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Benzodiazepine Peptidomimetic Inhibitors of Farnesyltransferase

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Abstract—A structural survey of protein Zn^{2+} binding geometries was instigated based upon the functional requirement of Ras farnesyltransferase for Zn^{2+}. The Cys-X-X-Cys motif found in Zn^{2+}-binding proteins such as aspartate transcarbamylase was used as a template to devise a bidentate-coordination model for Cys-A_1-A_2-X peptide inhibitors. Accordingly, replacement of the central dipeptide with the hydrophobic scaffold 3-amino-1-carboxymethyl-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one (BZA) yielded a peptidomimetic inhibitor, Cys(BZA)Met, of moderate potency (IC_{50} = 400 nM). N-Methylation of the cysteine amide improved potency almost 1000-fold (IC_{50} = 0.3–1 nM). The increased affinity presumably correlates with a preferred conformation of the inhibitor which maximizes a hydrophobic interaction between the scaffold and the enzyme, and the proper presentation of cysteine and methionine to allow bidentate coordination at Zn^{2+}. These non-peptide inhibitors have been shown to block farnesylation of the Ras protein in intact cells and provide lead compounds for the development of new cancer therapeutic agents.

Introduction

The message triggered by the binding of an extracellular growth factor to its membrane receptor is normally conducted to the nucleus by a coordinated series of protein–protein interactions.[1,2] In one such pathway, the Ras protein functions as a molecular switch, interconverting between active and inactive forms through the binding and hydrolysis of GTP.[3,4] Oncogenic mutation of the *ras* gene reduces the GTPase activity of the protein, causing it to remain 'locked' in the active, or GTP-bound state. Ras mutations are associated with ~30 % of all human tumors and are most prevalent in tumors of the pancreas and colon. Novel therapeutic strategies therefore seek to suppress tumor growth by inhibiting the action of constitutively active Ras.[5,6]

The transforming activity of mutant Ras requires membrane association, which is enabled by the post-translational addition of a hydrophobic 'anchor' to the protein.[7–9] The enzyme farnesyltransferase catalyzes the attachment of a farnesyl group to an unpaired cysteine residue near the C-terminus of Ras;[10–12] subsequent enzymes complete the modifications that enable Ras to associate with the membrane. Farnesyltransferase is a member of a recently-discovered family of Zn^{2+}-dependent prenyltransferase enzymes which catalyze the attachment of lipophilic moieties to the carboxy-terminal domains of certain intracellular proteins. Farnesyltransferase recognizes a characteristic Cys-A_1-A_2-X motif in protein substrates (A_1 and A_2 represent amino acids bearing aliphatic residues; X a C-terminal methionine or serine),[13,14] attaching the C-15 isoprene group to cysteine via a thioether linkage. A similar motif is recognized by geranylgeranyltransferase,[15,16] which transfers a C-20 isoprenoid to protein substrates terminating in Cys-A_1-A_2-Leu.[17] Farnesyltransferase is a heterodimeric protein (subunits designated as α and β) with an apparent molecular weight of approximately 100 kDa.[18,19] Farnesylpyrophosphate, the source of the farnesyl group, has been proposed to bind to the α subunit. Cross-linking studies have shown that protein substrate binding is localized at the β subunit, and is zinc-dependent.[12] Magnesium is required for farnesyl transfer, and is assumed to coordinate the pyrophosphate group to facilitate nucleophilic displacement of the farnesyl moiety by the cysteine thiolate.[11,18]

Tetrapeptides containing the Cys-A_1-A_2-X motif can act as competitive inhibitors of farnesyltransferase.[10,13,14] Effective binding to the enzyme requires a free thiol at the N-terminal cysteine and a free carboxylate at the C-terminal residue. Similar requirements are observed for inhibitors of geranylgeranyltransferase although with different C-terminal sidechain specificities.[14,19] Tetrapeptides terminating in methionine typically bind to farnesyltransferase with significantly higher affinities than the corresponding analogs terminating in serine. Most of these tetrapeptides are themselves substrates for the enzyme; a notable exception[20,21] is the peptide Cys-Val-Phe-Met which, by virtue of a free N-terminal amine and an aromatic residue at A_2, is a potent non-substrate inhibitor with a K_i of approximately 60 nM. Due to problems with cell uptake and stability, CVFM and related tetrapeptides have little effect on intact cells. We therefore have undertaken a medicinal chemistry program aimed at generating cell-permeable compounds that inhibit intracellular farnesylation.[22] These compounds can be used

to examine the physiological effects of such inhibition on tumor growth and metastasis, and to investigate the possible toxicity of systemic inhibition of farnesylation.

