PharmacologyOnLine December 30, 2014

Archives • 2014 • vol.3 • 209-215

TRYPTOPHAN-BASED LINEAR DIPEPTIDES AND 1,4-DIKETOPIPERAZINES AS SIMPLIFIED SCAFFOLD OF THE NATURAL CYCLOTETRAPEPTIDE FR235222

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Abstract

Design, synthesis and biological evaluation of simplified linear and cyclic peptidomimetic analogues of FR235222 (**1**), natural immunosuppressant and HDAC inhibitor, bearing hydroxyketone moiety as more stable zinc binding group, have been reported. Linear dipeptides (**6a-b**) show significant antiproliferative activities and are chosen to be promising lead compounds for further optimization, in order to elucidate molecule-enzyme surface recognition.

Key words: Peptides, HDAC inhibitors, Cancer, Peptidomimetics

Introduction

Histones play an important role as a regulator of the gene-expression profile in various tissue types. In details, histone tails, protruding out of the nucleosomes, undergo post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, glycosylation, biotinylation and carbonylation [1, 2]. Acetylation is the most studied chemical modification, which occurs on lysine residues in the amino-terminal regions of histones H2A, H2B, H3 and H4, and it is strictly related to the "active" transcriptional state of DNA, euchromatin. The functional state of ipo/iper-acetylated chromatin is regulated by catalytic activity of two families of enzymes: Histone Acetyl transferase (HATs) and Histone Deacetylases (HDACs). By targeting these key epigenetic modulators it has been possible to affect important downstream cellular events [3]. In particular HDAC inhibitors revealed clinical significance in tumor regression, growth arrest, cell proliferation [4-7] and apoptosis. Recently, the immunosuppressant fungal metabolite FR235222 (**1**) emerged as potent natural inhibitor of mammalian HDACs [8-10]. It acts as reversible zinc chelator and belongs to the class of cyclic tetrapeptide HDAC inhibitors, some of which have been already shown to act as effective anticancer agent [11]. This compound, therefore, represents an attractive basis for new drug design and its total synthesis has already been published by us and others [12, 13]. Conformational and docking studies [12, 14] were useful to understand the interaction between this natural ligand and its biological target and to propose a general binding mode of this molecule to the enzyme active site. The following key features are postulated to be involved in HDAC inhibition (Figure 1):

a) the α-hydroxyketone functionality, which represents the pharmacophore, responsible for the crucial zinc ion chelation event;

b) the carbon chain of the Ahoda residue, which constitutes the linker domain and has the appropriate length for the correct insertion onto the long, narrow channel of the enzyme active site, and for projecting the zinc chelating element at an optimal distance for metal interaction;

c) the cyclopeptide core, which plays the role of a surface recognition domain.

Considering our large interest in discovering and synthesizing small molecules related to FR235222 structure and pharmacological properties, and having in mind the accomplishments we achieved in previous studies, [15-18] we prepared an appropriate cyclic scaffold, mimetic of tetrapeptide-*core* of FR235222, simple to synthetize and modify afterwards. At first the new CAP proposed was represented by diketopiperazine nucleus, which exemplify a smallest cyclic peptide possible. The choice of the two amino acids was based on the evidence that by substituting *L*-Iva of the natural peptide FR235222 with *L*-Trp, an interesting increase in histone acetylation is exerted (figure 2) [15]. The Trp in this position modified the potency of the compounds suggesting that the cyclopeptide-based CAP group contributes to the high affinity binding to HDAC. Thus, we picked the Trp as key amino acid linked to the FR235222 pharmacophore, the Ahoda residue.

Herein, we report the synthesis and the biological evaluation of tryptophan-based cyclic dipeptides (diketopiperazine, **7-8** scheme 1) and the corresponding linear dipeptides (**6a-b,** scheme 1), in order to elucidate the significance of cyclic peptide core as good CAP group for HDAC inhibition (figure 3).

Methods

Compounds **6a-b, 7-8** were prepared following standard processes and completely characterized through ^{1}H, ^{13}C NMR, and high resolution mass spectrometry.

