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Benzodiazepine Peptidomimetics: Potent Inhibitors of Ras Farnesylation in Animal Cells

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Oncogenic Ras proteins transform animal cells to a malignant phenotype only when modified by farnesyl residues attached to cysteines near their carboxyl termini. The farnesyltransferase that catalyzes this reaction recognizes tetrapeptides of the sequence *CAAX*, where C is cysteine, *A* is an aliphatic amino acid, and *X* is a carboxyl-terminal methionine or serine. Replacement of the two aliphatic residues with a benzodiazepine-based mimic of a peptide turn generated potent inhibitors of farnesyltransferase [50 percent inhibitory concentration (IC_{50}) < 1 nM]. Unlike tetrapeptides, the benzodiazepine peptidomimetics enter cells and block attachment of farnesyl to Ras, nuclear lamins, and several other proteins. At micromolar concentrations, these inhibitors restored a normal growth pattern to Ras-transformed cells. The benzodiazepine peptidomimetics may be useful in the design of treatments for tumors in which oncogenic Ras proteins contribute to abnormal growth, such as that of the colon, lung, and pancreas.

Oncogenic Ras proteins are causally implicated in certain human malignancies (*1*). Poised at the inner surface of the plasma membrane, Ras proteins normally respond to growth stimuli like epidermal and platelet-derived growth factors by exchanging guanosine triphosphate (GTP) for constitutively bound guanosine diphosphate (GDP), thereby triggering cell division. The signal is terminated when the Ras protein hydrolyzes its bound GTP to GDP in a reaction that is stimulated by a guanosine triphosphatase (GTPase) activating protein (GAP) (*2*). About 50% of human colon carcinomas and 90% of pancreatic carcinomas produce mutant Ras proteins that bind GTP but cannot hydrolyze it (*1*). The mutant proteins are constitutively active, and this constant signal, coupled with other regulatory abnormalities, leads to malignant transformation.

The function of normal and oncogenic Ras proteins is absolutely dependent on the posttranslational attachment of a 15-carbon isoprenoid moiety, farnesyl, through a thioether linkage to a cysteine near the COOH-terminus of the protein (*3*). This modification is catalyzed by a heterodimeric Zn^{2+}-dependent enzyme designated CAAX farnesyltransferase (*4*, *5*). The enzyme uses farnesyl pyrophosphate as a donor and attaches a farnesyl group to the cysteine residue at the fourth position from the COOH-terminus of various proteins, including all four Ras proteins, nuclear lamins A and B, skeletal muscle phosphorylase kinase, and three retinal proteins (the γ subunit of transducin, the α subunit of cyclic guanosine monophosphate phosphodiesterase, and rhodopsin kinase) (*6*). The COOH-termini of all of these substrates share the tetrapeptide sequence CAAX, where C is cysteine, A stands for aliphatic residues, and X is methionine or serine (*7*). This CAAX motif appears to be the sole recognition site for the enzyme; hence, addition of CAAX sequences to the COOH-termini of other proteins renders them substrates for farnesylation (*8*). Moreover, in vitro the enzyme attaches a farnesyl group to tetrapeptides that conform to the CAAX consensus (*7*, *9*).

In the cell, farnesylation is the first step in a sequence of modifications that renders the COOH-terminus of the Ras protein hydrophobic. Farnesylation is followed by proteolytic removal of the terminal three amino acids and methylation of the free COOH group on the farnesylated cysteine (*3*). These reactions are necessary for Ras to become attached to the inner surface of the plasma membrane.

Oncogenic Ras proteins lose their transforming ability when farnesylation is prevented, either by mutation of the CAAX sequence or by blocking synthesis of farnesyl pyrophosphate with an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (*3*, *10*). Under some circumstances the cytosolic nonprenylated form of Ras may

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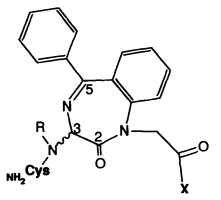
inhibit the action of the activated GTP-bound protein (*10*). These findings raise the possibility that inhibitors of farnesyltransferase may block the growth of malignant cells that are dependent on activated Ras proteins. Although such inhibitors could also block the farnesylation of other essential proteins such as normal Ras and nuclear lamins, dependence on oncogenic Ras might render tumor cells more sensitive than normal cells to the action of a farnesyltransferase inhibitor.

One route to the development of inhibitors of the *CAAX* farnesyltransferase is through the tetrapeptide binding site. Many tetrapeptides that conform to the *CAAX* consensus act as alternative substrates in vitro, thereby competitively inhibiting the farnesylation of Ras proteins (*4*, *9*). Tetrapeptides such as Cys-Val-Phe-Met (CVFM), which contain an aromatic residue in the third position of the *CAAX* sequence, inhibit farnesyltransferase without themselves becoming farnesylated (*9*). The resistance of these tetrapeptides to farnesylation depends not only on the aromatic residue, but also on the presence of a charged NH_2-terminus (*9*). Unfortunately, such tetrapeptides are ineffective when added to intact cells, either because they are taken up inefficiently or because they are rapidly degraded, or both. Therefore, we set out to create peptidomimetic analogs (*11*) that would inhibit the enzyme in vivo as well as in vitro.