Biological Effects

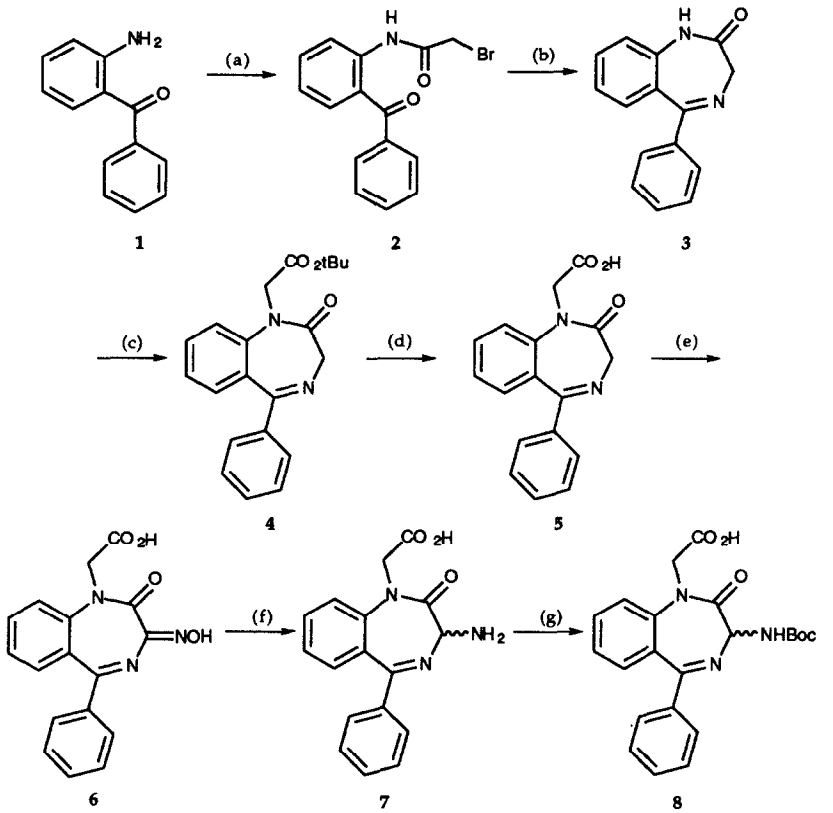
Assay of CAAX farnesyltransferase activity

Inhibition of recombinant farnesyltransferase was evaluated in the laboratories of Drs Michael S. Brown and Joseph L. Goldstein[23] using assays described previously.[13,20–22] Briefly, the amount of [^{3}H]farnesyl transferred from all-*trans*-[^{3}H]farnesylpyrophosphate to recombinant p21H-*ras* was measured using a filter binding assay. IC_{50} values generated in this competition assay are reported as the concentration of inhibitor required to reduce farnesylation of Ras by 50 %.

Chemistry

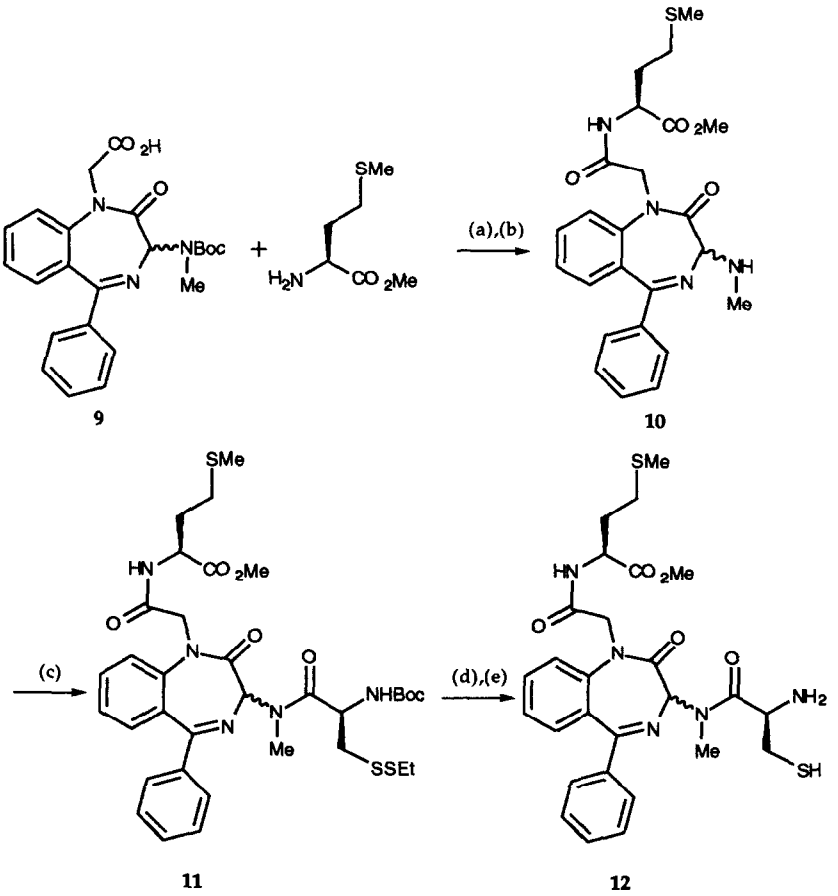
The structure of peptide inhibitors of farnesyltransferase corresponds to the CA_1A_2X carboxy-terminal sequence of the protein substrates of the enzyme. Rapid construction of these inhibitors was accomplished via solid-phase synthetic techniques using BOP, BOP-Cl, or PyBrOP couplings and *t*-butyloxycarbonyl amino protecting strategies.[24] Special emphasis was given to peptide inhibitors containing aromatic residues at one of the two internal positions (A_1A_2) as substitution of phenylalanine at A_2 had produced potent non-substrate inhibitors.[20,21]

Based on the turn-like structure proposed for the tetrapeptides (see Results and Discussion), we sought to replace the central hydrophobic residues with a rigid non-peptide framework that would present the N- and C-terminal cysteine and methionine in an approporiate orientation while providing hydrophobic groups as surrogates to the A_1 and A_2 sidechains. Of several such peptidomimetic structures,[25–28] the synthesis of N-Boc-3- amino-1- carboxymethyl-2,3- dihydro-5- phenyl-1*H*-1,4-benzodiazepin-2-one[22] (**8**, BZA) suitable for incorporation via solid-phase methods is shown in Scheme I. Inhibitors were prepared via standard coupling methodologies on solid-support in a manner identical to the synthesis of the tetrapeptides. The BZA scaffold was synthesized as a mixture of enantiomers at C-3, yielding inhibitors as a mixture of diastereomers (designated as **A** and **B**) separable via HPLC. Alteration of the amide backbone via N-methylation was accomplished via treatment of Boc-(NH)BZA (**8**) with NaH and MeI and coupling as before. Improvement of cellular uptake was accomplished by prodrug modification of the peptidomimetic inhibitor,[29] Cys(N-Me)BZA-Met, as the carboxy-terminal methyl ester.[22] Synthesis via solution-phase coupling of the components was accomplished as shown in Scheme II.



