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PEPTIDOMIMETIC INHIBITORS OF P21RAS FARNESYLTRANSFERASE: HYDROPHOBIC FUNCTIONALIZATION LEADS TO DISRUPTION OF P21RAS MEMBRANE ASSOCIATION IN WHOLE CELLS

Yimin Qian,† Michelle A. Blaskovich,¶ Churl-Min Seong,† Andreas Vogt,¶ Andrew D. Hamilton*† and Said M. Sebti*¶

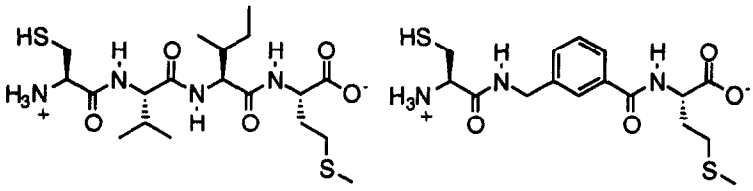
†Department of Chemistry and ¶Department of Pharmacology,
University of Pittsburgh,
Pittsburgh, PA 15260 USA

Abstract

In this paper we describe the synthesis of several hydrophobic inhibitors of p21ras farnesyltransferase. These peptidomimetic structures, containing cysteine and methionine residues separated by an aromatic spacer, are functionalized as their methyl esters and N,S-bis-benzyloxycarbonyl (Cbz) derivatives and are shown to penetrate NIH 3T3 cells to disrupt p21 ras plasma membrane association.

Introduction

The family of ras oncogenes and their ras protein products have been the subject of intense interest in recent years.[1] Of particular importance has been the recognition that mutated ras oncogenes are present in 50% of colon cancers and more than 90% of pancreatic cancers.[2] Furthermore, ras proteins have been shown to play a critical role in signal transduction as a molecular switch between growth factor receptors and the signalling pathway leading to cell proliferation.[3] Ras proteins are small (MW 21 KD), membrane-bound, guanine nucleotide binding structures that function by cycling between an active GTP-bound and an inactive GDP-bound state. In mutated p21ras the GTPase activity is much reduced and the protein remains locked in the active GTP-bound form, leading to uncontrolled cell proliferation. However, p21ras is not itself a membrane-associated protein. For proper function it requires post-translational modification to increase its lipophilicity. One of the key events in p21ras localization to the plasma membrane is farnesylation of the cysteine residue found in the carboxyl terminal tetrapeptide CAAX (C=cysteine, A=aliphatic amino acid, X=Ser or Met), that is conserved in all ras proteins. This step is catalyzed by a two subunit, zinc metalloenzyme, farnesyltransferase (FTase).[4] The dependence of the transforming activity of p21ras on farnesylation has led to an intense search for inhibitors of FTase in anticipation that they may have therapeutic potential as antitumor agents.[5]



1 2

Simple tetrapeptides such as Cys-Val-Ile-Met **1** (the carboxyl terminal sequence of K_B p21ras) inhibit FTase but also act as substrates and are farnesylated.[6] The use of these tetrapeptides to inhibit p21 ras FTase in intact cells is further hampered by low cellular uptake and rapid proteolysis. In an attempt to surmount these problems we[7,8] have recently designed tetrapeptide mimics of Cys-Val-Ile-Met where the central two amino acids are replaced by a hydrophobic spacer, such as 3-aminomethylbenzoic acid (3-AMBA) as in **2**.[9] This modification eliminates peptidic amide bonds from the molecule and offers several points for further functionalization (N- and C- terminus, phenyl ring and benzylic CH_2) to increase its membrane permeability. We have recently shown that **2** is a potent inhibitor of FTase but is not a substrate, since no farnesylation was detected in the presence of farnesyl pyrophosphate.[7] In the present paper we report that **2** is a poor inhibitor of FTase in whole cells but that simple hydrophobic modification leads to novel agents that are effective in disrupting p21ras plasma membrane association in vivo.