Inhibitor	R	X
BZA-1	H	Met_{COOH}
BZA-2	CH_3	Met_{COOH}
BZA-3	CH_3	Ser_{COOH}
BZA-4	CH_3	Leu_{COOH}
BZA-5	CH_3	Met_{COOMe}
BZA-6	CH_3	Met_{CONH_2}

Fig. 1. Structure of the *CAAX* peptide analogs incorporating BZA (*36*) as a dipeptide turn mimic. These compounds were prepared by manual solid-phase synthesis or in solution by standard peptide synthesis methodologies (*37*) as a separable mixture of diastereomers (of opposite configuration at C-3), and each isomer was purified by HPLC (*33*).

On the basis of the functional requirement of farnesyltransferase for Zn^{2+} (*5*) and an analysis of Zn^{2+}-binding geometries from the Cambridge database (*12*), we focused on the -Cys-*X*-*X*-Cys- motif found in several Zn^{2+}-binding proteins and Zn^{2+}-finger peptides as a model of the *CAAX* tetrapeptide in the bound state. In aspartate transcarbamylase, the two cysteine sulfhydryls coordinate to a single Zn^{2+} atom enforcing a tetrapeptide loop or turn (*13*). We used this geometry to design a model in which the NH_2-terminal cysteine of the *CAAX* peptide coordinates through a sulfhydryl to Zn^{2+} and the tetrapeptide loops around to allow additional coordination to Zn^{2+} through the terminal carboxylate.

On the basis of this model, we designed and synthesized nonpeptide scaffolds that could replace all or part of the tetrapeptide while enforcing the turn-like structure and providing critical NH_2- and COOH-terminal interactions. One such class of compounds consisted of molecules in which the aliphatic *AA* portion of the *CAAX* tetrapeptide was replaced with 3-amino-1-carboxymethyl-5-phenyl-benzodiazepin-2-one (BZA) (Fig. 1). We refer to this family of compounds as benzodiazepine peptidomimetics. The hydrophobic BZA scaffold mimics a natural dipeptide turn, defines a favorable presentation of the cysteine and COOH-terminal (*X*) residues, and yields potent inhibitors of *CAAX* farnesyltransferase.

Several BZA peptidomimetic compounds were synthesized with standard solid-phase methods (Fig. 1) (*14*). The BZA scaffold was prepared as a racemic mixture, yielding the benzodiazepine-substituted peptides as separable mixtures of two diastereomers (differing only in configuration at C-3). All of the peptide analogs eluted from a high-performance liquid chromatography (HPLC) column as two peaks, designated as A and B, and were tested independently as prenyltransferase inhibitors. The first of this series, BZA-1A and -1B were relatively poor inhibitors of farnesyltransferase as compared with CVFM, the best inhibitor that was previously known (Table 1). *N*-Methylation of BZA-1B to produce BZA-2B increased the potency by more than 400 times. This increase was specific for the B stereoisomer. We propose that the high affinity of BZA-2B is a result of the increase in both conformational rigidity and hydrophobicity of the (*N*-Me) BZA-substituted inhibitor. The improvement observed after *N*-methylation implies that the cis conformation about this amide bond is preferred.

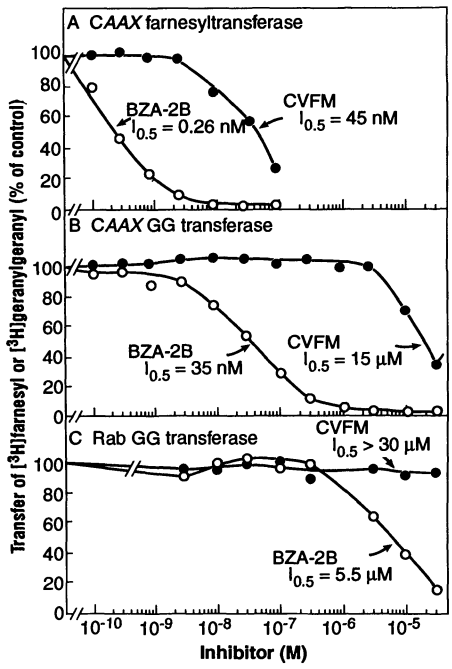


Fig. 2. Differential inhibition of *CAAX* farnesyltransferase (**A**), *CAAX* GG transferase (**B**), and Rab GG transferase (**C**) by BZA-2B (○) and CVFM (●). The assays mixtures contained (in a final volume of 50 μl) various components (*38*). After incubation for 30 min at 37°C, the amount of [^{3}H]prenyl group transferred to the appropriate protein substrate [H-Ras in (A), H-Ras (CVLL) in (B), Rab1A in (C)] was measured by precipitation with either SDS–trichloroacetic acid (A and B) (*4*) or ethanol-HCl (C) (*37a*). Immediately before use, each compound was dissolved at a concentration of 10 mM in 100% DMSO; serially diluted in solution containing 2.5% DMSO, 10 mM DTT, and 0.5% octyl β-D-glucoside; and added to the 50-μl reaction mixture in a volume of 20 μl. Each value represents a single incubation, except for the values taken as 100%, which are the average of duplicate incubations. The 100% of control values were 9.1, 3.0, and 6.7 pmol of [^{3}H]farnesyl or [^{3}H]geranylgeranyl transferred per tube in (A), (B), and (C), respectively.

