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Peptide chemistry applied to a new family of phenothiazine-containing inhibitors of human farnesyltransferase

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Abstract: Novel phenothiazine derivatives bearing an amino acid residue were synthesized via peptide chemistry, and evaluated for their inhibitory potential on human farnesyltransferase. The phenothiazine unit proved to be an important bulky unit in the structure of the synthesized inhibitors. Propargyl ester **20** bearing a tyrosine residue exhibited the best biological potential *in vitro* in the present study. Further syntheses and biological evaluation of phenothiazine derivatives are necessary in order to gain a full view of SAR in this family of farnesyltransferase inhibitors.

Key-words: farnesyltransferase inhibitor, phenothiazine, peptide coupling, activated ester

Over the years, farnesyltransferase (FTase) has generated much attention as a major target in the development of new anticancer agents.[1] It could be interesting also against Progeria and parasitic protozoa diseases such as malaria, Chagas diseases or Leishmanias.[2] This heterodimeric zinc metalloenzyme is one of the three prenyltransferases which catalyses covalent attachment of a prenyl unit (C15) from a farnesylpyrophosphate (FPP) to the free thiol group of the C-cysteine found in the terminal CAAX motif (where A are aliphatic amino acids, and X is a serine, a methionine or a glutamine for farnesyltransferase, and a leucine or an isoleucine for type I geranylgeranyltransferase(GGTase))[3] of a set of membrane small G-proteins. Many of these proteins, such as lamin A and B, Rac, Ras, RhoB or RhoE actively involved in many important cellular signaling pathways and in carcinogenesis. This protein farnesylation is critical for membrane binding and the biological function of G-proteins.[4] As one of the most important G-protein, Ras proteins have a well-established role in oncogenesis, and function as switches that control growth signal from cell surface receptors to nuclear transcription factors. It has been described that gene mutational activation of the Ras occurs in about 20% of pancreatic and colorectal adenocarcinoma,[5] as well as in many other human cancers.[6]

Inhibition of protein farnesyltransferase prevents membrane localization of Ras, and so constitutes a valid target for the conception of new cytostatic anticancer drugs,[7] and recent reviews report many data on SAR in these series.[8] The main FTase inhibitors (FTIs) that have undergone clinical development are non peptidic compounds such as Tipifarnib (R-115777),[9,10] BMS-214662[11] or Lonafarnib (SCH-66336)[10,12] (Figure 1).

A recent biological screening of the chemical library of our Organic Chemistry Department (Iasi) allowed us to discover new FTIs hits, such as compounds **IV** and **V** heretofore unencountered, bearing a bulky phenothiazine group to be placed in the A2 binding site.[13] Interestingly, precursors **1a** and **2a** also present low inhibition of human farnesyltransferase (Figure 1). Thus, it was decided to explore the FTase inhibition potential of tricyclic scaffolds bearing a chain with a terminal propargylic amide **1a** and ester **2a**. These compounds **VI** were mainly obtained either by exchanging the phenothiazine ring by a carbazole, or by increasing the length of the chain by the incorporation of glycine or tyrosine unit, able to form new hydrogen bonds or stacking interactions (Figure 1).