(a) $BrCH_2COBr$, CH_2Cl_2/H_2O; (b) NH_3, CH_3OH; (c) Cs_2CO_3, NMP, *t-butyl*-$OCOCH_2Br$; (d) TFA, CH_2Cl_2; (e) $KOC(CH_3)_3$, isobutyl nitrite, glyme; (f) H_2, Raney Ni, MeOH; (g) Boc_2O, NaOH, THF/H_2O.

Scheme 1. Synthesis of *N*-Boc-3-amino-1-carboxymethyl-2,3-dihydro-5-phenyl-1H-1,4-benzodiazepin-2-one.



(a) DIPC, HOBt, CH_2Cl_2; (b) TFA, CH_2Cl_2; (c) N-Boc-S-(S-Et)cysteine, EDC, HOBt, DMF; (d) TFA, CH_2Cl_2; (e) DTT, CH_3CN/H_2O, pH 8-8.5.

Scheme 2. Synthesis of the Methyl Ester of Cys(N-Me)BZAMet.

Results and Discussion

Because of the near absolute requirement for cysteine and methionine at the N- and C-termini, early investigations focused on modifying the central hydrophobic residues. The affinities of several N-acetylated tetrapeptide inhibitors are collected in Table 1, and all proved to be suitable as substrates for the enzyme as shown previously for N-acetylated inhibitors[21] (except **18** and **19** which were not tested). An interesting structure–activity trend emerged from these studies. In the native protein substrates, A_1 and A_2 are aliphatic residues. Replacement of either A_1 or A_2 by an aromatic residue (**14–17**, Table 1) retains or even enhances affinity for farnesyltransferase relative to acetyl-CVIM (**13**). Replacing both A_1 and A_2 with aromatic residues (**18** and **19**) results in a significant decrease in binding affinity. These results suggest that the sidechains of A_1 and A_2 might occupy a single hydrophobic 'pocket' which could not simultaneously accommodate two aromatic rings.

The conformational profiles of acetyl-CVFM and related peptides were initially examined by sequentially replacing each residue with a corresponding D-amino acid; all of these compounds were inactive (IC_{50} > 10 μM). Local conformational constraints had previously been added to the backbone by replacing A_1 or A_2 with proline.[20] The inactivity of the resulting compounds (IC_{50} ~ 6–7 μM) was difficult to interpret due to the absence of a hydrophobic sidechain. To overcome this problem, A_2 was replaced by (L)-1,2,3,4-tetrahydro-3-isoquinolinecarboxylic acid (Tic), which serves as a conformationally-constrained phenylalanine surrogate, yet preserves the *trans* amide linkage between A_1 and A_2. The resulting peptide, **20**, was a more potent inhibitor of Ras farnesylation than its unconstrained counterpart, **14**.

Table 1. Tetrapeptide inhibitors of farnesyltransferase. IC_{50} values determined as described previously [21]

	Compound	IC_{50} (nM)
13	acetyl-CVIM	150
14	acetyl-CVFM	250
15	acetyl-CIFM	180
16	acetyl-CYIM	50
17	acetyl-CYVM	140
18	acetyl-CYFM	>1,000
19	acetyl-CFFM	2,200
20	acetyl-CV(Tic)M	11

abbreviations: Tic: 3-carboxy-tetrahydroisoquinoline

Additional structural clues were provided by mechanistic considerations of farnesyl transfer by the enzyme.[11,12,30] The zinc-dependent binding of the tetrapeptides suggested that the N-terminal cysteine sidechain is directly

coordinated to a zinc atom within the β subunit of the enzyme, which contains an odd number of cysteine residues. The substrates for CAAX prenyltransferase enzymes all require a free C-terminal carboxylate, suggesting that this moiety is directly coordinated with one of the divalent cations of the catalytic apparatus. We assumed that the C-terminal carboxylate of the tetrapeptide was coordinated to the Zn^{2+} ion. Based on analyses of phosphate/Mg^{2+} coordination geometries found in the Cambridge Crystallographic Database[31] and the requirement that the farnesyl group be appropriately positioned for nucleophilic attack by the cysteine thiolate, we reasoned that the Zn^{2+} and Mg^{2+} ions were in close proximity, possibly sharing at least one carboxylate ligand. Thus, although we assumed bidentate coordination of Zn^{2+} by the tetrapeptide, a similar relative geometry of the thiolate and carboxylate moieties would have been required had the latter been coordinated to Mg^{2+} instead.

