Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 22, pp. 2677-2682, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00482-3

LOCAL CONSTRAINED SHIFTY PSEUDOPEPTIDES INHIBITORS OF RAS-FARNESYL TRANSFERASE

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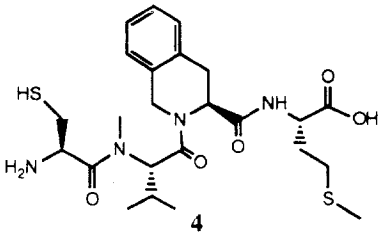
Abstract: Pseudopeptide analogues related to the C-terminal tetrapeptide of *ras*-protein (Cys-Val-X-Met) were synthesized and evaluated for inhibition of *ras* farnesyl transferase (FTase). We demonstrate that the introduction of a shifty amino acid related to Cys instead of Cys-Val and a tetrahydroisoquinoline carboxylic acid (TIC) instead of Phe lead to potent inhibitors of FTase on isolated enzyme or on cell based tests. One of the pseudopeptides, conceived as a prodrug, suppressed specifically the ability of *ras* transformed cells to form colonies in soft agar.

The ras oncogene[1] is found mutated in approximately 25 % of overall human cancers. A mutated *ras* has been linked to 50% of colon cancers[2] and 90 % of pancreas cancer[2]. Many laboratories have recently focused on the mechanism of *ras*-induced cellular transformation in anticipation that this mechanism might be useful in the identification of novel anticancer therapeutic approaches[3]. In our laboratory, we are investigating the post-translational modifications of the ras gene product, the *ras* protein. The C-terminal CAAX sequence, where C is Cys, A is an aliphatic amino acid and X is any amino acid, is highly conserved in the different *ras* proteins. *Ras* proteins undergo several post-translational modifications before reaching their site of action in the cell membrane. The first and most important modification is the farnesylation of the thiol group of the cysteine located at the fourth amino acid position of the *ras* C-terminus[4a]. Ras farnesyl transferase (FTase) is the enzyme that catalyses this event. It has been previously shown[4b] that the inhibition of farnesylation of *ras* proteins preclude their migration to their site of action in the cell membrane and prevent their cell transforming activity. Therefore, inhibitors of *ras* farnesyl transferase represent a new family of potential antitumor agents against the mutated oncogenic Ras-proteins. Some inhibitors of the bovine FTase related to the tetrapeptide C-terminus of *ras* have been recently published[3,5] (see **1-3** in table 1) Those compounds displayed significant activity on isolated bovine FTase, however, they exhibited poor activities in cell based assays[5]. In our studies analogue **1** displayed a weaker activity on human FTase[6] as compared to bovine FTase:

Table 1: Isolated human and bovine enzyme FTase inhibitory activity of CAAX box analogs

Symbol	Product	IC_{50} (Bovine FTase[3,5]) nM	IC_{50} (Human FTase[6]) nM
1	Cys-Val-Phe-Met	57, 60	1000
2	CysΨ(CH_2NH)-Val-Phe-Met	23	-
3	CysΨ(CH_2NH)-ValΨ(CH2NH)Phe-Met	21	-
4	Cys-(NMe)Val-Tic-Met	-	5

To explain the lack of significant activity of **2-3** in cell based assays[5], among other factors such as low internalization and/or metabolic unstability, we suggest a low recognition of the analogues by the human FTase. Herein, we have synthesized a local constrained analogue of **1** in which tetrahydroisoquinoline carboxylic acid (Tic) was introduced instead of Phe and N-MeVal instead of Val by solid phase peptide synthesis (SPPS) as previously described[6-7], such introduction led to the potent inhibitor **4** on isolated enzyme assay[6] (see table 1):