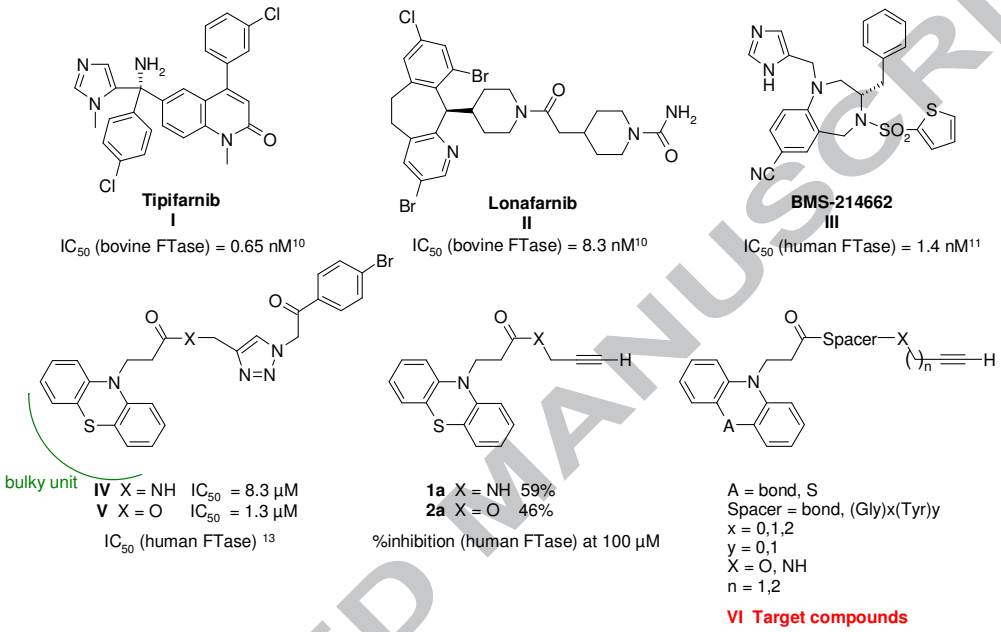
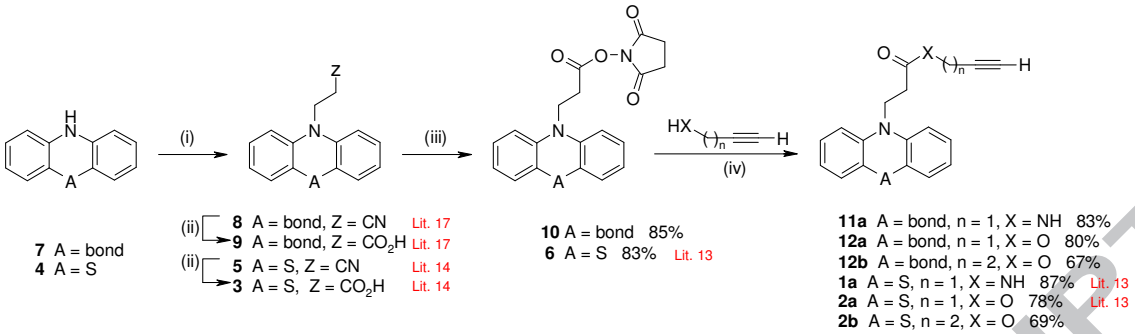