HDAC profiling was performed in the presence of a 50 µM solution of the fluorogenic tetrapeptide RHKK(Ac) substrate (from p53 residues 379–382) or in the presence of a 50 µM solution of its diacetylated analogue RHK(Ac)K(Ac) for HDAC8. Upon its deacetylation, the fluorophore was released giving rise to fluorescence emission, which was detected by a fluorimeter, and the IC_{50} values of the compounds were calculated from the resulting sigmoidal dose-response inhibition slopes. [19] The cytotoxic effects of the most promising compound (1-100 µM) in DMSO solution were evaluated on U937 cell lines by MTT assay. The assay is based on the observation that viable cells have the ability to metabolize a water-soluble tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma), into an insoluble formazan salt. Reduction of cell viability by more than 30% is considered a cytotoxic effect, otherwise the Ic_{50} value is considered not available (NA). The concentration of each compound which inhibit cell viability of 50% (Ic_{50}) was determined using Prism 5.0

(GraphPad Software Inc.). IC_{50} values were presented as means ± SEM of three independent experiments carried out by triplicate.

In vitro activity of linear dipeptides analogues: 50 pmol of an acetylated peptide of histone H4 (Ac-S-G-R-G-K-G-G-K-G-L-G-K-G-G-A-K(ac)-MCA, generous gift of Dr. M. Yoshida) was incubated in a total volume of 10 µL with 0,5 µg of a HeLa nuclear extract (BioVision, Mountain View, CA, USA) in 20 mM Tris, 150 mM NaCl, pH 8 for 1h at 37 °C in presence or absence of inhibitors. The reaction was stopped by heating the samples for 5 min at 95 °C. 1 mL of each sample was spotted onto an H4 proteinchip (Ciphergen, Fremont, CA, USA) and allowed to dry. Spots were then washed twice with 5 µL of 5 mM HEPES. Finally 2 x 0.8 µL of 20% CHCA (in 50% acetonitrile and 0.5% TFA) were spotted onto each spot. Peptide masses were acquired by SELDI-TOF (Ciphergen, Fremont, CA, USA) mass spectrometry and IC_{50} values were calculated using the peak areas in the following way: % of deacetylation was calculated as the percent of deacetylated peptide with respect to the sum of deacetylated and acetylated peptides. Inhibitory activities were then calculated as percent inhibition of the deacetylation reaction.

Chemistry

The backbone of diketopiperazine - dipeptides **5a-b** - was synthesized by coupling L-Tryptophan methylesther (TrpOMe) and (2*S*,9*R*)-2-amino-9-hydroxy-8-oxodecanoic acid (Ahoda), protected on the amine group, using standard coupling reagents (scheme 1). Cyclization of these dipeptides into corresponding diketopiperazine was not easily performed due to the hindered lateral chain of L-trp and TBDMS (Tert-butyl dimethylsilane); O-protecting group. Thus, extensive methodology was carried out in order to obtain desired products in good yield and purity.

Several reactions were carried out in order to attempt cyclization of dipeptides **5a-b** into diketopiperazine **7**. MW-assisted transfer hydrogenation and final TBAF-mediated deprotection furnished desired cyclized product **7** in only 2 minutes (Scheme 1) [20]. Further decoration of diketopiperazine **7** was performed by MW-assisted alkylation of **7** into tribenzyl derivative **8** in good yield.

With the aim of collecting more information regarding structure activity relationship of cyclic products **7-8**, also linear dipeptides **6a-b** were screened *in vitro* as HDAC inhibitors.

Biochemistry

Compounds **6a-b, 7** and **8** were screened for their antiproliferative activity in U937 cells and the level of H4 acetylation was detected, data shown in table 1. [19]

Results and Discussion

Diketopiperazine derivatives (**7-8**) did not present any representative antiproliferative activity, neither increment of H4 acetylation level, even if the selected scaffold was supposed to better reproduce the cyclic core of FR235222 CAP, improving also its pharmacokinetic profile. Actually, both of classes did not reveal any significant activity on the histone H4 acetylation level. The reason of these unpredicted biological results for Trp-diketopiperazine **7**, could be associated to the incorrect accommodation of the zinc-binding group into the channel of the histone deacetylase active site, due to weak interactions with the rim of the enzyme. On the other hand, inactivity of tribenzylated diketopiperazine **8** might be ascribed either to its very low solubility in cellular media or to the stumbling block represented by three benzyl groups, which led to a distortion of the cap, that enable the hydroxyketone to chelate zinc ion in the active site of the enzyme. Surprisingly, linear dipeptides **6a-b** show a significant biological results with IC_{50} of 53 and 44 µM respectively for **6a** and **6b**. These results could be explained with the increased number of hydrogen bond donors and acceptors that results into a better interaction with the catalytic site.