Our working model was derived from the 'Cys-X-X-Cys' tetrapeptide motif found in aspartate transcarbamylase,[32] alcohol dehydrogenase,[33-35] and 'Zinc-finger' domains.[36,37] In this motif, a Zn^{2+} ion is simultaneously coordinated by the N- and C-terminal cysteine sidechains, establishing a turn-like backbone conformation (Figure 1A). We proposed a similar conformation of the acetyl-CVFM tetrapeptides in which the C-terminal carboxylate of methionine occupies the second Zn^{2+} coordination site. This model was supported by several of the structure–activity trends mentioned above. First, the backbone conformation is consistent with an all-L-amino acid configuration based on Ramachandran analysis. Secondly, the turn-like structure requires a packing of the hydrophobic sidechains of the A_1 and A_2 residues. Such packing is favored for aliphatic–aliphatic, aromatic–aliphatic, or aliphatic–aromatic sidechain combinations. When A_1 and A_2 are both aromatic, however, it is not possible to closely pack the sidechains without either perturbing the backbone conformation or inducing eclipsed sidechain torsions. Finally, the backbone angles of the A_2 residue in the Cys-X-X-Cys motif are very close to the angles enforced when A_2 is replaced by tetrahydroisoquinoline (Figure 1B). As stated previously, a similar turn-like structure would have been proposed had we assumed that the C-terminal carboxylate was coordinated to Mg^{2+}.

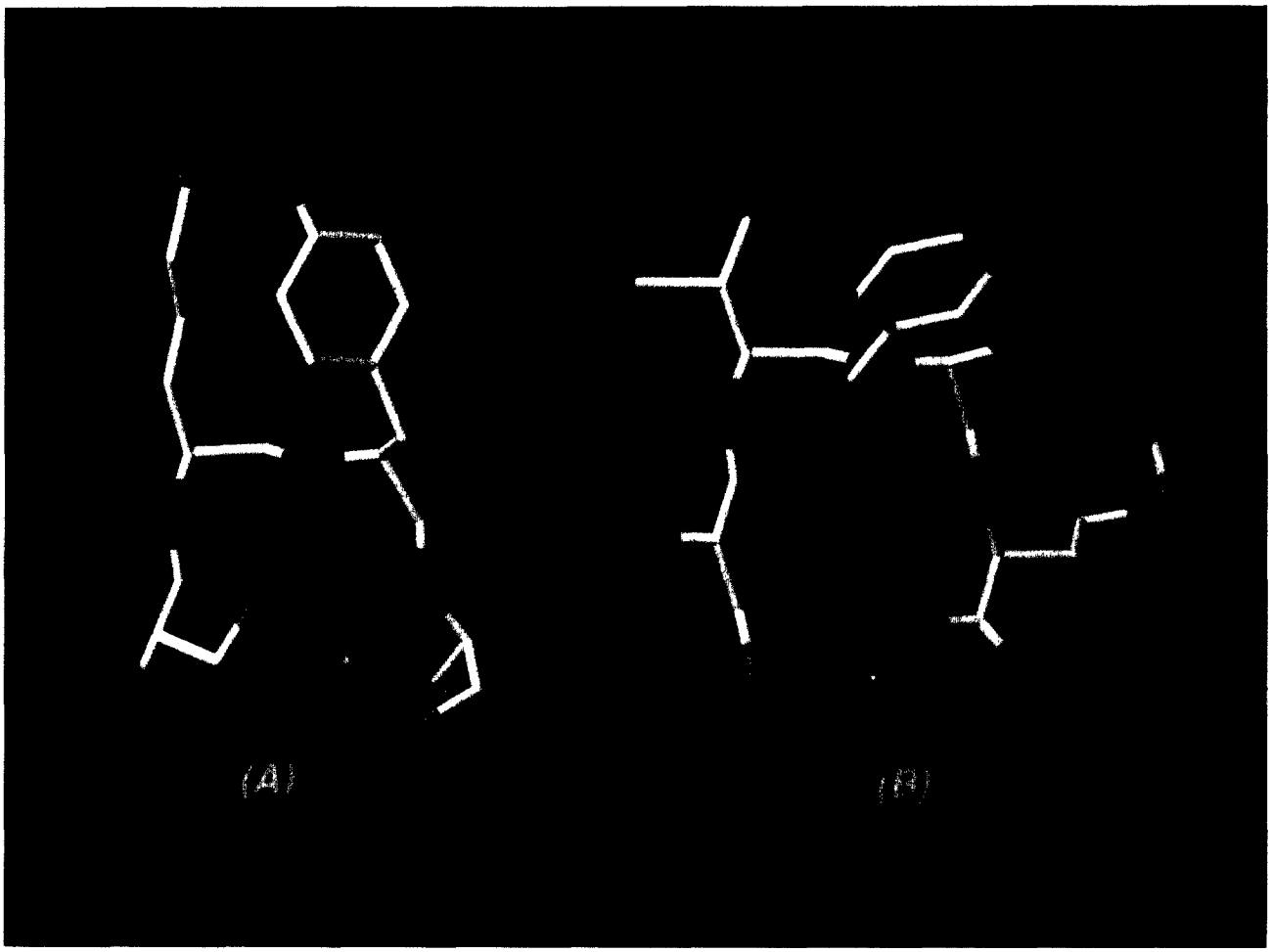
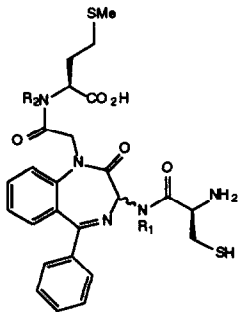


Figure 1. **(A):** Cys-Lys-Tyr-Cys loop from aspartate transcarbamylase[32], illustrating the bidentate coordination of Zn^{+2} by the cysteine sidechains. A similar "CXXC" motif is found in zinc-finger domains. **(B):** Proposed bidentate coordination model for the potent tetrapeptide inhibitor Cys-Val-Tic-Met. Tic constrains the backbone conformation of the third residue to match the conformation found in the "CXXC" loop. The turn-like conformation suggested by this model is enforced when the central hydrophobic residues A_1A_2 are replaced by the rigid BZA scaffold.