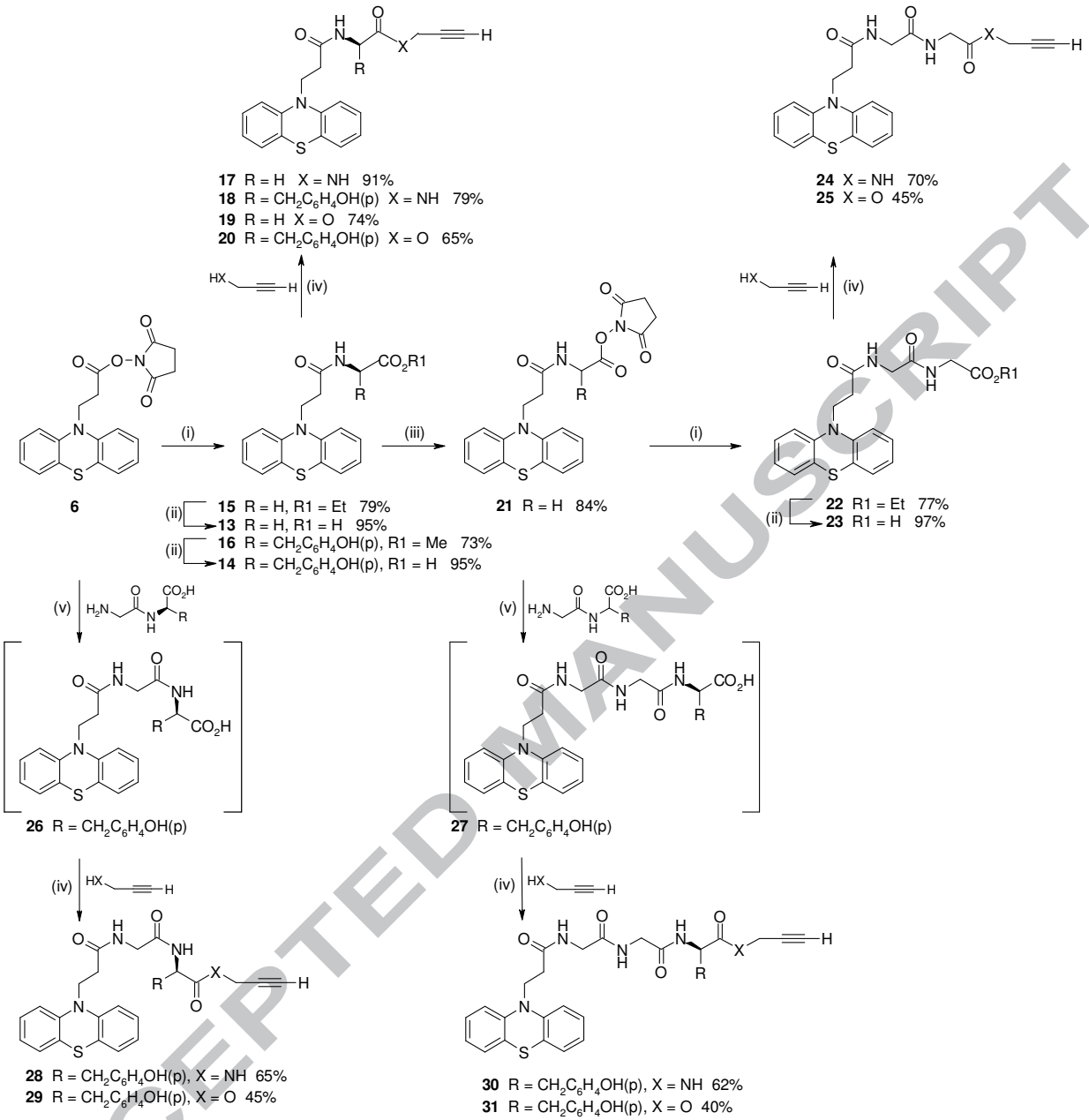
Figure 1. Structure of known FTIs **I-III**,[10,11] of FTIs synthesized by our team **IV**, **V**,[13] and of target compounds **VI**.

We first studied the nature of the heterocycle (carbazole *versus* phenothiazine) while the number of methylene units X was varied from n = 1 to n = 2; the general synthesis of these compounds is outlined in Scheme 1. Starting acid **3** was obtained by condensation of phenothiazine **4** with acrylonitrile in the presence of Triton B[14] then by hydrolysis of nitrile **5** to acid **3** with aqueous sodium hydroxide in methanol.[14] EDCI [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] coupling[15] of carboxylic acid **3** with *N*-hydroxysuccinimide led to activated ester **6**,[13,16] and then reaction with propargylamine or propargyl alcohol in presence of triethylamine gave propargylic amide **1a** and ester **2a** respectively.[13,17] The same sequence allow to obtain the ester **2b** (n = 2) in 69% yield, and applied to carbazole **7**[18] led to 85% of hydroxysuccinyl ester **10**, and then to the corresponding amide **11a** (n = 1, 83%) and esters **12a** (n = 1, 80%) and **12b** (n = 2, 67%) (Scheme 1).



Scheme 1. Reagents and conditions: (i) Triton B, 0 °C to reflux, 2 h; (ii) aqueous NaOH, MeOH, reflux, 15 h; (iii) 1.2 equiv *N*-hydroxysuccinimide, 1.2 equiv EDCI, CH_2Cl_2, rt, 24 h; (iv) 1.7 equiv propargylamine, propynol or butynol, 2.2 equiv triethylamine, CH_2Cl_2, rt, 24 h.