Conclusion

In conclusion we have developed new tryptophan-based CAP groups that confer different activities depending on the cyclic or linear structure of the inhibitor. Based on these preliminary biological results, the molecular simplification of cyclotetrapeptide core of FR235222 into diketopiperazine scaffold (compounds **7-8**) revealed to be not effective, whilst the linear precursors **6a-b** disclosed promising antiproliferative properties. A little chemical collection of modified Trp-based HDAC inhibitors has been prepared for further investigation on the surface recognition motif and biological evaluation is currently underway and will be reported in due course.

Acknowledgments

Authors thank Sigma-Tau S.p.A. Pomezia (Roma) for partial financial support and for the biological assays, performed by Reaction Biology (US).

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Figure 1. FR235222 key features involved in HDAC inhibition

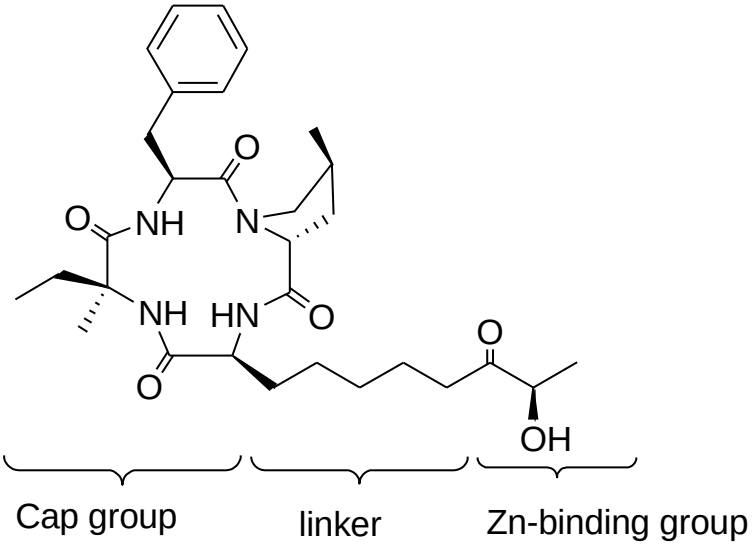


Figure 2. Comparison between FR235222, **1** and its analogue **2**. The substitution of L-Iva with L-Trp increased three times the activity (from 60 nM to 20 nM).