We chose to use this model to explore whether the central hydrophobic residues could be replaced by a 'turn mimic' which would enforce the required geometry of the N- and C-terminal 'Zn^{2+} ligands' and provide hydrophobic groups to fill the 'cavity' occupied by the A_1 and A_2 sidechains. The dipeptide isostere BZA was chosen as a replacement for A_1 and A_2. Similar strategies in which key structural elements are replaced with rigid peptide mimics have been the subject of several recent reviews.[38,39] The corresponding peptide,[22] Cys-BZA-Met (**21**, Table 2), while not extremely potent, represented a lead suitable for further optimization. In contrast to the tetrapeptides, either isomer at C-3 of the diazepine, which would correspond to the α-C of the tetrapeptide A_1 residue, is tolerated. N-methylation of the two remaining amide bonds was undertaken to improve the cellular penetration of the inhibitor through a disruption of the hydrogen-bonding network at each amide.[40] Surprisingly, not only were such modifications tolerated, N-methylation of the cysteine-BZA amide linkage yielded ~1000-fold increase in binding affinity of one of the diastereomers, **22B**. We believe that the increased potency is a result of the greater proportion of the *cis* configuration of the N-methylated amide bond. Indeed, examination of the ^{1}H-NMR spectrum of **22B** (500 MHz, D_2O) reveals the N-CH_3 protons (3.53 and 3.06 ppm) and the ring α-CH proton at C-3 (5.85 and ~5.3 ppm) as two separate resonances, consistent with a mixture of both *cis* and *trans* amide configurations (data not shown). Additionally, MM2 calculations indicate that N-methylation of the amide bond would not dramatically alter the conformational profile of a *trans* amide linkage to a degree that would result in a 1000-fold change in affinity, although such a possibility cannot be eliminated. Using a similar strategy, replacement of the A_1A_2 dipeptide with the conformationally flexible isostere 3-aminomethylbenzoic acid yielded a potent, selective inhibitor of farnesyltransferase as recently descibed.[41]

Table 2. Effect of amide bond N-methylation on the potency of benzodiazepine peptidomimetic inhibitors of farnesyltransferase. IC_{50} values determined as described previously.[22] Compounds prepared as separable mixtures of diastereomers at C-3, designated as "A" and "B"



Compound	R_1	R_2	IC_{50} (nM)
21A	H	H	400
21B			400
22A	CH_3	H	800
22B			0.3 - 1 [a]
23A	H	CH_3	>10,000
23B			2,000
24A	CH_3	CH_3	300
24B			2

[a]precise determination difficult due to lower limit of assay.[48]

In studies shown previously,[22] peptides such as CVFM are ineffective in blocking farnesylation in intact cells, while incubation of Cys-(NMe)BZA-Met at 250 μM showed a marked reduction of intracellular farnesylation. The effectiveness of this inhibitor compared with unmodified tetrapeptides may be due to increased resistance to degradation. Additionally, these inhibitors may more effectively penetrate the cell membrane. Masking the carboxy-terminus as the methyl ester yielded a 10-fold increase in potency. This more lipophilic inhibitor is presumably converted to the required C-terminal acid within the cell. Other successful modifications of CAAX-based inhibitors to reduce proteolytic degradation and improve membrane permeability have been described.[42]

These new compounds provide reagents for testing the biological effects of inhibiting farnesylation. We have shown previously that prolonged incubation of Cys-(NMe)-BZA with H-*ras*(Gly12Val)-transformed rat-1 fibroblasts caused a reduction in growth rate and reversion of the transformed phenotype associated with cells harboring mutant Ras.[22] While wild-type and src-transformed cells were unaffected by this treatment, the effects of systemic inhibition of farnesylation must be carefully examined *in vivo* before clear conclusions regarding the effective therapeutic use of such compounds can be addressed. It is apparent, however, that cells bearing mutant Ras are more sensitive to treatment with these compounds. Such an approach may allow the development of novel cancer therapeutic agents. The efficacy of this strategy against solid tumors bearing multiple genetic mutations in addition to the mutant *ras* gene must be evaluated. Finally, important toxicity issues regarding the blockage of farnesylation of other proteins (such as the nuclear lamins[43,44] and retinal proteins[45-47]) must be addressed.

Experimental

2-Bromoacetamidobenzophenone (2)

A solution of bromoacetyl bromide (100 mL, 1.15 mol) dissolved in dichloromethane (300 mL) was added over 30 min to a solution of 2-aminobenzophenone (197 g, 1.0 mol) dissolved in dichloromethane (1.3 L) and water (100 mL) at −10 °C under vigorous mechanical stirring. The resulting mixture was stirred for an additional 1 h at −5 °C then warmed to ambient temperature. The layers were separated and the organics were washed with dilute sodium bicarbonate, then dried over sodium sulfate. Evaporation afforded 309.8 g (0.97 mol, 97 %) of 2-bromoacetamidobenzophenone **2** as off-white crystals. ^{1}H-NMR (300 MHz, $CDCl_3$): 11.5 ppm (1H, br s), 4.0 ppm (2H, s).