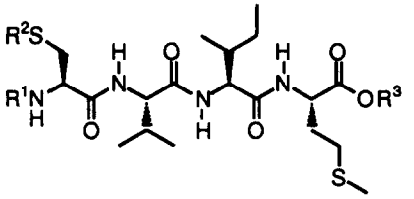
Materials and Methods

Peptide **1** and peptidomimetic **2** were synthesized as described before.[7] The fully functionalized peptide **1a** and peptidomimetic **2a** were prepared by reactions of bis-Cbz-cysteine with Val-Ile-Met methyl ester and 3-aminomethylbenzoylmethionine methyl ester, respectively (using DCC and HOBt as coupling conditions). In a typical coupling reaction, 1.16 mmol of 3-aminomethylbenzoylmethionine methyl ester and 1.16 mmol of bis-Cbz-cysteine was dissolved into 50 mL of dichloromethane. Under an ice bath, 1.27 mmol of DCC and 1.27 mmol of HOBt was added to this solution. The resulting mixture was stirred over night at room temperature. The white precipitate was removed and the filtrate was washed and dried. After the evaporation of the solvent, the residue was purified by flash column chromatography (silica gel, ethyl acetate / hexane = 2 / 3) to give a pale yellow solid of pure **2a** (0.55 g, yield: 71%). m.p. 103-104 °C. The mono-Cbz derivative **2c** was prepared from **2a** by treatment with NaOMe in MeOH. The corresponding N-acetyl derivative **2d** was prepared from the N-acetyl-S-trityl, methyl ester derivative of **2** by sequential deprotection (first using $HgCl_2$ and H_2S, then precipitated by HCl-saturated ether).[10] The final functionalized peptidomimetic was the methyl ester **2b** which was synthesized from the N-Boc-S-trityl, methyl ester derivative of **2** with the same deprotection method as mentioned for **2d**.

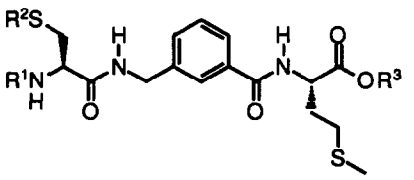
Inhibition of p21ras FTase in vitro was carried out exactly as described previously for p21ras FTase from a human colon carcinoma cell.[7] Briefly, p21ras FTase was purified from NIH 3T3 cells and an aliquot was mixed with the target peptidomimetic before adding to the following reaction mixture: 50 mM Tris (PH 7.5), 50 μM $ZnCl_2$, 20 mM KCl, 1 mM DTT, 20 μM recombinant p21 H-ras (from Dr. Crowl,

Hoffman-LaRoche), 10 pmols [^{3}H]FPP. After incubation for 30 min at 37 °C, the reaction was stopped and filtered on glass fiber filters.[7]

Inhibition of p21ras processing and plasma membrane association was carried out as follows: NIH 3T3 cells with 80% confluency were grown in Dulbecco modified Eagle's medium containing 10% fetal bovine serum. Peptidomimetic stocks were prepared by mixing 100 μM of inhibitors with 10 mM of DTT in DMSO. The NIH 3T3 cells were treated with various peptides and peptidomimetics for 18 hrs, then lysed and immunoprecipitated with a monoclonal antibody (Y13-259) against p21ras as described previously.[11] The farnesylated and non-farnesylated p21ras in the immunoprecipitates were separated on a 12.5% SDS-PAGE, then transferred to nitrocellulose and immunoblotted with Y13-259 antibody. The membrane-bound farnesylated (m) and cytosolic non-farnesylated (c) p21ras were detected with a peroxidase-conjugated secondary antibody.



1a. $R^1 = R^2 = Cbz$, $R^3 = CH_3$



2a. $R^1 = R^2 = Cbz$, $R^3 = CH_3$

2b. $R^1 = R^2 = H$, $R^3 = CH_3$

2c. $R^1 = Cbz$, $R^2 = R^3 = H$

2d. $R^1 = Acetyl$, $R^2 = H$, $R^3 = CH_3$

Results and Discussion

We first determined the ability of peptidomimetic **2** and its parent compound Cys-Val-Ile-Met **1** to inhibit p21ras membrane association. NIH 3T3 cells were grown to subconfluency and treated with either **1** or **2** (250 and 500 μM) for 18hr as described above. The cells were also treated with lovastatin (60 μM), which is known to decrease cellular farnesyl pyrophosphate concentrations and which we and others have shown to inhibit p21 ras plasma membrane anchoring.[11] The cells were then lysed and the cleared lysates were immunoprecipitated with anti-p21ras antibody (Y13-259). Immunoprecipitates were then separated on SDS-PAGE and immunoblotted with Y13-259 antibody as described above. Figure 1 shows control cells that were treated with vehicle (lane 2) containing only membrane p21ras. As expected from previous work,[11] lovastatin which was used as positive control, inhibited p21ras plasma membrane association (Figure 1, lane 1). Farnesylated p21ras migrates faster than non-farnesylated cytosolic p21ras on SDS-PAGE.[11] When cells were treated with Cys-Val-Ile-Met **1** and its mimetic **2**, no inhibition of p21ras plasma membrane association was observed (Figure 1, lanes 3 and 7, respectively). Several reasons could account for this lack of inhibition of p21ras plasma membrane association. It is possible that even though **2** lacks peptidic amide bonds, it is still degraded. It is also possible, however, that permeability of this

molecule in plasma membranes is low which would result in inefficient cellular uptake. Contributing factors to low cellular uptake could be charged groups such as the methionine carboxylate and the terminal amine.