The bioactivities of compounds possessing a carbazole sub-unit proved to be inferior to that of corresponding phenothiazine derivatives (see later), thus the new syntheses were performed in these series. Activated ester **6** was condensed with glycinyl or tyrosyl esters to give acids **13** and **14** after saponification of the esters **15** and **16**. Acids **13** and **14** were directly condensed with propargyl amine or alcohol in the presence of EDCI, thus furnishing the compounds **17**-**20** with a Gly (x = 1, y = 0) or Tyr (x = 0, y = 1) linker, in yields from 65% to 91%. Again, acid **13** was utilized in a new sequence of activation, reaction with ethyl glycinate, saponification, and then EDCI condensation with propargyl amine or alcohol. Propargylic amide **24** and ester **25**, with a glycylglycyl (x = 2, y = 0) (Gly-Gly) linker were thus obtained in cumulated 44% and 28% yields respectively (Scheme 2). One-pot reactions were also utilized starting from activated esters **6** and **21** whose the sequential condensation firstly with GlyTyrOH (Et_3N) then with propargyl amine or alcohol (EDCI) in dimethylformamide allowed obtaining propargylic products **28**-**31** with a Gly-Tyr (x = 1, y = 1) or Gly-Gly-Tyr (x = 2, y = 1) respectively, in 40% to 65% yield (Scheme 2).

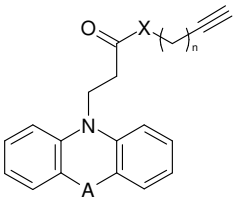


Scheme 2. Reagents and conditions: (i) 1.2 equiv L-glycine ethyl ester hydrochloride or L-tyrosine methyl ester hydrochloride, 1.2 equiv triethylamine, CH_2Cl_2, rt, 24 h; (ii) NaOH 2N, 80 °C, 2-5 h; (iii) 1.2 equiv *N*-hydroxysuccinimide, 1.2 equiv EDCI, CH_2Cl_2, rt, 24 h; (iv) 1.7 equiv propargylamine or propargyl alcohol, 1.2 equiv EDCI, DMF, rt, 24 h; (v) 1.0 equiv glycine-L-tyrosine, 2.2 equiv triethylamine, DMF, rt, 5 h.

The activity of all the synthesized phenothiazines was evaluated on human FTase.[19] Results are presented in Tables 1-3 and in Figure 2. The importance of the nature of the tricycle was considered firstly. The replacement of the phenothiazine unit by a carbazole motif was detrimental to the affinity for the protein (*e. g.*: compound **2a** *vs* **12a** (Table 1)). The influence of the length between the amide or ester function and the acetylene group was also studied. A methylene linkage (n = 1) led to compounds with better inhibitory activity than an ethylene one (n = 2) (*e. g.*: compound **2a** *vs* **2b** (Table 1)). The methylene

unit was thus conserved in the skeleton of amides and esters bearing an amino acid residue (Table 2). In this series, compounds with a glycyl spacer were inactive. A tyrosyl spacer provides the best pharmacomodulation in the present work (compound **20**, Table 2). The replacement of the mono amino acid spacer by a di- or tri- amino acid led to a slight loss of activity. The target compounds **18**, **20**, **24** and **25** were synthesized starting from intermediary carboxylic acids **14** and **23** that were also evaluated for their ability to inhibit human FTase (Tables 2 and 3). Interestingly, they exhibited similar potency as corresponding final products (*e. g.*: acid **14**: IC_{50} (FTase) = 30.3 ± 2.6 µM *vs* propargyl derivative **20**: IC_{50} (FTase) = 18.0 ± 2.4 µM, and acid **23**: IC_{50} (FTase) = 80.5 ± 6.7 µM *vs* propargyl derivative **25**: IC_{50} (FTase) = 78.7 ± 5.5 µM, Table 2). This comparison was not possible for the intermediary acids for the synthesis of compounds **28**-**31** since they were obtained via a one-pot reaction, without isolating the acid precursors. However, starting esters **15**, **16** and **22** were also tested and did not show noticeable inhibitory activity (Table 3).