Table 1. Inhibition of *CAAX* farnesyltransferase by benzodiazepine peptidomimetics. The values for 50% inhibition of the farnesylation of H-Ras were obtained in experiments in which each peptide was tested at multiple concentrations as described in Fig. 2A.

Compound		IC_{50} (nM)	Number of experiments
Name	Structure		
CVFM	CysValPheMet	39 (14–64)*	11
BZA-1A†	Cys(BZA)Met-COOH	380, 450	2
BZA-1B	Cys(BZA)Met-COOH	430, 450	2
BZA-2A	Cys(N-Me)(BZA)Met-COOH	350, 390	2
BZA-2B	Cys(N-Me)(BZA)Met-COOH	0.85 (0.26–1.8)	11
BZA-3A	Cys(N-Me)(BZA)Ser-COOH	>10,000	2
BZA-3B	Cys(N-Me)(BZA)Ser-COOH	8.2 (6.2–10)	3
BZA-4A	Cys(N-Me)(BZA)Leu-COOH	84, 100	2
BZA-4B	Cys(N-Me)(BZA)Leu-COOH	1.3 (0.5–2.1)	4
BZA-5B	Cys(N-Me)(BZA)Met-COOMe	32, 50	2
BZA-6B	Cys(N-Me)(BZA)Met-$CONH_2$	6.6, 19	2

*Mean (and range) of values from the indicated number of experiments done over a 3-month period. †The designations A and B refer to the two diastereomers with opposite configurations at C-3 (*33*).

We prepared a series of analogs of BZA-2B in which the COOH-terminal amino acid was varied (Table 1). BZA-3B, terminating in serine, was only one-sixth as potent as the compounds terminating in methionine (BZA-2B) or leucine (BZA-4B). The COOH-terminal amide of BZA-2B, designated BZA-6B, had only one-tenth the potency of BZA-2B, indicating the importance of the COOH-terminal negative charge as observed with simple tetrapeptide inhibitors (*7*, *9*). COOH-terminal esterification (*15*), as in BZA-5B, also decreased the activity in vitro, but greatly improved potency in intact cells (see below).

We compared the inhibitory activity of BZA-2B on the three known protein prenyltransferases (Fig. 2). The compound inhibited modification of Ras by recombinant rat CAAX farnesyltransferase by 50% at 0.26 nM. The concentration of CVFM required to achieve the same inhibition was about 200 times greater (Fig. 2A). Two other prenyltransferases, both of which transfer 20-carbon geranylgeranyl (GG) groups, have been identified in rat brain. One of these, CAAX GG transferase, attaches GG groups to proteins that terminate in CAAX sequences in which X is leucine (*16*, *17*). The other enzyme, Rab GG transferase, recognizes a different class of substrates that do not terminate in CAAX sequences (*18*). BZA-2B inhibited the CAAX GG transferase at a concentration more than 100 times greater than that required to inhibit CAAX farnesyltransferase [50% inhibitory concentration (IC_{50}) = 35 nM] (Fig. 2B) and was even less active in inhibiting Rab GG transferase (IC_{50} = 5.5 μM) (Fig. 2C). CVFM was a poor inhibitor of CAAX GG transferase and caused no detectable inhibition of Rab GG transferase.

BZA-2B, which contains a COOH-terminal methionine, and BZA-4B, which contains leucine, were equally potent in inhibiting CAAX farnesyltransferase (IC_{50} ~1 nM) (Table 1). This result was unexpected because leucine-terminated peptides are much less effective than methionine-terminated peptides in inhibiting farnesylation of H-Ras (*7*). Apparently, the enzyme does not discriminate between COOH-terminal leucine and methionine residues in the benzodiazepine peptides, indicating that important binding determinants are defined by the cysteine and benzodiazepine substituents. In contrast, the CAAX GG transferase was about five times more sensitive to the leucine-terminated inhibitor (BZA-4B) than to BZA-2B (IC_{50} = 7 nM as compared with 36 nM) (*19*).

In addition to inhibiting the farnesylation of H-Ras, BZA-2B inhibited incorporation of [^{3}H]farnesyl pyrophosphate into a biotinylated heptapeptide that terminated in CVIM (*19*), which corresponds to the sequence of K-RasB (*20*). The IC_{50} value was approximately the same as that observed with H-Ras as substrate. The BZA-2B compound appeared to be a pure enzyme inhibitor and was not itself farnesylated (*18*), as indicated by a thin-layer chromatography assay (*9*).

To study farnesylation in intact cells, we used Met18b-2 cells, a line of Chinese hamster ovary (CHO) cells that takes up [^{3}H]mevalonate efficiently owing to the production of a mevalonate transport protein (*21*). The [^{3}H]mevalonate is converted into [^{3}H]farnesyl and [^{3}H]geranylgeranyl, which are then attached to proteins (*21*). To prevent isotopic dilution of the [^{3}H]mevalonate, we added compactin, which blocks the synthesis of unlabeled mevalonate in the cells (*21*). The cells were incubated with the benzodiazepine peptides for 2 hours and then labeled with [^{3}H]mevalonate for 4 hours, after which the prenylated proteins were visualized by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. A control tetrapeptide, Ser-Val-Ile-Met (SVIM), which does not inhibit prenyltransferases, did not alter