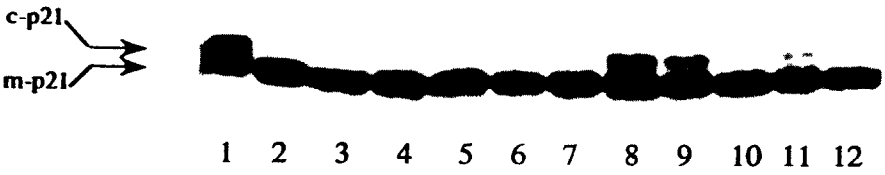


Figure 1: Inhibition of p21ras membrane association by CAAX peptides and peptidomimetics. NIH 3T3 cells were treated by various peptides and peptidomimetics and the ability of these inhibitors to block p21ras farnesylation and membrane association in whole cells was determined as described in Materials and Methods. lane 1, lovastatin (60 μM); lane 2, control; lane 3, **1** (500 μM); lane 4, **1** (250 μM); lane 5, **1a** (500 μM); lane 6, **1a** (250 μM); lane 7, **2** (500 μM); lane 8, **2a** (500 μM); lane 9, **2a** (250 μM); lane 10, **2c** (500 μM); lane 11, **2b** (500 μM); lane 12, **2b** (250 μM).

In order to improve the membrane permeability of **2** we have investigated the attachment of hydrophobic groups onto the amino terminus, the cysteine sulfur and the carboxyl terminus. Benzyloxycarbonyl (Cbz) groups were chosen for the N- and S- sites and methyl ester for the carboxyl site due to their lipophilicity, their ease of synthesis and their potential for cleavage by endogenous protease or esterase enzymes within the cell. However, we do not have enough evidence to prove that Cbz groups are removed by cellular enzymes. Compounds **1a-2d** were synthesized as described above.

The ability of the compounds to inhibit p21ras FTase in vitro was determined by measuring the transfer of [^{3}H] farnesyl from [^{3}H]-FPP to recombinant p21ras. Underivatized Cys-Val-Ile-Met **1** and its mimetic Cys-3-AMBA-Met **2** inhibited p21ras FTase with IC_{50} values of 0.34 μM and 0.38 μM, respectively (Table 1 and Figure 2). However, none of the functionalized derivatives (**1a** and **2a-d**) showed good inhibition (IC_{50} values of greater than 10 μM, Table 1). This is consistent with previous reports that a free terminal carboxylate was required for inhibition of farnesylation in tetrapeptide based derivatives. We next determined the ability of these derivatives of Cys-Val-Ile-Met and its peptidomimetic to inhibit p21ras FTase in intact cells using the method described above. NIH 3T3 cells treated with **1a** (500 and 250 μM) contain only membrane bound p21ras (Figure 1, lane 5 and 6, respectively). In contrast, cells treated with **2a** (500 and 250 μM) show both cytosolic and membrane bound p21ras (Figure 1, lane 8 and 9, respectively). Thus, N,S-di-Cbz-Cys-3-AMBA-Met-OCH_3 **2a** was able to inhibit p21ras FTase in intact cells. Since this molecule is unable to inhibit p21ras FTase in vitro, the data suggest that it is converted by proteases to more active compounds. Furthermore, Figure 1 (lane 11) also shows that simple neutralization of the methionine carboxylate with a methyl ester as in **2b** resulted in a prodrug capable of inhibiting p21ras plasma membrane association. However, this inhibition occurred only at 500 μM but not at 250 μM (Figure 1, lane 11 and 12, respectively). The fact that **2a** was able to inhibit p21ras plasma membrane association at 250 μM (Figure 1, lane 9) suggests that the Cbz groups enhance cellular uptake

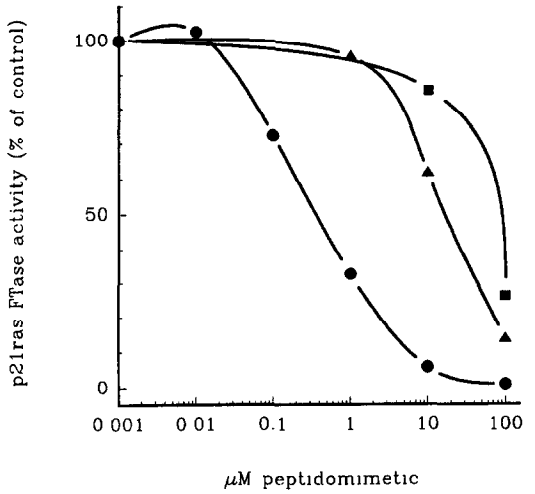


Figure 2: In vitro inhibition of FTase from NIH 3T3 cells. The ability of p21ras CAAX peptidomimetics to inhibit p21ras FTase from NIH 3T3 cells was determined as described in Materials and Methods. **2** (●), **2a** (■), **2b** (▲)

and may also increase resistance to degradation. Interestingly, N-acetyl-Cys-3-AMBA-Met-OCH_3 **2d** did not inhibit p21ras membrane association (data not shown) due possibly to the difficulty of cleaving the N-acetyl amide bond and the high IC_{50} value of **2d** in p21ras FTase assays in vitro (Table 1). Also, simple Cbz derivatization of the N-terminus of Cys-3-AMBA-Met **2c** (Figure 1, lane 10) was insufficient as a prodrug strategy. Thus, both Cbz and methyl ester groups appear to play an important role for biological activities in whole cells.

