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Communications to the Editor

2-Substituted Piperazines as Constrained Amino Acids. Application to the Synthesis of Potent, Non Carboxylic Acid Inhibitors of Farnesyltransferase

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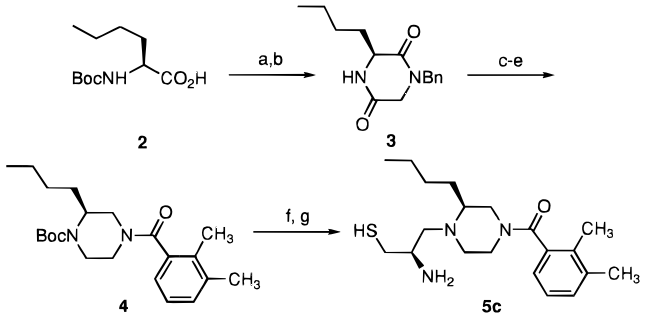
Mutations in the *ras* oncogene are present in many human cancers, including 30−50% of colon and 90% of pancreatic cancer.[1,2] The Ras protein undergoes a series of post-translational modifications, including S-farnesylation of a cysteine residue located in the C-terminal tetrapeptide, a region of the molecule referred to as the "CaaX box" (C = cysteine, a = any aliphatic amino acid, X = a prenylation specificity residue).[2] The enzyme farnesyltransferase (FTase) utilizes farnesyl diphosphate (FPP) to farnesylate the cysteine thiol of CaaX sequences, in which X is usually serine, methionine, or glutamine.[3−5] Since farnesylation of mutant Ras proteins is required for cell transformation,[6] inhibition of FTase represents a pharmacological target for cancer chemotherapy.[7] FTase inhibitors have been shown to inhibit protein farnesylation in cell culture and to suppress *ras*-transformed cell growth without affecting normal cell growth.[8] Efficacy of an FTase inhibitor in animal models of cancer has been demonstrated by the inhibition *ras*-dependent tumor growth in nude mice[9] and by the regression of tumors in Ha-*ras*-transgenic mice (Oncomouse).[10] Current medicinal chemistry efforts are focused on obtaining an inhibitor suitable for drug development.

Scheme 1. Synthesis of 2-Alkyl-4-acylpiperazine FTase Inhibitors[a]



[a] (a) $BnNHCH_2CO_2C_2H_5$, DCC, CH_2Cl_2, 18 h (100%); (b) HCl, $CHCl_3$; $NaHCO_3$, H_2O (100%); (c) $LiAlH_4$, THF, reflux, 15 h; Boc_2O, CH_2Cl_2, 2 h (89%); (d) H_2 10% Pd/C, CH_3OH, 16 h (100%); (e) 2,3-dimethylbenzoic acid, EDC·HCl, HOBT, DMF, 3 h (88%); (f) TFA, CH_2Cl_2, 0.75 h; *N*-Boc-*S*(Tr)-L-cysteinal, $NaBH(OAc)_3$, $ClCH_2CH_2Cl$, 0−20 °C, pH 6, 18 h (67%); (g) 4 equiv of Et_3SiH, TFA, CH_2Cl_2, 0.75 h (55%).

Tetrapeptides corresponding to the C-terminus of Ras inhibited farnesylation of the full length protein by acting as alternate substrates.[3,11,12] Modifications to either the a_2 residue of the Ca_1a_2X box or to the tetrapeptide backbone eliminated substrate activity while retaining inhibitory activity.[13−20] Two critical binding elements present in these inhibitors were the N-terminal thiol group and the C-terminal carboxylic acid. Since FTase is an intracellular enzyme, the utility of FTase inhibitors in treating cancer is highly dependent on their ability to cross the cell membrane and inhibit intracellular protein farnesylation. Although potent inhibitors of FTase *in vitro*, a major limitation of the carboxylic acids was poor cell membrane permeability; an ester prodrug strategy was required in order to obtain activity in cell culture.[14,16,19,21,22] The limitations of prodrugs prompted the design of inhibitors which do not depend upon a carboxylic acid for *in vitro* potency. For example, the pseudodipeptide amide **1a**

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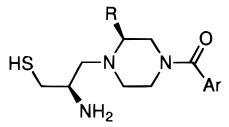
Table 1. FTase Inhibition and Selectivity of Piperazines *in Vitro* and Inhibition of Ras Processing in Cell Culture