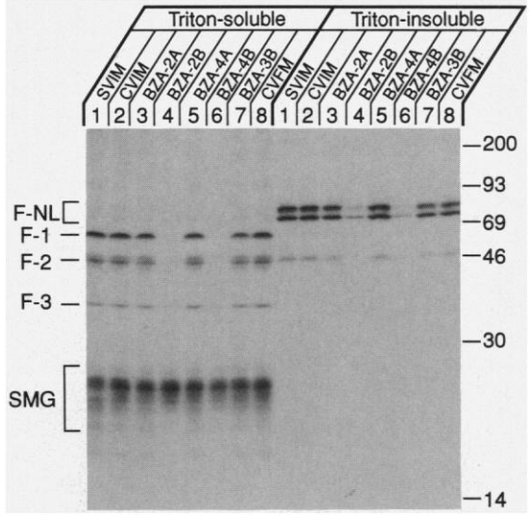


Fig. 3. Inhibition of [^{3}H]mevalonate incorporation into prenylated proteins in monolayers of hamster Met18b-2 cells by BZA-2B and BZA-4B. On day 0, stock cultures of Met18b-2 cells (*21*) were seeded at a density of 3 × 10^5 cells per 60-mm dish in 3 ml of medium A [Dulbecco's modified Eagle's medium (DMEM)–Ham's F12 medium (1/1, DMEM/F12) containing penicillin (100 U/ml), and streptomycin (100 μg/ml)] supplemented with 5% (v/v) fetal calf serum (FCS). On day 3, each monolayer was re-fed with 1 ml of medium A supplemented with 1% FCS (dialyzed against 0.15 M NaCl), 100 μM compactin (*39*), and 250 μM of the indicated compound added in 10 μl of a 100% DMSO–10 mM DTT solution. (Immediately before addition to the medium, each compound was dissolved at a concentration of 25 mM in 100% DMSO–10 mM DTT). After a 2-hour incubation at 37°C, each monolayer received 100 μCi of [^{3}H]mevalonolactone (60 Ci/mmol, American Radiolabeled Chemicals, Inc.) added in 100 μl of the above medium, and the incubation was continued for 4 hours. The cells were harvested and disrupted in buffer containing 1% (v/v) Triton X-100, after which a detergent-soluble fraction (supernatant, lanes 1 through 8) and insoluble fraction (pellet, lanes 1 through 8) were prepared (*40*). A portion of each fraction (90 μg of protein) was subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel. The gel was treated with ENTENSIFY (NEN–DuPont), dried, and exposed to Kodak XOMAT-AR film for 9 hours at −80°C. [^{14}C]methylated molecular size standards (Amersham) are shown at the right (in kilodaltons). F-NL, farnesylated nuclear lamins (*25*). F-1, F-2, and F-3, farnesylated proteins of unknown function (*19*, *22*). SMG, small (20 to 27 kD) G proteins, most of which are geranylgeranylated (*23*, *24*).

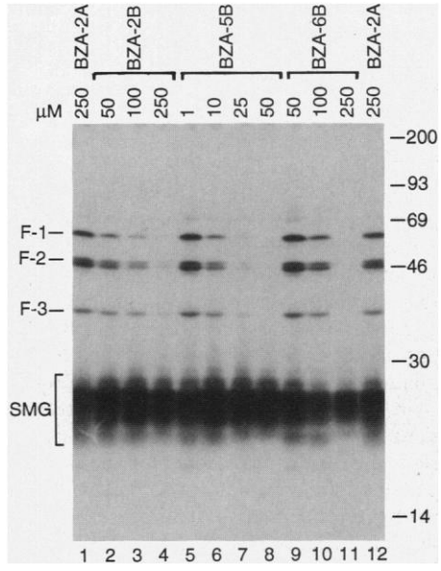


Fig. 4. Dose dependence of inhibition of [^{3}H]mevalonate incorporation into prenylated proteins in Met18b-2 cells by BZA-2B, its methyl ester (BZA-5B), and its COOH-terminal amide (BZA-6B). Cells were set up for experiments on day 0 as described (Fig. 3). On day 3, monolayers were re-fed with 1 ml of medium A containing 1% dialyzed FCS, 100 μM compactin, and 10 μl of a 100% DMSO–10 mM DTT solution containing varying concentrations of the indicated compound. After a 2-hour incubation at 37°C, each dish received 100 μCi of [^{3}H]mevalonolactone, and the incubation was continued for 4 hours. The cells were disrupted in Triton X-100 (*40*), and the detergent-soluble fraction was subjected to SDS electrophoresis. The gel was exposed to film for 40 hours at −80°C. The designations F-1, F-2, F-3, and SMG at the left are as described in Fig. 3. Molecular size standards are shown at the right (in kilodaltons).

the pattern of farnesylated proteins in the cells and was used as a control for all experiments. In the presence of SVIM, [^{3}H]mevalonate was incorporated into Triton-soluble and Triton-insoluble proteins (Fig. 3). The Triton-soluble proteins marked F-1, F-2, and F-3 (unknown functions) are farnesylated (*19*, *22*). The labeled bands of 20 to 27 kD consist largely of small molecular size GTP-binding proteins (G proteins) (*23*), the vast majority of which are geranylgeranylated (*24*). The major proteins in the Triton-insoluble pellet are the nuclear lamins A and B, which are farnesylated (*25*).