2,3-Dihydro-5-phenyl-1H-1,4-benzodiazepin-2-one (3)

A suspension of 2-bromoacetamidobenzophenone **2** (275 g, 0.86 mol) in methanol (1 L) was treated with a solution

of saturated ammonia in methanol (3 L), and the resulting solution was stirred at ambient temperature for 6 h, then heated at reflux for an additional 4 h. After cooling, water (500 mL) was added, and the solution was concentrated to about 1 L in volume, yielding 200.7 g (0.85 mol, 98 %) crystalline 2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one **3**. ^{1}H-NMR (300 MHz, $CDCl_3$): 9.5 ppm (1H, br s), 4.35 (2H, br s).

tert-Butyl-2,3-dihydro-5-phenyl - 1H-1,4- benzodiazepin -2-one-1-acetate (4)

A 1 L round-bottomed flask was equipped with a magnetic stirring bar and nitrogen inlet and was sequentially charged with 100 g (0.423 mol) of 2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one **3**, 600 mL of 1-methyl-2-pyrrolidinone, 97 mL (117 g, 0.601 mol) of *tert*-butyl bromoacetate, and 194 g (0.595 mol) of cesium carbonate. After stirring overnight at ambient temperature, the reaction mixture was diluted with 2 L water and extracted with ethyl acetate (3 × 600 mL). The combined organic extracts were washed with water (4 × 300 mL) and brine (200 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to provide 202 g crude **4**. This material was recrystallized from hexanes/ethyl acetate to provide 123 g (0.351 mol, 83 %) of *tert*-butyl-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetate **4** as a white crystalline solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 4.8 ppm + 3.8 ppm (2H, d, J = 11 Hz), 4.5 ppm + 4.2 ppm (2H, d, J = 17 Hz), 1.5 ppm (9H, s).

2,3- Dihydro -5- phenyl- 1H -1,4- benzodiazepin -2- one -1-acetic acid (5)

A solution of *tert*-butyl-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetate **4** (58 g, 0.166 mol) in trifluoroacetic acid (TFA, 100 mL) was stirred overnight, followed by evaporation and retreatment with TFA (100 mL). The mixture was concentrated under reduced pressure and the residue was dissolved in dichloromethane, then washed sequentially with water and brine. The organics were dried over anhydrous sodium sulfate and evaporated to yield 48.4 g 2,3-dihydro-5-phenyl-1*H* -1,4-benzodiazepin-2-one-1-acetic acid **5** (0.164 mol, 99 %) as a yellow foam which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$ + 1 drop DMSO-d_6): 10.8 ppm (1H, br s), 4.8 ppm + 3.85 ppm (2H, d, J = 13.2 Hz), 4.6 ppm + 4.4 ppm (2H, d, J = 17.4 Hz).

3-Oximino-2,3-dihydro-5-phenyl-1H-1,4-benzodiazepin-2-one-1-acetic acid (6)

A solution of 2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **5** (30 g, 0.102 mol) in glyme (1 L) was cooled to –5 °C and deoxygenated with nitrogen. Solid potassium *tert*-butoxide (47.7 g, 0.43 mol) was added portionwise and the resulting red solution was stirred for 30 min at 0–5 °C. A solution of isobutyl nitrite (13.8 mL, 0.116 mol) in glyme (20 mL) was added, producing an orange–yellow suspension. The mixture was neutralized after $^1/_2$ h with acetic acid (200 mL) and concentrated to afford crude **6** as a yellow solid which was used directly in the following reaction. ^{1}H NMR (300 MHz, DMSO-d_6): 11.0 ppm (1H, s), 4.3 ppm (2H, s).

3- Amino -2,3- dihydro -5- phenyl-1H-1,4-benzodiazepin-2-one-1-acetic acid (7)

A solution of 3-oximino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **6** (33 g, 102 mmol) in methanol (200 mL) containing 2 mL acetic acid was hydrogenated over Raney Nickel (1:1 by weight to oxime, washed twice with water then once with ethanol) at 65 psi and ambient temperature for 1 ^{1}/2 days. The catalyst was removed by suction filtration through celite, and the solution concentrated to yield crude 3-amino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **7** which was used directly in the following reaction. ^{1}H-NMR (300 MHz, DMSO-d_6): 4.35 ppm + 4.05 ppm (2H, d, J ~ 15 Hz), 4.35 ppm (1H, s), 3.4 ppm (br).

3- (tert-Butoxycarbonyl) amino-2,3-dihydro-5-phenyl -1H-1,4-benzodiazepin-2-one-1-acetic acid (8)

A solution of the above 3-amino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **7** (~102 mmol) in tetrahydrofuran (100 mL) and water (100 mL) was cooled to 0 °C and di-*tert*-butyl dicarbonate (28.8 g, 132 mmol) was added under a nitrogen atmosphere followed by 1 N sodium hydroxide until the pH of the solution was ~10. The solution was allowed to come to ambient temperature, stirred overnight, cooled again to 0 °C and acidified (pH ~ 3) with dropwise addition of concentrated sulfuric acid. The solution was diluted with ethyl acetate, partitioned, and the organic extract dried over sodium sulfate and concentrated. The residue was recrystallized from 120 mL methanol yielding 18 g (44 mmol, 43 %) of 3-(*tert*-butoxycarbonyl) amino- 2,3 - dihydro - 5 - phenyl -1*H*-1,4-benzodiazepin-2-one-1-acetic acid **8** as cream needles. ^{1}H NMR (300 MHz, $CDCl_3$): 11.7 ppm (1H, br), 6.55 ppm (1H, d, J ~ 8 Hz), 5.4 ppm (1H, d, J ~ 8 Hz), 4.6 ppm (2H, s), 1.4 ppm (9H, s). MS [$(M + H)^+$]: 410.1.