Table 1: Inhibition of p21ras FTase in vitro and inhibition of membrane association in vivo.

peptide / peptidomimetic		FTase inhibition IC_{50} [μM]	membrane association inhibition
1	Cys-Val-Ile-Met	0.34	-
1a	N,S-di-Cbz-Cys-Val-Ile-Met-OCH_3	90	-
2	Cys-3-AMBA-Met	0.38	-
2a	N,S-di-Cbz-Cys-3-AMBA-Met-OCH_3	100	++
2b	Cys-3-AMBA-Met-OCH_3	18	+
2c	N-Cbz-Cys-3-AMBA-Met	14	-
2d	N-Acetyl-Cys-3-AMBA-Met-OCH_3	100	-

These results clearly demonstrate that p21ras CAAX peptidomimetic inhibitors of p21ras FTase can be induced to inhibit ras processing in intact cells by hydrophobic functionalization.[12] Similar derivatization of the parent Cys-Val-Ile-Met tetrapeptide (as in **1a**) showed no effect on p21ras membrane association (Figure 1, lane 5 and 6), possibly due to protease degradation. Both replacement of the central dipeptide by a hydrophobic, non-peptidic spacer as well as Cbz derivatization and neutralization of the methionine carboxylate appear essential for this effect.

Acknowledgment

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References

1. Grand, R.J.A.; Owen, D. *Biochem. J.* **1991**, *279*, 609.
2. Barbacid, M. *Annu. Rev. Biochem.* **1987**, *56*, 779.
3. Stokoe, D.; MacDonald, S.G.; Cadwallader, K.; Simons, M.; Hancock, J.F. *Science (Washington, D.C.)* **1994**, *264*, 1463.
4. Reiss, Y.; Goldstein, J.L.; Seabra, M.C.; Casey, P.J.; Brown, M.S. *Cell*, **1990**, *62*, 81.
5. For a recent review see: Gibbs, J.B.; Oliff, A.; Kohl, N.E. *Cell*, **1994**, *77*, 175.
6. Goldstein, J.L.; Brown, M.S.; Stradley, S.J., Reiss, Y.; Gierasch, L.M. *J. Biol. Chem.* **1991**, *266*, 15575.
7. Nigam, M.; Seong, C.M.; Qian, Y.; Hamilton, A.D.; Sebti, S.M. *J. Biol. Chem.* **1993**, *268*, 20695.
8. Qian, Y.; Blaskovich, M.; Saleem, M.; Seong, C.M.; Wathen, S.P.; Hamilton, A.D.; Sebti, S.M. *J. Biol. Chem.* **1994**, *269*, 12410.
9. For other approaches to FTase inhibition see: Leftheris, K.; Kline, T.; Natarajan, S.; DeVirgilio, M.K.; Cho, Y.H.; Pluscec, J.; Ricca, C.; Robinson, S.; Seizinger, B.R.; Manne, V.; Meyers, C.A. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 887. Graham, S.L.; deSolms, S.J.; Giuliani, E.A.; Kohl, N.E.; Mosser, S.D.; Oliff, A.; Pompliano, D.L.; Rands, E.; Brslin, M.; Deana, A. A.; Garsky, V.M.; Scholtz, T.H.; Gibbs, J.B.; Smith, R.L. *J. Med. Chem.* **1994**, *37*, 725. James, G.L.; Goldstein, J.L.; Brown, M.S.; Rawson, T.E.; Somers, T.C.; McDowell, R.S.; Crowley, C.W.; Lucas, B.K.; Leuinson, A.D.; Marsters, J.C. *Science*, **1993**, *260*, 1937. Garcia, A.M.; Rowell, C.; Ackermann, K.; Kowalczyk, J.J.; Lewis, M.D. *J. Biol. Chem.* **1993**, *268*, 18415.
10. Photaki, I.; Taylor-Papadimitriou, J.; Sakarellos, C.; Mazarakis, P.; Zervas, L. *J. Chem. Soc. (C)*, **1970**, 2683.
11. Jani, J.P.; Tkalcevic, G.; Sebti, S.M. *J. Cellular Pharmacology*, **1994**, *1*, 67.
12. The intermediate compound N-Cbz-Cys-3-AMBA-Met-OMe may be sufficiently functionalized to retain inhibition activity in intact cells at 250 μM, and we are currently investigating this possibility.

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