At a concentration of 250 μM, CVIM and CVFM, two tetrapeptides that inhibit CAAX farnesyltransferase in vitro (*9*), did not alter prenylation in the intact cells (Fig. 3). On the other hand, two benzodiazepine peptidomimetics, BZA-2B and BZA-4B, markedly decreased the labeling of all three Triton-soluble farnesylated proteins (F-1 to F-3) and reduced moderately the labeling of the farnesylated lamins (F-NL). The inhibitors had little effect on the geranylgeranylated small molecular size G proteins (SMG). The less active diasteromers of these compounds (BZA-2A and BZA-4A) did not inhibit prenylation in intact cells. BZA-3B, the serine-terminating benzodiazepine, did not inhibit prenylation in intact cells (Fig. 3) even though it was a moderately potent inhibitor in vitro (Table 1).

In Met18b-2 cells, BZA-5B (the methyl ester of BZA-2B) was a much more potent inhibitor than the parent compound in the intact cell even though it was less potent in the in vitro assay (Fig. 4). It inhibited farnesylation of proteins F-1 to F-3 at 10 μM and almost completely inhibited their farnesylation at 25 μM. Ten times more BZA-2B than BZA-5B was required to produce the same inhibition. BZA-6B, the amidated version of BZA-2B, was more potent than the parent compound, but not as potent as the methyl ester derivative. The control benzodiazepine, BZA-2A (250 μM), did not inhibit farnesylation. Inhibition of protein farnesylation in intact Met18b-2 cells by BZA-2B persisted for 24 hours and was reversed within 4 hours when the inhibitor was removed by washing of the cells (*19*).

To demonstrate the inhibition of farnesylation of Ras proteins directly, we incubated Met18b-2 cells with [^{3}H]mevalonate and then immunoprecipitated the cell extracts with a monoclonal antibody that reacts with all four Ras proteins (Fig. 5). Increasing concentrations of BZA-5B did not detectably inhibit the incorporation of radioactivity into the abundant small molecular size G proteins, most of which are geranylgeranylated. At 50 μM, however, the compound abolished incorporation of [^{3}H]mevalonate into immunoprecipitated Ras proteins. Inhibition was readily detectable at 10 μM. SVIM and BZA-2A, which do not inhibit [^{3}H]mevalonate incorporation into farnesylated proteins (Figs. 3 and 4), also did not inhibit the farnesylation of Ras proteins (Fig. 5).

Rat1 fibroblasts transformed with an activated mutant of H-Ras(Gly12Val) grow in multilayered clumps, indicative of malignant transformation (Fig. 6A). Incubation with BZA-2B (200 μM) for 5 days reversed the transformed phenotype (Fig. 6B), whereas BZA-2A had no effect (Fig. 6A). This change in morphology is similar to that seen after injection of antibodies to Ras into Ras-transformed cells (*26*). Rat1 fibroblasts transformed with the Src oncogene also grew in a multilayered pattern (Fig. 6C), but this pattern of growth was not affected by BZA-2B (Fig. 6D). The compound also had no apparent effect on the morphology of untransformed Rat1 fibroblasts (Fig. 6, E and F).

The effect of farnesyltransferase inhibition on cell growth was examined after the same three cell lines were seeded at low density and allowed to grow for 10 days in the absence or presence of various concentrations of BZA-5B. In the absence of the inhibitor, all three cell lines grew logarithmically (Fig. 7). In the presence of BZA-5B, the growth of Ras-transformed Rat1 fibroblasts was inhibited in a time- and dose-dependent manner, reaching ~90% inhibition after 10 days in the presence of 25 μM BZA-5B (Fig. 7B). The growth of Src-transformed (Fig. 7D) and untransformed Rat1 cells (Fig. 7F) was not affected at concentrations of BZA-5B up to 25 μM. The other isomer, BZA-5A, had no inhibitory effect on the growth of any of the three cell lines at 25 μM (*19*).

In similar experiments of 5 days duration, BZA-5B (75 μM) inhibited the

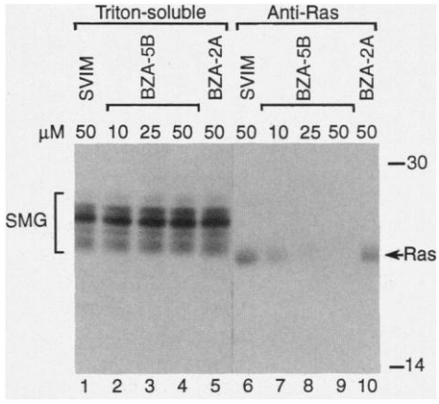


Fig. 5. Inhibition of [^{3}H]mevalonate incorporation into Ras proteins in Met18b-2 cells by BZA-5B. Cells were set up for experiments on day 0 as described (Fig. 3). On day 3, monolayers were treated with varying concentrations of the indicated compound and labeled with [^{3}H]mevalonolactone as in Fig. 3. The cells were disrupted in Triton X-100 (*40*), and a portion of the detergent-soluble fraction (50 μg of protein) was analyzed by SDS-PAGE (lanes 1 through 5) as in Fig. 3. Another portion (300 μg of protein) from each dish was also analyzed by immunoprecipitation with a monoclonal antibody to Ras (*41*), followed by electrophoresis (lanes 6 through 10). Lanes 1 through 5 were exposed to film for 16 hours at −80°C; lanes 6 through 10 for 72 hours. The designation SMG is as described in Fig. 3. Molecular size standards are shown on the right (in kilodaltons).