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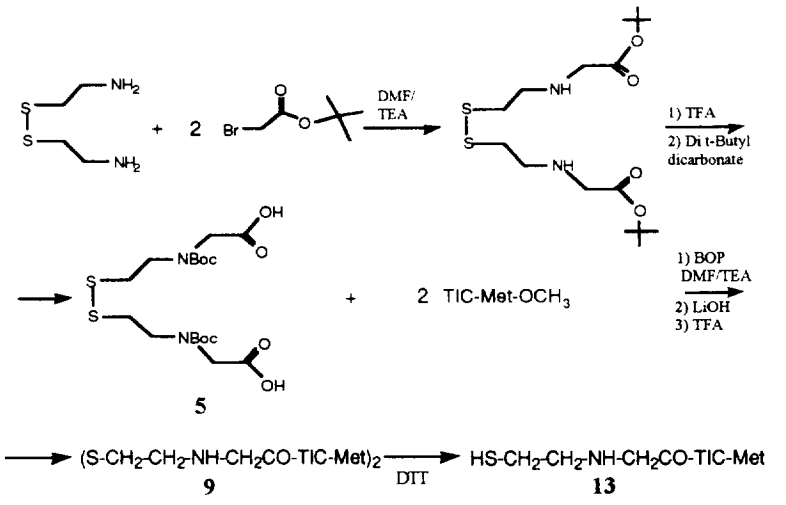
While this work was in progress, another group[8] has shown that peptide Ac-CVTicM is twenty fold more active than Ac-CVFM. On the other hand, we have introduced an additional constraint by the introduction of NMeVal instead of Val. This additional constraint yielded a product with increased activity on isolated enzyme assays. Based on the latest results, with the aim of getting increased cell internalization and metabolic stability, we have designed and synthesized novel peptide and pseudopeptide analogues related to **4** as inhibitors of human FTase:

1- *Introduction of a shifty amino acid related to Homocys instead of Cys-Val*: the introduction of shifty amino acids into peptides to obtain the so-called "peptoides" by i*n situ* synthesis of the shifty amino acid on a solid phase growing peptide, has been recently reported by several groups[9-10]. However, a general solution synthesis of shifty amino acids convenient for thiol containing chains was not reported to date. The shifty amino acid related to cys will be an unstable *gem* thiol/amine, therefore, we have synthesized the closer related shifty amino acid derived from homocysteine:

Homocysteine **Shifty-homocysteine**

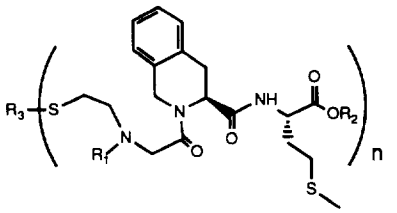
Shifty homocystine was synthesized in solution by alkylation of the dimeric cystamine with tert-butyl bromo acetate in DMF in the presence of triethylamine (TEA) at room temperature, bis,Boc-(Shifty)Homocystine was purified by flash chromatography on a silica column and analysed by NMR and mass spectroscopy. Peptide coupling of bis,Boc-(Shifty)Homocystine was carried out with benzotriazole-1-yloxytris-(dimethylamino) phosphonium hexafluorophosphate[11] (BOP reagent) in DMF in the presence of TEA. Ester

hydrolysis with 4 N LiOH in H_2O/acetone (1:1) at 0°C for 30 min. afforded the free carboxylic acid derivatives, finally, dimers were reduced to monomers with dithiothreitol (DTT) as previously described[12]. Peptides were purified by semipreparative HPLC with a Bio-Sil C18 HL 90-10 (250x10 mm) column from Bio-Rad with a linear gradient of H_2O/CH_3CN, from 100 % H_2O to 100 % CH_3CN in 30 min:



In table 2 are outlined the activities of the shifty peptides related to **9** and **13**:

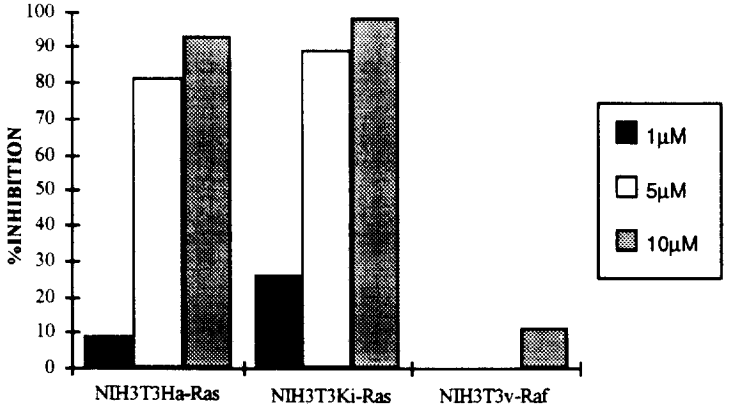
Table 2: Isolated enzyme[6] and cell[13] FTase inhibitory activity of shifty peptides:



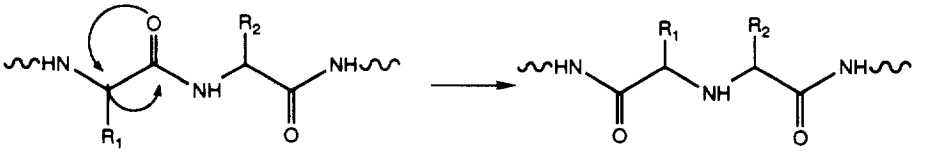
Symbol	n	R_1	R_2	R_3	IC_{50} in vitro (nM)	Cells (μM) +DTT	Cells (μM) -DTT
6	2	Boc	Me	-	» 10^5	»10^3	10^3
7	2	H	Me	-	145	100	100
8	2	Boc	H	-	»10^5	»10^3	»10^3
9	2	H	H	-	50	»10^3	»10^3
10	1	Boc	Me	H	»10^5	-	-
11	1	H	Me	H	290	-	-
12	1	Boc	H	H	»10^5	-	-
13	1	H	H	H	100	-	-

Substitution of the Cys-Val fragment in the CAAX sequence by shifty homocysteine does not affect significantly the inhibitory activity of the products on isolated enzyme (see product **13**). Moreover, a prodrug form of the carboxyl terminus is crucial for obtaining active products on cell based assays (see **7**). Finally, product **7** which is formulated as a prodrug form of both carboxyl C-terminus (ester) and thiol (disulfure bridge) display activity on cell based assays either under reducing (+DTT) conditions (product **11** is formed *in situ*) or under non reducing conditions (-DTT) in which the reduced form **11** is not present in the extra cellular media. As previously reported, a free thiol group is absolutely necessary for obtaining biologically active products[14], therefore, the prodrug form of the thiol group must undergo *in situ* reduction once the peptide has crossed the cell membrane (cytosol is a reducing medium). Product **7** suppressed as well the anchorage independent growth of NIH 3T3 cells transformed with oncogenic Ha-Ras as that of the oncogenic Ki-Ras, but had no effect on the growth of NIH 3T3 cells transformed by v-Raf oncogene[15] (see table 4).

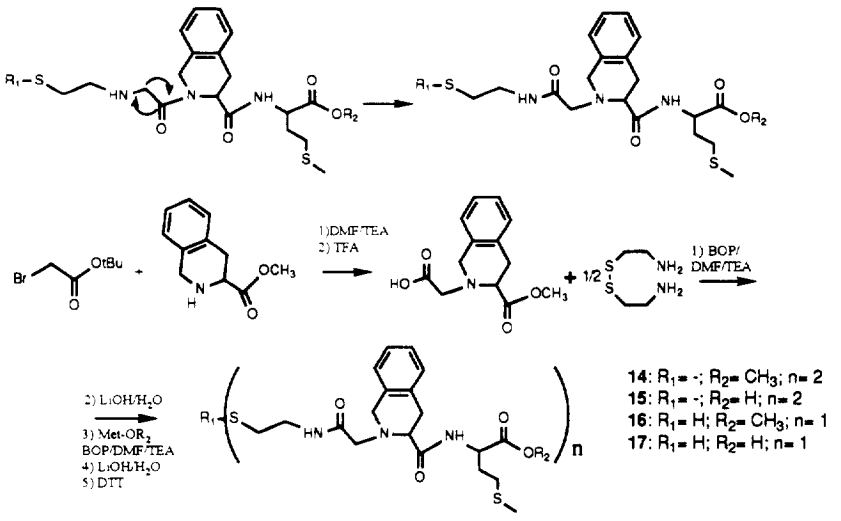
Table 4: inhibition of the growth of *ras*-transfected cells by compound **7**: NIH 3T3 Ha-Ras, NIH 3T3 Ki-Ras or NIH 3T3 v-Raf cells were seeded at a density of 5000 cells per plate (30 mm in diameter) in a 0.3 % top agarose layer over a bottom 0.5 % agarose layer. Both layers contained the compound. The number of colonies was determined 21 days later after seeding cultures (for more details see note 15):