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			IC_{50} (nM)		IC_{50} (μM)
compd	R	Ar	FTase[a]	GGTase I[b]	Ras processing[c]
5a	n-C_4H_9	phenyl	24 ± 4 (2)	ND[d]	ND
5b	n-C_4H_9	1-naphthyl	1 ± 1 (3)	160 ± 40 (2)	0.5
5c	n-C_4H_9	2,3-dimethylphenyl	5 ± 2 (2)	360 (1)	0.5
5d	H	2,3-dimethylphenyl	160 ± 30 (2)	2800 (1)	10
5e	C_2H_5	2,3-dimethylphenyl	68 ± 10 (2)	ND	5
5f	$CH_3OCH_2CH_2$	1-naphthyl	3 ± 2 (5)	10000 ± 1000[e] (2)	0.5
5g	c-Pr-OCH_2CH_2	1-naphthyl	1 ± 2 (2)	3900[e] (1)	0.5

[a] Concentration of compound required to inhibit 50% of FTase-catalyzed incorporation of [^{3}H]FPP into recombinant human Ha-Ras protein. Assay results are reported ± SEM for the number of determinations in parentheses and were obtained using the protocol described in ref 11. [b] Inhibition of bovine type-I GGTase, except where noted. [c] Inhibition of post-translational processing of Ras protein in RAT1 cells transformed with viral Ha-*ras*. Assay conditions are described in ref 34. [d] Not determined. [e] Inhibition of human recombinant GGTase I.

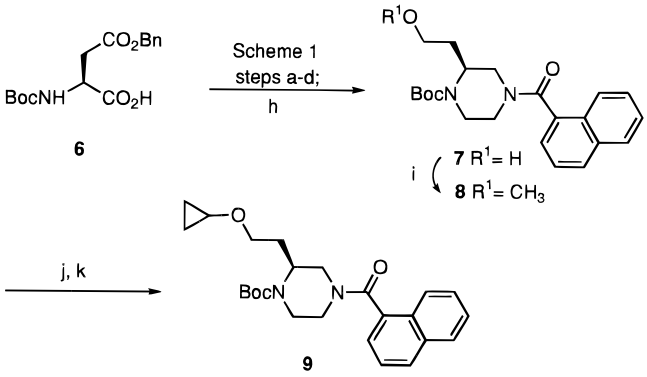
(IC_{50} of 23 nM) was designed to mimic the Ca_1a_2 portion of the Ca_1a_2X box.[23] Unfortunately, assays in cell culture with **1a** and related compounds were largely hampered by nonmechanism-based cytotoxicity. We therefore concentrated our efforts upon improving the potency and tolerability of the non carboxylic acid inhibitors.

Chemistry. 2-Alkylpiperazines were synthesized essentially according to the procedure described for the synthesis of enantiomerically pure 2-methylpiperazine.[24] For example, *N*-Boc-norleucine **2** was coupled to ethyl *N*-benzylglycinate, followed by N-deprotection and cyclization to the diketopiperazine **3** (Scheme 1). LAH reduction then furnished the *N*-benzylpiperazine. The N-benzyl protecting group was removed by catalytic hydrogenation, and the free amine was acylated with 2,3-dimethylbenzoic acid to give **4**. Reductive alkylation with (*S*)-*N*-Boc-*S*-trityl cysteine aldehyde was best accomplished under conditions described for the reductive alkylation of weakly basic amines.[25] Simultaneous removal of the *N*-Boc and *S*-trityl protecting groups with Et_3SiH and TFA furnished (2-amino-3-mercaptopropyl)-piperazine **5c**. Compounds **5a**,**b**,**e** were synthesized in similar fashion from the appropriate amino acids. Compound **5d** was prepared from comercially available *tert*-butyl 1-piperazinecarboxylate.