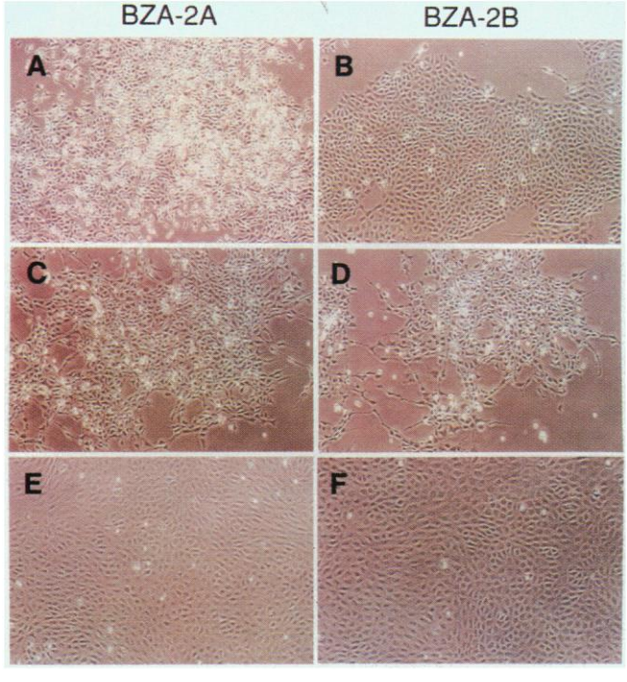


Fig. 6. Morphology of H-Ras(Gly12Val)–transformed Rat1 fibroblasts (**A** and **B**), Src-transformed Rat1 fibroblasts (**C** and **D**), and untransformed Rat1 fibroblasts (**E** and **F**) incubated in the presence of either BZA-2A (A, C, and E) or BZA-2B (B, D, and F). On day 0, cells (*42*) were plated in monolayer culture at 3 × 10^3 cells per well (24-well plates) in 1 ml of DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.7% DMSO, and 0.5 mM DTT in the presence of 200 μM of the indicated compound. On day 3, cells were re-fed with the same medium. On day 5, the cells were photographed under contrast at a magnification of 100×.

growth of H-Ras(Gly12Val)–transformed mouse myoblasts (C41 cell line) by 80%, but had no effect on the growth of the parental untransformed myoblasts (C2 cell line). BZA-5B also reversed the morphological phenotype of NIH 3T3 cells transformed by the K-RasB of Kirsten murine sarcoma virus (DT-1 cell line) (*19*).

The lack of reversion of the Src-transformed phenotype in Rat1 fibroblasts was unexpected, because a neutralizing antibody to Ras and a dominant negative H-Ras mutant blocked transformation by Src in NIH 3T3 cells (*27*). The current experiments were done with cells that were growing actively in high concentrations of serum. These cells, which are receiving multiple growth signals, seem to be able to tolerate an inhibition of farnesyltransferase. Whether these findings can be generalized to other cells in other growth conditions remains to be demonstrated.

The benzodiazepine peptidomimetics are peptidomimetic inhibitors of farnesyltransferase that block the activity of the enzyme in intact cells. Protein farnesyltransferase inhibitors produced by a strain of *Streptomyces* have been identified (*28*). These compounds, related in structure to the antibiotic manumycin, had inhibitory constants in the range of 1 to 5 μM and appeared to act competitively with regard to the farnesyl pyrophosphate substrate. Although these compounds were not demonstrated to inhibit farnesylation in intact animal cells, the most potent one decreased the size of a Ras-dependent tumor in nude mice. The mechanism for this in vivo effect was not elucidated.

Other microbial inhibitors of protein farnesyltransferase include a series of gliotoxins (inhibitory constants in the range of 1 μM) and pepticinnamins (*29*). The latter are pentapeptides with inhibitory constants as low as 0.3 μM. Neither of these classes of compounds has been shown to be effective in intact cells. A (α-hydroxyfarnesyl)phosphonic acid acutely causes a partial inhibition of Ras farnesylation in intact cells (*30*). The effects of this compound on other prenylation reactions in vivo were not tested, and it is not known whether this compound would affect the Ras-transformed phenotype.

The high potency of the benzodiazepine peptidomimetics is consistent with our initial hypothesis that the tetrapeptide substrate binds to the farnesyltransferase in a turn conformation, which would place the NH_2-terminal cysteine and the COOH-terminal methionine in close approximation to where both might interact with the Zn^{2+} atom that is known to be essential for peptide binding (*5*). These findings therefore raise the possibility that a large variety of inhibitors could be synthesized from mimics of the dipeptide turn motif.

Our experiments suggest that benzodiazepine peptidomimetics can reverse the phenotype of Ras-transformed cells at concentrations that permit the long-term growth of normal cells. This raises the possibility that partial inhibition of farnesyltransferase may allow cells to synthesize sufficient farnesylated nuclear lamins and other proteins to allow growth while blocking the action of mutant oncogenic Ras proteins. It is also possible that GTPase-defective Ras proteins act as dominant negative regulators of cell growth when they are rendered cytosolic by inhibition of farnesyltransferase. Untransformed cells, lacking a mutant Ras protein, would be protected from this inhibitory effect.