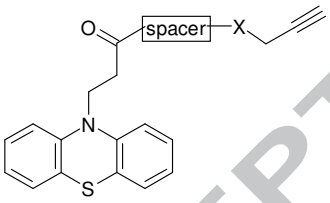
Table 1. Inhibitory activities of propargyl derivatives **1a**, **2a-b**, **11a**, and **12a-b** on human farnesyltransferase



Compound	A	X	N	% Inh (FTase)[a,b]
1a	S	NH	1	59
2a	S	O	1	46
2b	S	O	2	9
11a	bond	NH	1	40
12a	bond	O	1	13
12b	bond	O	2	0

[a] Inhibition ratio of protein farnesyltransferase at a 100 µM concentration.
[b] Values represent mean of two experiments.

Table 2. Inhibitory activities of propargyl derivatives bearing amino acid(s) residue **17-20**, **24-25**, and **28-31** on human farnesyltransferase



Compound	Spacer	X	% Inh (FTase)[a,b]	IC_{50} (µM ± SD[c])[b]	R^{2d}
17	Gly	NH	42	n. d. [e]	-
19	Gly	O	25	n. d.	-
18	Tyr	NH	43	n. d.	-
20	Tyr	O	90	18.0 ± 2.4	0.935
24	Gly-Gly	NH	42	n. d.	-
25	Gly-Gly	O	59	78.7 ± 5.5	0.939
28	Gly-Tyr	NH	68	24.4 ± 3.3	0.898
29	Gly-Tyr	O	69	36.3 ± 0.9	0.994
30	Gly-Gly-Tyr	NH	82	39.7 ± 1.0	0.994
31	Gly-Gly-Tyr	O	70	30.1 ± 2.7	0.909

[a] Inhibition ratio of protein farnesyltransferase at a 100 µM concentration.
[b] Values represent mean of two experiments.
[c] SD: standard deviation.
[d] R^2: regression factor.
[e] Not determined.

Table 3. Inhibitory activities of some intermediary esters (**15**, **16**, **22**) and carboxylic acids (**13**, **14**, **23**) on human farnesyltransferase

Compound	% Inh (FTase)[a,b]	IC_{50} (µM ± SD[c])
13	48	n.d.[d]
14	74	30.3 ± 2.6
15	41	n.d.
16	33	n.d.
22	42	n.d.
23	60	80.5 ± 6.7

[a] Inhibition ratio of protein farnesyltransferase at a 100 µM concentration.

[b] Values represent mean of two experiments.
[c] SD: Standard deviation.
[d] Not determined.

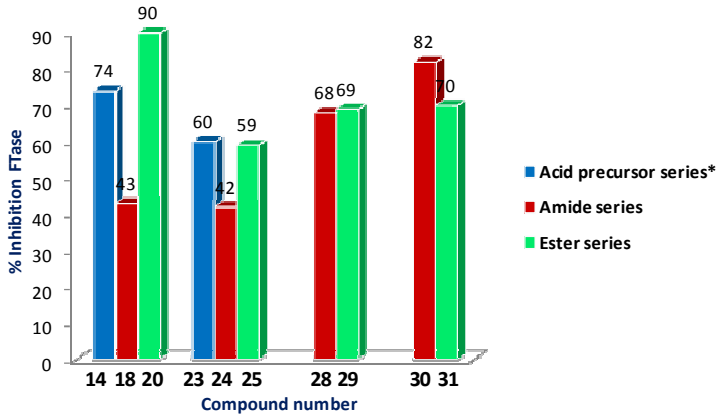


Figure 2. Comparative inhibitory activity on human FTase of amides (in red), esters (in green) and their acid precursors (in blue) at a 100 µM concentration. *Esters and amides **28-31** were synthesized via a one-pot procedure, without isolating the corresponding acid precursors.