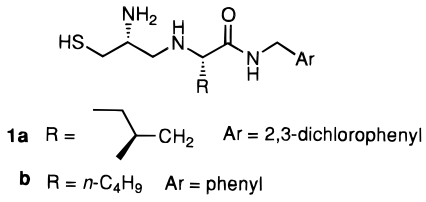
The benzyl ester of *N*-Boc-L-aspartic acid (**6**) was converted to the (2-hydroxyethyl)piperazine **7** following steps a−d in Scheme 1 and subsequent coupling to 1-naphthoic acid (Scheme 2). O-Alkylation of **7** with methyl iodide in the presence of sodium hydride furnished methyl ether **8**. Alcohol **7** was also converted to the vinyl ether with mercuric acetate in ethyl vinyl ether; the vinyl ether was subsequently reacted with diiodomethane and diethylzinc to give cyclopropylether **9**.[26] Both **8** and **9** were further elaborated to **5f** and **5g**, respectively, according to steps f and g, Scheme 1.

Results and Discussion. It is well established that the introduction of conformational constraints into peptides can dramatically affect receptor binding affinity.[27] Pseudodipeptide FTase inhibitors **1a**,**b** have access to a plethora of conformations, and structural information relating to their binding conformation(s) was not available. We hypothesized that in a compound such as **1b** (IC_{50} = 900 nM[23]), replacing the aliphatic amino acid with piperazine would be one way of

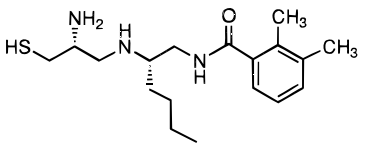
Scheme 2. Synthesis of (2-Alkoxyethyl)piperazine Intermediate[a]



[a] (h) 1-Naphthoic acid, EDC·HCl, HOBT, DMF (90%); (i) CH_3I, NaH, DMF, 0 °C, 1 h (85%); (j) $C_2H_5OCHCH_2$, $Hg(O_2CCH_3)_2$, 20 °C, 18 h; (k) CH_2I_2, $(C_2H_5)_2Zn$, Et_2O, 30 °C, 1 h (34%, two steps).



stabilizing and testing an approximately extended conformation. Appropriate substitution on the piperazine ring might also exploit the hydrophobic a_1 binding site occupied by Ca_1a_2X analogs. In subsequent studies, a series of *N*-acylpiperazines emerged as the most active compounds from among a number of piperazine-constrained analogs. For example, *N*-acylpiperazine **5a** was found to be 40-fold more potent than **1b** (Table 1). Variation of the *N*-acyl substituent led to further improvements in potency, *e.g.* 1-naphthoylpiperazine **5b**, which had an IC_{50} of 1 nM. To further ascertain the importance of conformational constraint upon activity, the ring-opened analog **10** was synthesized and found to be 650-fold less active than the constrained analog **5c**. Shortening or eliminating the C-2 piperazine alkyl substituent (**5d**,**e**) led to a decrease in activity, consistent with this group occupying a lipophilic a_1 binding pocket. Inverting the stereochemistry of the butyl group in **5c** led to a 2-fold decrease in potency (data not shown).



10

The piperazines **5** were not substrates of FTase. Kinetic analysis of **5c** binding to FTase revealed a competitive mode of inhibition with respect to the Ras substrate (K_i = 1 nM). The closely related prenyl transferase geranylgeranyltransferase I (GGTase I) recognizes CaaX sequences wherein X is leucine.[11,28,29] It is interesting to note that piperazines **5** selectively inhibited FTase compared to GGTase, despite the lack of a structural element corresponding to the specificity-determining X residue (Table 1). This phenomenon has also been observed in two other series of FTase inhibitors.[23,30] Simple alkyl-substituted piperazines such as **5b** exhibited up to 160-fold selectivity for inhibition of FTase.