2- *Introduction of a carboxy-retroinverso peptide isoster*: in our attempts to obtain potent and stable analogues we have designed and synthesised a new peptide bond isoster (pseudopeptide)[16] in which the C-α is interchanged with the carbonyl of the peptide bond. We designated this modification as "carboxy retro-inverso" pseudopeptide in analogy with the wellknown "retro-inverso" pseudopeptide in which the peptide bonds were reversed[17]:



"Natural peptide backbone" "Carboxy retro-inverso pseudopeptide"



The carboxy retro-inverso pseudopeptides **14-15** are related to the dimeric forms **6-9** and the carboxy retro-inverso **16-17** are related to the monomeric forms **10-13**. The carboxy retro-inverso analogues displayed low activity on the isolated enzyme (IC_{50}=2 μM for **17**), and they are all inactive in cell based assays. The only difference between **7** and **14** is the reversal of the methylene and carbonyl groups. The lack of activity in product **14** may be attributed to conformational and/or structural reasons. Structure-activity relationship studies (SAR) together with NMR and molecular dynamics models for elucidating structural and/or conformational differences between **7** and **14** are currently underway.

In summary, we have demonstrated that tetrahydroisoquinoline analogues of C-V-X-M are preferred inhibitors of human FTase rather than the Phe derivatives. Introduction of a shifty amino acid derived from Hcys instead of Cys-Val lead to a potent inhibitor of FTase able to revert specifically the phenotype of Ras transformed cells. Moreover, we have shown that the thiol group may be masked without loosing the activity of the products on cell based assays. Finally, the introduction of a carboxy retro-inverso bond isoster instead of the shifty Hcys yielded inactive products. We are currently investigating whether there are conformational and/or structural differences between the active shifty pseudopeptides and the inactive carboxy retro-inverso species, the effect of such differences on the biological activity remains to be elucidated. Additional modifications starting from molecular dynamics models of the shifty Hcys-Tic-Met are currently underway.

Acknowledgement: this work was done as a part of the "Bio Avenir" program supported by Rhone Poulenc, with the participation of the French Ministry of Research and the French Ministry of Industry. We thank Dr. Martine Danzer and Dr. Frederic Herman for structural analyses and to O. Boniface, R. Boulay, Nicole Baudouy and Maria Le Roux for expert technical assistance.

Notes and References:

1- Barbacid, M., *Annu. Rev. Biochem.* **1987**, *56*: 779-827

2- Lowy, D., (ed) *Seminars in Cancer Biology* **1992**, *3*, 167-247, Academic Press

3- Brown, M. S., Goldstein, J. L., Paris, K. J., Burnier, J. P., and Masters, J. C.; *Proc. Natl. Acad. Sci.* **1992**, *89*, 8313

4- a)Reiss, Y., Goldstein, J. L., Seabra, M. C., Casey, P. J., and Brown, M. S. *Cell* **1990**, *62*, 81-88 b) Jackson, J.H., Cochrane, J.L., Bourne, J.R., Solski, P.A., Buss, J.E. and Der, C.J. *PNAS* **1990**, *87*, 3042-3046

5- Garcia, A. M., Rowell, C., Ackerman, K., Kowalczyk, J. J., and Lewis, M. D.; *J. Biol. Chem.* **1993**, *25*, 18415-18418