To further expand the understanding of the experimental results, molecular modeling studies for esters **20**, **29** and **31** were carried out in the active site of protein FTase. Docking results indicated that indeed they can be placed in this site (Figure 3a-c).

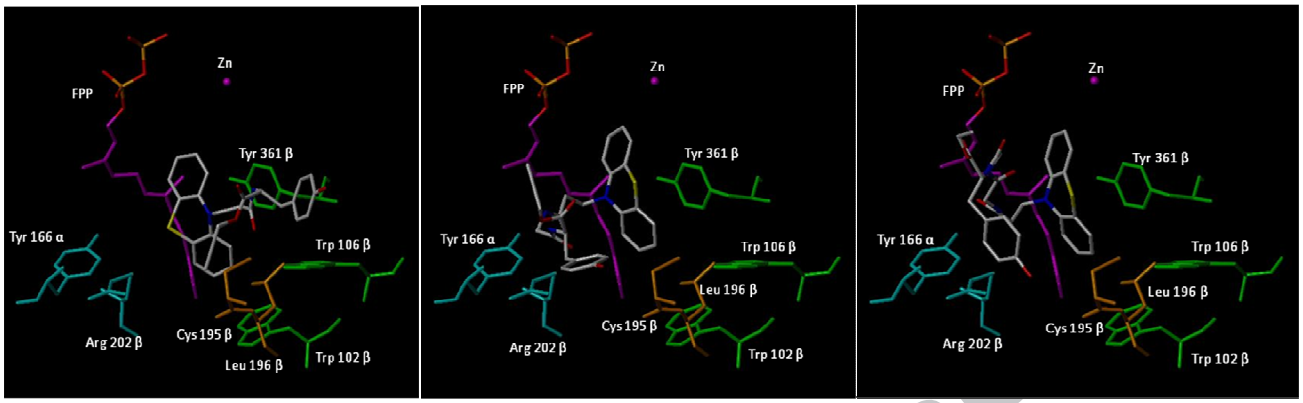
Farnesyltransferase structure was taken from the 1LD7[20] entry of the RCSB Protein Data Bank[21] due to the structural proximity of the co-crystallized ligand with our own molecules. Both the ligand and the water molecules were removed from the enzyme, before validating the protocol by docking the cocrystallized ligand back into the binding site, defined as a 10Å sphere around the position of the ligand in the complex. A formal charge of +2 was assigned to the farnesyltransferase zinc cation to ensure it would be correctly handled for the docking. The molecules under investigation were built from the standard fragments library of Sybyl 6.9.1[22] and their geometry optimized using Gasteiger-Hückel partial charges and a dielectric constant set to 4.30 solutions were generated for each compound using GOLD 5.1,[23] then ranked by PLP fitness score after assessing the backward compatibility versus the previous in house GoldScore/X-Score consensus scoring scheme. The consistency of the results was checked visually and in case of several clusters of solutions, the most representative solution of each was selected as the results of the docking run.

The best conformation obtained for compound **20** (Figure 3(a)) is different from those of compounds **29** and **31** (Figure 3(b) and (c)). However, compound **20** has a larger cluster of conformations; this may be related to its slightly higher potency. No hydrogen bonds are established, only stacking interactions are observed.

Esters **29** and **31** occupy roughly the same place of the pocket with the bulky tricycle in front of the FPP chain. The two nitrogen atoms from the two amide functions of ester **29** establish two hydrogen bonds with His 201 and Lys 164. However, these interactions are represented on a secondary potential conformation. In the same way, ester **31** bearing a longer chain presents another conformation (with only three solutions) where the phenol group forms a hydrogen bond with Asn 127 and the two nitrogen atoms from the two last amide functions form hydrogen bonds with Lys 164.

Also, the docking studies showed that the presence of the propargylic ester was not needed for the biological activity. This is in accordance with our biological assay results since carboxylic acids **14** and **23** have the same inhibitory potential as final corresponding esters **20** and **25** (Figure 2). However, it is well known that carboxylic acids are generally more active than corresponding esters on the protein

farnesyltransferase, esters being more appropriate for the cellular evaluation. We thus synthesized them in this perspective.