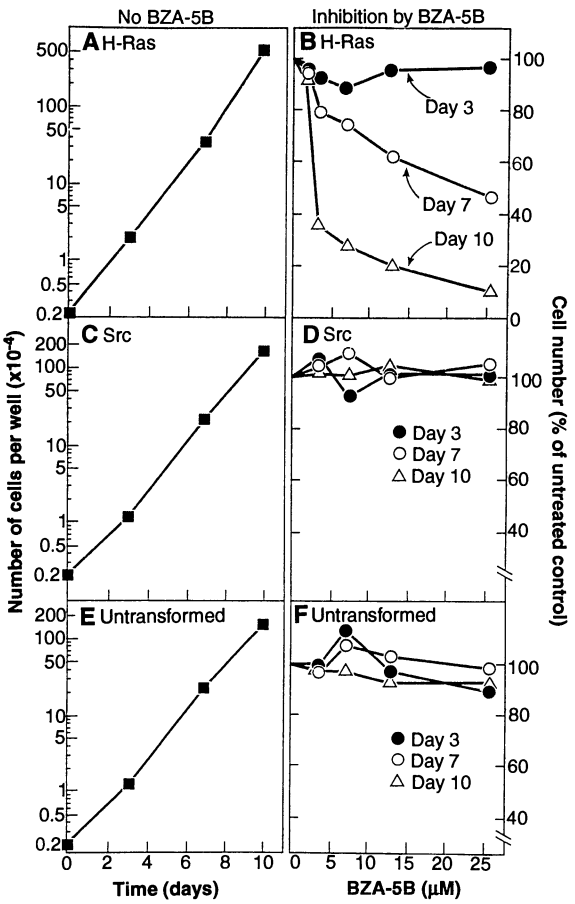


Fig. 7. Inhibition of cell growth of H-Ras(Gly12Val)–transformed Rat1 fibroblasts (**A** and **B**), but not Src-transformed Rat1 fibroblasts (**C** and **D**), or untransformed Rat1 fibroblasts (**E** and **F**). On day 0, cells (*42*) were plated in monolayer culture at 2 × 10^3 cells per well (24-well plates) in 1 ml of DMEM supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.5 mM DTT, 0.025% DMSO, and the indicated concentration of BZA-5B. On days 3 and 7, cells were re-fed with the same medium. At the indicated time, cells were harvested by trypsinization and counted in a Coulter counter. (A, C, and E) Growth rate of each cell line in the absence of BZA-5B. (B, D, and F) Inhibition of growth as a function of BZA-5B concentration at each time point. The values taken as 100% correspond to the appropriate cell numbers in (A), (C), and (E) at the indicated time. Each value represents a single incubation.

