounds that were shown to be potent inhibitors of FPTase were also evaluated for FPTase inhibitory activity in cells by a cell-based radiotracer assay for farnesyl transferase inhibition (CRAFTI).[13] In this assay conducted in cultured H-ras transformed Rat1 cells, the concentration of compound required to displace 50% of a highly potent radiolabeled FPTase inhibitor[14] from FPTase was determined and reported as an IC_{50} value. Reported values are the average of at least two determinations. All of the potent in vitro inhibitors of FPTase also possessed potent FPTase inhibitory activity in our cell-based CRAFTI assay (Table 1).

Several of the more potent inhibitors of GGPTase (Table 1, **9**, **12**, **13**, **22**, and **23**) were evaluated for their GGPTase inhibitory activity in cells, as measured by the inhibition of geranyl-geranylation of the C-terminal CAAX sequence of Rap 1a in PSN-1 cells (Table 1).[15] Reported values are the average of at least two determinations. All four compounds exhibited potent Rap 1a activity in PSN-1 cells, with the potency correlating directly with the observed GGPTase potency in the enzyme assay.

Further biochemical evaluation[16] of a number of potent inhibitors (**9**, **12**, **13**, **15**, **16**, **20**, and **23**) demonstrated that all of the potent GGPTase inhibitors were GGPP competitive and non-competitive with CAAX peptide.[17] In contrast, evaluation of the mechanism of FPTase inhibition indicated that these compounds were in general non-competitive with respect to FPP, and competitive with CAAX peptide. All of the GGPP competitive compounds displayed a marked dependence on phosphate anions to achieve maximal potency, with ATP among the most effective anions.[5] The combination of inhibitor and phosphate was found to compete effectively with GGPP binding and to lower IC_{50} values by as much as 200-fold when compared to inhibitor alone.[5] These experimental findings suggest that this group of compounds is likely to occupy at least part of the prenyl binding region of the GGPTase active site. Phosphates are believed to competitively bind in the pyrophosphate site of the GGPTase binding site, thereby displacing GGPP and facilitating binding of the inhibitors. This group of compounds also displayed slow-binding

Table 1. FPTase and GGPTase inhibitory and cell culture activity for **2** and **6**–**23**



Compd[a]	R	Ar	FPTase IC_{50} (nM)[b]	GGPTase IC_{50} (nM)[b]	CRAFTI IC_{50} (nM)[b]	Rap 1a EC_{50} (nM)[b]
2	H	Ph	62.5	60.6	21.8	ND
6	H	2-MePh	6.4	9.1	62.9	ND
7	H	3-MePh	34	76	166	ND
8	H	2-(OMe)Ph	3.1	0.84	4, 27 ($n=2$)	ND
9	H	2-(OEt)Ph	1.6	0.32	4.52	71
10	H	2-Cl Ph	6.8	0.68	7.95	ND
11	H	2-(MeOCH2)Ph	6.1	0.32	9.1	ND
12	H	2-(OCF3)Ph	0.36	0.16	2.2	18
13	H	2-(F3CCH2)Ph	2.5	4.82	0.85	50
14	H	2-(MeSO2)Ph	99	18	ND	ND
15	H	2-(OMe)-Pyridin-3-yl	18	48	13	ND
16	H	2-(OEt)-Pyridin-3-yl	5	8.7	8.67	ND
17	H	2,3-Methylenedioxy Ph	15.5	19.8	10.9	ND
18	H	3-(OCF3)Ph	13.4	74	12.1	ND
19	H	3,4-Methylenedioxy Ph	102	121	ND	ND
20	Me	Ph	19	789	ND	ND
21	Me	2-(OEt)Ph	1.5	80	1.13	ND
22	Me	2-(MeOCH2)Ph	17	178	3.17	680
23	Me	2-(OCF3)Ph	0.33	53	0.34	660

[a]All compounds tested in biological assays were fully characterized and exhibited satisfactory analytical (NMR, HPLC purity >98%, HRMS) data.
[b]Details of assay conditions are provided in the text and references cited therein. All cited values are the average of at least two determinations.

characteristics on GGPTase, demonstrating a 20-fold enhancement in GGPTase inhibitory potency after a 30-min preincubation of GGPTase and inhibitor (prior to addition of GGPP).[5] This procedure was necessary to obtain accurate GGPTase inhibition values for compounds in this class. The reaction mechanism of many prenyl-protein transferases utilizes a carbocation-pyrophosphate intermediate. The combination of slow-binding kinetics and phosphate dependence suggests that these inhibitors may be acting as transition state analogues, where the phosphate may be a mimic of the pyrophosphate leaving group.[5]

In summary, we have discovered a series of highly potent, dual FPTase/GGPTase inhibitors. Representative compounds were shown to be potent inhibitors of both enzymes in cell culture, and were shown to be GGPP competitive for GGPTase inhibition but CAAX competitive for FPTase inhibition. These compounds represent a new class of highly potent dual prenylation inhibitors, and may be important experimental tools for elucidating the role of prenylation inhibitors as novel chemotherapeutic agents.

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8. Satisfactory analytical data (NMR, HPLC purity >98%, HRMS) were obtained for all final compounds that were tested in biological assays. Full experimental details for the synthesis of these compounds are presented in the following patent: Hartman, G. D.; Lumma, W. C.; Sisko, J. T.; Smith, A. M.; Tucker, T. J.; Stokker, G. E. PCT International Application WO 00/51611, 2000.
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10. The reductive amination procedure was adapted from a procedure described in the following paper: Mattson, R. J.; Pham, K. M.; Leuck, D. J.; Cowen, K. A. *J. Org. Chem.* **1990**, *55*, 2552.
11. Experimental details of the FPTase assay procedure are presented in the patent application cited in ref 8.
12. Experimental details of the GGPTase assay procedure are presented in refs 5 and 8.
13. Experimental details of the cell based radiotracer assay for farnesyl transferase (CRAFTI) are presented in the patent application cited in ref 8.
14. The radiolabeled FPTase inhibitor used in this assay is [^{125}I]-4-{[5-(2*S*)-4-(3-iodophenyl)-2-[2-(methylsulfonyl)ethyl]-5-oxopiperazin-1-yl]methyl}benzonitrile. This compound has approximately 50,000 high-affinity binding sites (apparent K_d ~1 nM) in Rat1 cells.
15. Experimental details of the Rap 1a cell-based assay are provided in refs 5 and 8.
16. Details of the experimental techniques used in the enzyme kinetics analysis are detailed in ref 5.
17. The slope of a plot of log(IC_{50}) versus log[GGPP] in the presence of 5 mM ATP was used to determine the mechanism of GGPTase inhibition. Slope values were determined for the following compounds: **9**, slope = 1.05; **12**, slope = 0.84; **13**, slope = 0.99; **15,** slope = 0.84; **16**, slope = 0.90; **20**, slope = 0.63; **23**, slope = 0.67. All of the slope values are >0.50 and are strongly suggestive of a GGPP competitive inhibitor.