(a) (b) (c)

Figure 3. Docking of esters bearing a phenothiazine moiety in the active site of FTase: (a) compound **20**, (b) compound **29**, and (c) compound **31**.

In this study, we have synthesized and evaluated new phenothiazine derivatives bearing a glycine (Gly), a tyrosine (Tyr), a glycylglycine (Gly-Gly), a glycyltyrosine (Gly-Tyr) or a glycylglygyltyrosine (Gly-Gly-Tyr) unit as human farnesyltransferase inhibitors. These structural modifications furnished compounds that showed decreased inhibitory potency relative to our previously described phenothiazines.[13,24] However, most of these compounds possess *in vitro* inhibition activities in the micromolar range. Propargyl ester **20** bearing a tyrosine residue exhibited the most potent *in vitro* biological potential in the present study. Nevertheless, given these modest results obtained, non-specific interactions are not excluded.

The phenothiazine unit is an important bulky unit that can be placed in the A_2 binding site of the farnesyltransferase.[25] This fact was supported once again since the replacement of the phenothiazine by a carbazole unit was unfavorable to the affinity for the protein.

We are continuing synthesis and biological evaluation of additional phenothiazine analogs by focusing on other points of the molecule in order to gain a full view of the SAR in this family of farnesyltransferase inhibitors.

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Supplementary data

Supplementary data (synthesis details, physico-chemical characterization for all new compounds) associated with this article can be found in this section.

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Peptide chemistry applied to a new family of phenothiazine-containing inhibitors of human farnesyltransferase

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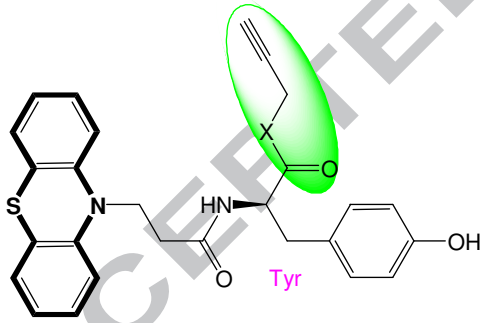
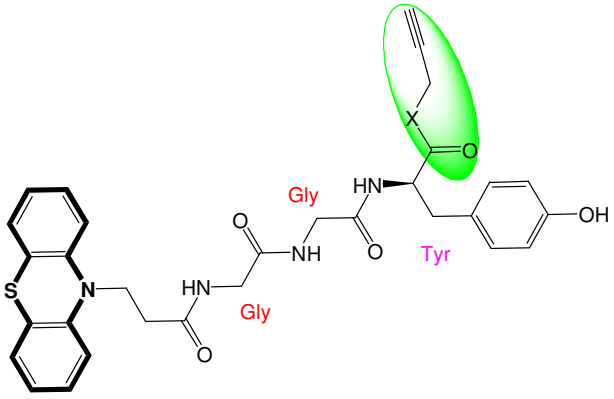
Abstract: Novel phenothiazine derivatives bearing an amino acid residue were synthesized via peptide chemistry, and evaluated for their inhibitory potential on human farnesyltransferase. The phenothiazine unit proved to be an important bulky unit in the structure of the synthesized inhibitors. Propargyl ester **20** bearing a tyrosine residue exhibited the best biological potential *in vitro* in the present study. Further syntheses and biological evaluation of phenothiazine derivatives are necessary in order to gain a full view of SAR in this family of farnesyltransferase inhibitors.



18: X = NH, % Inh (human FTase) at 100µM: 43

20: X = O, % Inh (human FTase) at 100µM: 90
IC_{50} (µM ± SD) = 18.0 ± 2.4



30: X = NH, % Inh (human FTase) at 100µM: 82
IC_{50} (µM ± SD) = 39.7 ± 1.0

31: X = O, % Inh (human FTase) at 100µM: 70
IC_{50} (µM ± SD) = 30.1 ± 2.7