3-(tert-Butoxycarbonyl)methylamino-2,3-dihydro-5-phenyl-1H-1,4-benzodiazepin-2-one-1-acetic acid (9)

An oven-dried, 100 mL round-bottomed flask was equipped with a magnetic stirring bar and nitrogen inlet and was sequentially charged with 4.63 g (11.3 mmol) of 3-(*tert*-butoxycarbonyl)amino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **8**, 50 mL of anhydrous tetrahydrofuran, and 2.80 mL (6.38 g, 45.0 mmol) of methyl iodide. The reaction flask was cooled to –5 °C in an ice/acetone bath and 1.18 g (29.5 mmol) of sodium hydride (60 % dispersion in mineral oil) was added in portions over a 5 min period (CAUTION: vigorous gas evolution). After 50 min, the reaction mixture was quenched with a 5 % (w/v) aqueous solution of citric acid, diluted with water and extracted with ethyl acetate (3 × 40 mL). The combined organics were washed with water (30 mL), brine (30 mL), dried with anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to provide 6.27 g of a viscous yellow oil. Flash chromatography of the crude material on 150 g of silica

using 43:55:2 ethyl acetate:hexanes:acetic acid as eluent yielded 4.54 g (10.7 mmol, 95 %) of 3-(*tert*-butoxycarbonyl) methylamino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **9** as a clear glass. ^{1}H NMR (300 MHz, $CDCl_3$): 8.4 ppm (1H, br), 5.8 ppm + 5.5 ppm (1H, 2 s), 4.7 ppm + 4.4 ppm (2H, d, J = 17.7 Hz), 3.35 ppm (3H, s), 1.45 ppm + 1.25 ppm (9H, 2 s). MS [(M + H)$^+$]: 424.1909; found $C_{23}H_{26}N_3O_5$.

Peptide synthesis

Peptides were synthesized via standard solid-phase methodologies.[24] 3-(*tert*-Butoxycarbonyl)methylamino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid (**9**, 860 mg, 2.0 mmol), benzotriazol-1-yloxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, 900 mg, 2.0 mmol), N-methylmorpholine (NMM, 225 µL, 2.0 mmol), and 1-hydroxybenzotriazole hydrate (HOBt, 280 mg, 2.0 mmol) in dimethylacetamide (DMA, 30 mL) were added to deprotected L-methionine-linked Merrifield resin (Bachem, 1.25 g, 0.71 meq/g, 12 h). After washing [DMA, then dichloromethane (CH_2Cl_2)] and deprotection (45 % TFA/5 % anisole/5 % EtSMe/CH_2Cl_2), the resin was neutralized (20 % Et_3N/CH_2Cl_2) and washed (CH_2Cl_2). Next, Fmoc-(S-trityl)-L-cysteine (2.1 g, 3.6 mmol), bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl, 0.99 g, 3.9 mmol), and diisopropylethylamine (1.4 mL, 7.8 mmol) were combined and added to the resin (CH_2Cl_2, 30 mL, 10 h). After removal of the Fmoc (20 % piperidine/DMA) and trityl (45 % TFA/ 5% EtSMe/5 % anisole/CH_2Cl_2) protecting groups the resin was washed with methanol, dried under vacuum, cleaved from the resin (32 mL, HF/10 % anisole/5 % EtSMe, 0 °C, 1 h) and purified via HPLC. Purification of 173 mg of crude material (Vydac C18, CH_3CN/H_2O/0.1 % TFA) afforded the product, N-[[3-[(2-amino-3-mercapto-1-oxopropyl)-methylamino] -2,3-dihydro-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-1-yl]acetyl]-L-methionine (**22**), as two separable diastereomers (opposite configuration at C-3 of the benzodiazepine) designated **22A** (39 mg) and **22B** (22 mg) corresponding to the early and late eluting peaks respectively. ^{1}H NMR (**22A**, 300 MHz, DMSO-d_6): 8.45 ppm (1H, d, J = 7.5 Hz), 8.2 ppm (br), 7.7 ppm – 7.4 ppm (m), 7.4 ppm – 7.2 ppm (m), 5.87 ppm (1H, s), 4.7 ppm – 4.4 ppm (m), 4.25 ppm – 4.15 ppm (m), 3.47 ppm (3H, s), 3.33 ppm (br), 3.05 ppm – 2.85 ppm (m), 2.5 ppm – 2.3 ppm (m), 2.0 ppm – 1.7 ppm (m), 1.97 ppm (3H, s). ^{1}H NMR (**22B**, 300 MHz, DMSO-d_6): 8.60 ppm + 8.47 ppm (1H, 2 d, J = 8 Hz), 7.7 ppm – 7.25 ppm (m), 5.80 ppm + 5.40 ppm (1H, 2 s), 4.72 ppm – 4.64 ppm (m), 4.3 ppm – 4.2 ppm (m), 3.48 ppm + 3.44 ppm (3H, 2 s), 3.3 ppm (br), 3.04ppm – 2.84 ppm (m), 2.5 ppm – 2.3 ppm (m), 2.0 ppm – 1.7 ppm (m), 2.0 ppm + 1.97 ppm (3H, 2 s). Mass (electrospray, M + H$^+$) calcd: 558.35 found: 558.3 (**22A**), 558.3 (**22B**).