6. Clerc, F.F., Guitton, J.D., Fromage, N., Lelievre, Y., Duchesne, M., Tocque, B., James-Surcouf, E., Commerçon, A. and Becquart, J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1779-84

7. Merrifield, R.B. *J. Amer. Chem. Soc.* **1963**, *85*, 2149

8- Marsters, J.C.; McDowell, R.S.; Reynolds, M.E.; Oare, D.A.; Somers, T.C.; Stanley, M.S.; Rawson, T.E.; Struble, M.E.; Burdick, D.J.; Chan, K.S.; Duarte, C.M.; Paris, K.J.; Tom, J.Y.K.; Wann, D.T.; Xue, Y.; Burnier, J.P. *Bioorg. Med. Chem.* **1994**, *2*, 949-957.

9- Simon, R. J., Kania, R. S., Zuckerman, R. N., Huebner, V. D., Jewell, D. A., Banville, S., Ng, S., Wang, L., Rosenberg, S., Marlow, C. K., Spellmeyer, D. C., Tan, R., Frenkel, A. D., Santi, D. V., Cohen, F. E., and Bartlett, P. A., *Proc. Natl. Acad. Sci.* **1992**, *89*, 9367

10- Turgeman, R., Bar-Akiva, G., Selinger, Z., Goldblum, A., and Chorev, M. *Proc. 13th Am. Pept. Symp.* **1993**, 293-295.

11. Castro, B., Dormoy, J. R., Dourtoglou, B., Evin, G., Selve, C., and Ziegler, J. C. *Synthesis* **1976**, 751

12. Cleland, W.W. *Biochemistry* **1964**, *3*, 480-2

13. Inhibition of Ras processing in intact cells was performed as followed: THAC cells (CCL39 cells transformed with activated Ha Ras[18] were treated with compounds in presence or in absence of DTT for 24 hr and then lysed in triton X114 buffer (20 mM Tris-HCl, 1% triton X114, 5 mM $MgCl_2$, 5 mM DTT pH=7.4). The farnesylated Ras protein was separated from the non-farnesylated Ras protein by phase extraction with triton X114[19]. The proteins were fractionated by 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred onto a polyvinylidene difluoride membrane (PVDF Millipore Corp.). The filters were incubated with specific anti-Ras monoclonal antibody (pan Ras Ab3, Oncogene Science) and then with ^{125}I protein A. Ras proteins were detected by autoradiography and the Ras specific bands were cut off and counted in a scintillation counter. The radioactivity of the bands corresponding to farnesylated and non-farnesylated Ras allow the determination of the % inhibition of Ras farnesylation.

14. Seabre, M.C., Reiss, Y., Casey, P.J., Brown, M.S., Goldstein, J.L. *Cell* **1991**, *65*, 429

15. Inhibition of the growth of Ras-transformed cells in soft agar by compound 7: NIH 3T3 cells transformed with either activated Ha-Ras, activated Ki-Ras or v-Raf were seeded at a density of 5000 cells per plate (30 mm in diameter) in a 0.3% top agarose layer (DMEM medium supplemented with 10% calf serum) over a bottom agarose layer (0.5%). Both layers contained 0.1% dimethylsulfoxide (DMSO) or the indicated concentration of compound 7 (dissolved in DMSO at 1000 times the final concentration used in the assay, the cells were not re-fed with compound 7 during the assay). The number of colonies was determined 21 days after seeding cultures in untreated and treated samples to determine the % of inhibition of colony growth.

16. Spatola, A.F. *Chem. Biochem. Amino Acids, Pept. Proteins* **1983**, *7*, 257-267

17. Chorev, M., Shavitz, R., Goodman, M., Minick, S. and Guillemin, R., *Science* **1979**, *204*, 1210.

18- Seuwen, K., Legerde, A., and Pouysegur, J. *EMBO J.* **1988**, *7(1)*, 161-8

19- Bordier, C., *J. Biol. Chem.* **1981**, *256 (4)*, 1604-7

(*Received in Belgium* 7 *April* 1995; *accepted* 5 *October* 1995)