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33. Purification by HPLC (Vydac C18, acetonitrile-H_2O-TFA) resolved each of the benzodiazepine-substituted peptides into two peaks consisting of each of two diastereomers with opposite configuration at C-3. These isomers were designated as A and B, corresponding to the early- and late-eluting peaks, respectively. Identification of the absolute stereochemistry at C-3 is in progress.
34. Recombinant C*AAX* farnesyltransferase was produced in a baculovirus expression system (Y. Reiss, M S Brown, J L. Goldstein, in preparation). Briefly, the cDNAs encoding the α and β subunits of rat C*AAX* farnesyltransferase were each excised from their parent vectors (*31*) and inserted separately into the corresponding sites of the baculovirus expression vector pVL1393 (*32*). Recombinant baculoviruses encoding both subunits were introduced together into Sf9 cells. Seventy-two hours after infection, the cells were harvested and disrupted in a Parr cell disruption bomb C*AAX* farnesyltransferase was isolated by chromatography on Q-Sepharose. The enzyme was judged to be ~90% pure by Coomassie blue staining after SDS PAGE.
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37. 3-Amino-1-carboxymethyl-5-phenyl-benzodiazepin-2-one was prepared by analogy to published procedures [M G. Bock *et al.*, *J. Org. Chem.* **52**, 3232 (1987)]. Subsequent chemistries used *N*-Boc-protected-BZA and followed established peptide synthesis methodologies (*14*) *N*-Boc-(*N*-Me)BZA was prepared from *N*-Boc-BZA by treatment with NaH and MeI. Protected amino acids and analogs were activated with BOP or BOP-Cl and coupled to suitably derivatized Merrifield resin (Bachem). Peptides were cleaved from the resin in HF–10% anisole–5% EtSMe at 0°C for 1 hour, purified by HPLC (*33*), and characterized by electrospray mass spectrometry (Sciex) The methyl ester derivative (BZA-5) was prepared by solution coupling of *N*-Boc-(*N*-Me)BZA to methionine methyl ester with DIPC followed by deprotection [trifluoroacetic acid (TFA)] and coupling of *N*-Boc-(*S*-ethylthio)cysteine with EDC in DMF.
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38. The assay mixture for C*AAX* farnesyltransferase contained (in a final volume of 50 μl) 50 mM tris-chloride (pH 7.5), 50 μM $ZnCl_2$, 3 mM $MgCl_2$, 20 mM KCl, 5 mM dithiothreitol (DTT), 0.4% (v/v) octyl β-D-glucoside, 1% (v/v) dimethyl sulfoxide (DMSO), 0.6 μM all-*trans*-[^{3}H]farnesyl pyrophosphate (9730 dpm/pmol; Dupont–New England Nuclear), 40 μM recombinant H-Ras (*4*), 10 ng of purified farnesyltransferase (*34*), and various concentrations of the indicated compound in (A). The assay mixture for C*AAX* GG transferase contained (in a final volume of 50 μl) 50 mM sodium Hepes (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 0.3 mM Nonidet P-40, 0.2% octyl β-D-glucoside, 1% DMSO, 0.5 μM all-*trans*-[^{3}H]geranylgeranyl pyrophosphate (33,000 dpm/pmol; American Radiolabeled Chemicals, Inc.), 5 μM recombinant H-Ras (CVLL) (*16*), 6.3 μg of partially purified C*AAX* GG transferase (*16*), and various concentrations of the indicated compound in (B). The assay mixture for Rab GG transferase contained (in a final volume of 50 μl) 50 mM sodium Hepes (pH 7.2), 5 mM $MgCl_2$, 5 mM DTT, 0.3 mM Nonidet P-40, 0.2% octyl β-D-glucoside, 1% DMSO, 0.5 μM [^{3}H]geranylgeranyl pyrophosphate (33,000 dpm/pmol), 2 μM recombinant Rab1A (*18*), 2 ng each of purified components A and B of Rab GG transferase (*18*), and the indicated compound in (C).
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40. Each cell monolayer was harvested by rinsing three times with 3 ml of 50 mM tris-HCl (pH 7.5), 0.15 M NaCl. Lysis buffer (300 μl) [0.5× Dulbecco's phosphate-buffered saline containing 1% Triton X-100, leupeptin (5 μg/ml), pepstatin (5 μg/ml), 0.5 mM phenylmethylsulfonyl fluoride, and 0.05 trypsin inhibitor units per milliliter of aprotinin] was added to each monolayer. After incubation on ice for 5 min, the lysates were centrifuged for 30 s in a microfuge at 12,000*g*. The resulting supernatant was transferred to a new tube, and each sedimented sample was resuspended in lysis buffer (60 μl). Protein concentrations were determined by the BCA protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's directions. Samples were mixed with 2× SDS sample buffer (*35*) and heated at 95°C for 5 min before electrophoresis.
41. Portions of the Triton X-100–soluble fraction (300 μg of protein) were incubated with a rat monoclonal antibody to Ras (1 μg) (Oncogene Science, Inc.) overnight at 4°C on a rotating platform. Immune complexes were precipitated by addition of 25 μl of protein A–agarose suspension that had been precoated with goat antibody to rat immunoglobulin G (Oncogene Science, Inc.) according to the manufacturer's directions. After a 30-min incubation at 4°C, the agarose beads were centrifuged and washed five times with 1 ml each of washing solution [50 mM tris-chloride (pH 7.5), 50 mM NaCl, 0.5% (w/v) deoxycholate, 0.5% (v/v) Nonidet P-40, and 0.1% (w/v) SDS]. Each sample received 75 μl of 1× SDS sample buffer (*35*) and was heated for 5 min at 95°C before electrophoresis.
42. The H-Ras–transformed cells were generated by transfection of Rat1 fibroblasts with an SV40-driven expression vector containing an *H-ras* gene in which glycine at amino acid 12 was replaced by valine [P. H. Seeburg, W. W. Colby, D. J. Capon, D. V. Goeddel, A. D. Levinson, *Nature* **312**, 71 (1984)]. Cells that overgrew the monolayer were extracted and plated into agar to obtain a cell line displaying a fully transformed phenotype. The Src-transformed cells were generated by transfection of Rat1 fibroblasts with a vector containing the v-*src* gene [M. A. Snyder, J. M. Bishop, W. W. Colby, A. D. Levinson, *Cell* **32**, 891 (1983)] as well as the *neo* gene that confers G418 resistance, both under control of SV40 early promoters. G418-resistant cell clones that displayed a transformed phenotype were used to generate a cell line.
43. We thank D. Noble-Morgan, T. Martin, S. Broz, D. Burdick, D. Oare, K. Paris, and M. Reynolds for technical assistance. Supported by NIH grant HL 20948, by a grant from The Perot Family Foundation, and by a postdoctoral fellowship from the Helen Hay Whitney Foundation (G.L.J.).

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Induction of Type I Diabetes by Interferon-α in Transgenic Mice

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Type I diabetes is an autoimmune disease involving an interaction between an epigenetic event (possibly a viral infection), the pancreatic β cells, and the immune system in a genetically susceptible host. The possibility that the type I interferons could mediate this interaction was tested with transgenic mice in which the insulin-producing β cells expressed an interferon-α. These mice developed a hypoinsulinemic diabetes associated with a mixed inflammation centered on the islets. The inflammation and the diabetes were prevented with a neutralizing antibody to the interferon-α. Thus, the expression of interferon-α by the β cells could be causal in the development of type I diabetes, which suggests a therapeutic approach to this disease.

Type I diabetes is caused by the progressive loss of pancreatic β cells and is associated with several autoimmune phenomena (*1*). Although particular alleles closely linked to the major histocompatibility complex (MHC) class II locus increase the risk of developing type I diabetes (*2*), studies of identical twins have implicated poorly characterized environmental factors in the initiation of the disease (*3*). Several reports have suggested that viral infections could be these environmental agents in type I diabetes (*4*). However, many different viruses are epidemiologically linked to diabe-

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