Ester synthesis

As shown in Scheme II, 3-(*tert*-butoxycarbonyl)-methylamino-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one-1-acetic acid **9** (2.3 g, 5.4 mmol), L-methionine methyl ester hydrochloride (2.7 g, 13.5 mmol), diisopropylcarbodiimide (DIPC, 1.04 mL, 6.6 mmol), and HOBt (0.9 g, 6.7 mmol) were combined in CH_2Cl_2 (20 mL). After 10 h, the reaction was diluted (CH_2Cl_2, 90 mL), washed with 0.1 N sulfuric acid then with brine, dried over anhydrous magnesium sulfate and concentrated to yield 3.5 g crude *N*-[[3-(*tert*-butoxycarbonyl)methylamino-2,3-dihydro-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-1-yl] acetyl]-L-methionine methyl ester. ^{1}H NMR (300 MHz, $CDCl_3$): 7.7 ppm – 7.2 ppm (m), 6.92 ppm + 6.81 ppm (1H, 2 d, J = 8.3 Hz), 5.73 ppm + 5.71 ppm (1H, 2 s), 4.8 ppm – 4.6 ppm (m), 4.3 ppm – 4.2 ppm (m), 3.71 ppm + 3.65 ppm (3H, 2 s), 3.36 ppm (3H, s), 2.46 ppm – 2.38 ppm (m), 2.3 ppm – 1.8 ppm (m), 2.05 ppm + 2.01 ppm (3H, 2 s), 1.48 ppm (9H, s).

The coupled material (0.75 g, ~1.3 mmol) was deprotected (30 % TFA, 30 mL, 3 h), concentrated, neutralized via partition between ethyl acetate (100 mL) and saturated sodium bicarbonate. The organics were washed with brine, dried over anhydrous sodium sulfate and purified (silica, CH_2Cl_2:MeOH 99:1 with 0.2 % Et_3N) to yield 0.55 g (1.17 mmol, 90 %) *N*-[[3-amino-2,3-dihydro-2-oxo-5-phenyl-1*H*-1, 4-benzodiazepin-1-yl]acetyl]-L-methionine methyl ester **10** as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): 9.2 ppm (1H, br), 8.37 ppm + 7.89 ppm (1H, 2 d, J = 7.8 Hz), 7.66 ppm – 7.14 ppm (m), 4.98 ppm (1H, s), 4.92 ppm – 4.3 ppm (m), 3.74 ppm – 3.70 ppm (m), 3.66 ppm + 3.60 ppm (1H, 2 s), 2.94 ppm + 2.88 ppm (3H, 2 s), 2.8 ppm – 1.9 ppm (m), 1.99 ppm + 1.96 ppm (3H, 2 s).

Reaction of **10** with Boc-(S-ethylthio)-cysteine (0.99 g, 3.6 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 0.68 g, 3.6 mmol), and HOBt (0.16 g, 1.2 mmol) in DMF (10 mL, 12 h) was followed by concentration, aqueous workup and chromatography (as above), to yield *N*-[[3-[(2-(*tert*-butoxycarbonyl)amino-3-mercapto-1-oxopropyl)methylamino]-2,3-dihydro-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-1- yl] acetyl] -L- methionine methyl ester **11** (0.82 g, 93 %). ^{1}H NMR (300 MHz, $CDCl_3$): 7.7 ppm – 7.22 ppm (m), 7.04 ppm + 6.95 ppm (1H, 2 d, J = 7.5 Hz), 5.82 ppm + 5.77 ppm (1H, 2 s), 5.75 ppm + 5.49 ppm (1H, 2 d, J = 8.9 Hz), 5.2 ppm – 4.66 ppm (m), 3.75 ppm + 3.72 ppm (3H, 2 s), 3.66 ppm + 3.65 ppm (3H, 2 s), 3.3 ppm – 1.2 ppm (m), 2.09 ppm + 2.04 ppm (3H, 2 s), 1.46 ppm + 1.42 ppm (9H, 2 s).

Removal of the Boc- (30 % TFA as above) and ethylthio- (60 mg dithiothreitol, 20 mL 50 % CH_3CN/H_2O, pH 7.5) protecting groups afforded *N*-[[3-(2-amino-3-mercapto-1-oxopropyl)methylamino]-2,3-dihydro-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-1-yl]acetyl]-L-methionine methyl ester **12**. Purification by HPLC (Vydac C18, CH_3CN/H_2O/TFA) resolved the diastereomers possessing opposite configuration at C-3. These isomers were designated **12A** and **12B** as before. Purification of 120 mg of the crude material yielded the two diastereomers **12A** (26 mg) and **12B** (30 mg). ^{1}H NMR (**12A**, 300 MHz, DMSO-d_6): 8.6 ppm (1H, d, J = 7.8 Hz), 8.25 ppm (br), 7.74 ppm – 7.22 ppm (m), 5.84 ppm (1H, s), 4.72 ppm – 4.24 ppm (m), 3.57 ppm (3H, s), 3.42 ppm (3H, s), 3.0 ppm – 1.75 ppm (m), 2.0 ppm (3H, s). ^{1}H NMR (**12B**, 300 MHz, DMSO-d_6): 8.78 ppm + 8.63 ppm (1H, 2 d, J = 7.8 Hz), 8.3 ppm + 8.16

ppm (2 br), 7.72 ppm – 7.24 ppm (m), 5.80 ppm + 5.40 ppm (1H, 2 s), 4.72 ppm – 4.22 ppm (m), 3.62 ppm + 3.60 ppm (3H, 2 s), 3.45 ppm (3H, s), 3.18 ppm – 1.74 ppm (m), 2.0 ppm + 1.98 ppm (3H, 2 s). Mass (electrospray, M + H^+) calcd: 572.2; found: 572.3 (**12A**), 572.3 (**12B**).

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