In an assay where Ha-*ras*-transformed cells were incubated with these inhibitors for 24 h and then lysed, denaturing gel electrophoresis of immunoprecipitated Ras protein showed inhibition of Ras processing (farnesylation). The most active compounds in this assay had IC_{50}s of ~0.5 μM (Table 1). A second cell assay measured the competence of these compounds in inhibiting transformed cell growth. Both the size and number of colonies of *ras*-transformed RAT1 cells were reduced in soft agar in the presence of 10 μM **5b** (Table 2). Furthermore, growth inhibition was selective for *ras*-transformed cells. The *raf* oncogene transforms cells in a farnesylation- and *ras*-independent manner, and the growth of these cells was not sensitive to **5b** up to a concentration of 10 μM. Growth inhibition was also not the result of cellular toxicity, as determined by viable staining with the dye MTT[31] (Table 2). An important goal was to inhibit cancer cell growth without engendering toxicity to normal cells, as assessed by comparison of the minimun inhibitory concentration (MIC) and the cytotoxic endpoint (CTE, concentration compatible with ≥90% cell survival). Unfortunately, the cytotoxicity profile of piperazine **5b** was similar to that of the dipeptide amides **1**. In both series of compounds, toxicity was not mechanism related, since the CTE did not correlate with FTase inhibitory potency. Cytotoxicity in cytolytic lymphocytes had previously been reported for a series of dipeptide ester substrates of dipeptidyl peptidase-1.[32] Cell toxicity was greatest when the dipeptide was comprised of hydrophobic residues, but was absent in analogs incorporating a hydrophilic residue such as serine. Application of these findings to the current series of FTase inhibitors led to the synthesis of compounds with polar functional groups in the piperazine C-2 substituent. While the serine-derived piperazines were no longer cytotoxic, they were also less active FTase inhibitors. Gratifyingly, however, the ethers **5f** and **5g** were potent inhibitors both *in vitro* and in cell culture and exhibited at least a 10-fold difference in concentration between the CTE and MIC in cell culture (Tables 1 and 2). These compounds additionally exhibited an increased selectivity (>3000-fold) for FTase inhibition *vs* GGTase I inhibition.

Table 2. Effect of FTase Inhibitors on the Anchorage Independent Growth of *ras*- and *raf*-Transformed Cells in Soft Agar

	soft agar MIC[a] (μM)		
compd	*ras*	*raf*	CTE[b] (μM)
5b	10	>10	10
5f	10	>50	≥100
5g	2.5	ND[c,d]	25

[a] Minimum inhibitory concentration (MIC) required to achieve a reduction in size and number of colonies of RAT1 v-*ras*- or RAT1 v-*raf*-transformed cells in soft agar relative to vehicle-treated control. Assay conditions are described in ref 21. [b] Highest nontoxic concentration in cultured RAT1 cells assessed by viable staining with MTT.[31] [c] Not determined. [d] No inhibition of colony formation was observed in a v-*mos*-transformed control cell line at 2.5 μM, the highest concentration tested.

To assay the effect of piperazine FTase inhibitors in an animal tumor model, **5f** was evaluated for its ability to block tumor growth in nude mice. The growth of tumors arising from Ha-*ras*-transfected RAT1 cells subcutaneously implanted in nude mice was significantly inhibited by treatment with **5f**. A dose of 45 mpk *ip* once daily for 5 days or *po* twice daily for 10−16 days resulted in 47% and 49% reductions in tumor weight, respectively, when compared to the vehicle-treated control group. Sections of tumor tissue taken at time of necropsy from untreated mice exhibited microscopic characteristics of an aggressive sarcoma. These sections showed a high degree of cellularity, with many mitotic figures indicative of rapidly proliferating tissue. In contrast, sections of tumor tissue from treated animals were distinctly less cellular and contained few or no mitotic figures. Thus **5f** not only inhibited tumor growth but also altered the histological appearance of the remaining tumor tissue. A similar effect has also been reported in animal studies of a prodrug CaaX peptidomimetic FTase inhibitor.[33] In contrast, the growth of tumors derived from a v-*mos* transformed RAT1 control cell line was not affected by similar *ip* administration of **5f**, thus confirming the specificity for *ras*-transformed cell growth inhibition observed in soft agar.

Conclusion. A series of piperazines **5** were designed as constrained analogs of dipeptide amide FTase inhibitors **1**. This conformational constraint increased potency by 40-fold and resulted in compounds with nanomolar IC_{50} values *vs* FTase. A key feature of these inhibitors is that a carboxylic acid is not required for high inhibition potency. Suppression of Ras processing in cell culture demonstrated the ability of piperazines **5** to inhibit intracellular protein farnesylation. Selective inhibition of colony formation of *ras*-transformed cells in soft agar reversed one of the key phenotypic changes associated with cancer cells. The piperazine **5f** significantly inhibited *ras*-dependent tumor growth in nude mice. On the basis of these results, piperazine FTase inhibitors represent a promising class of potential cancer chemotherapeutics.

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