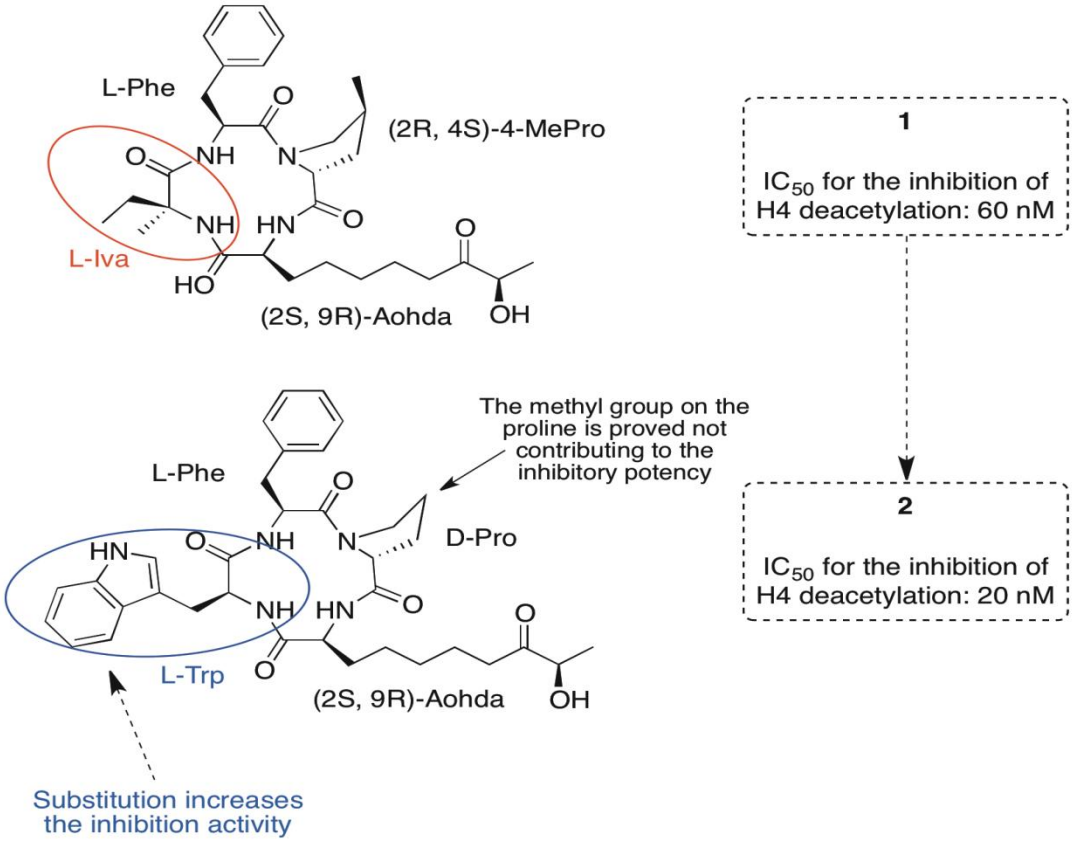
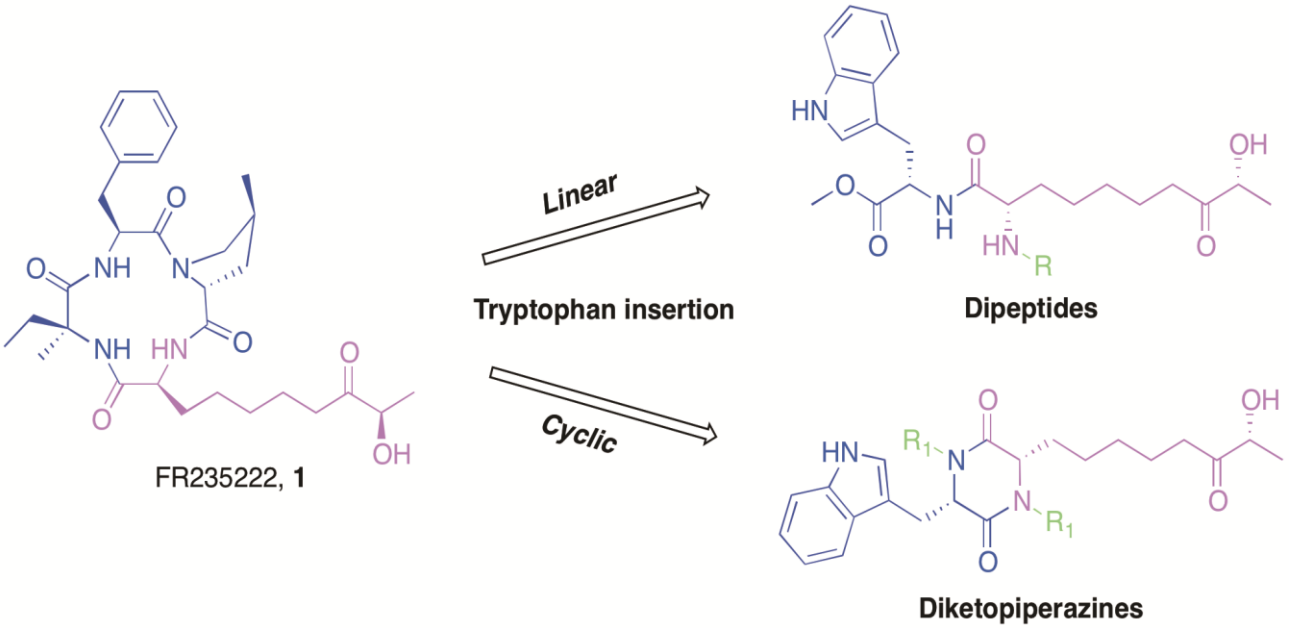
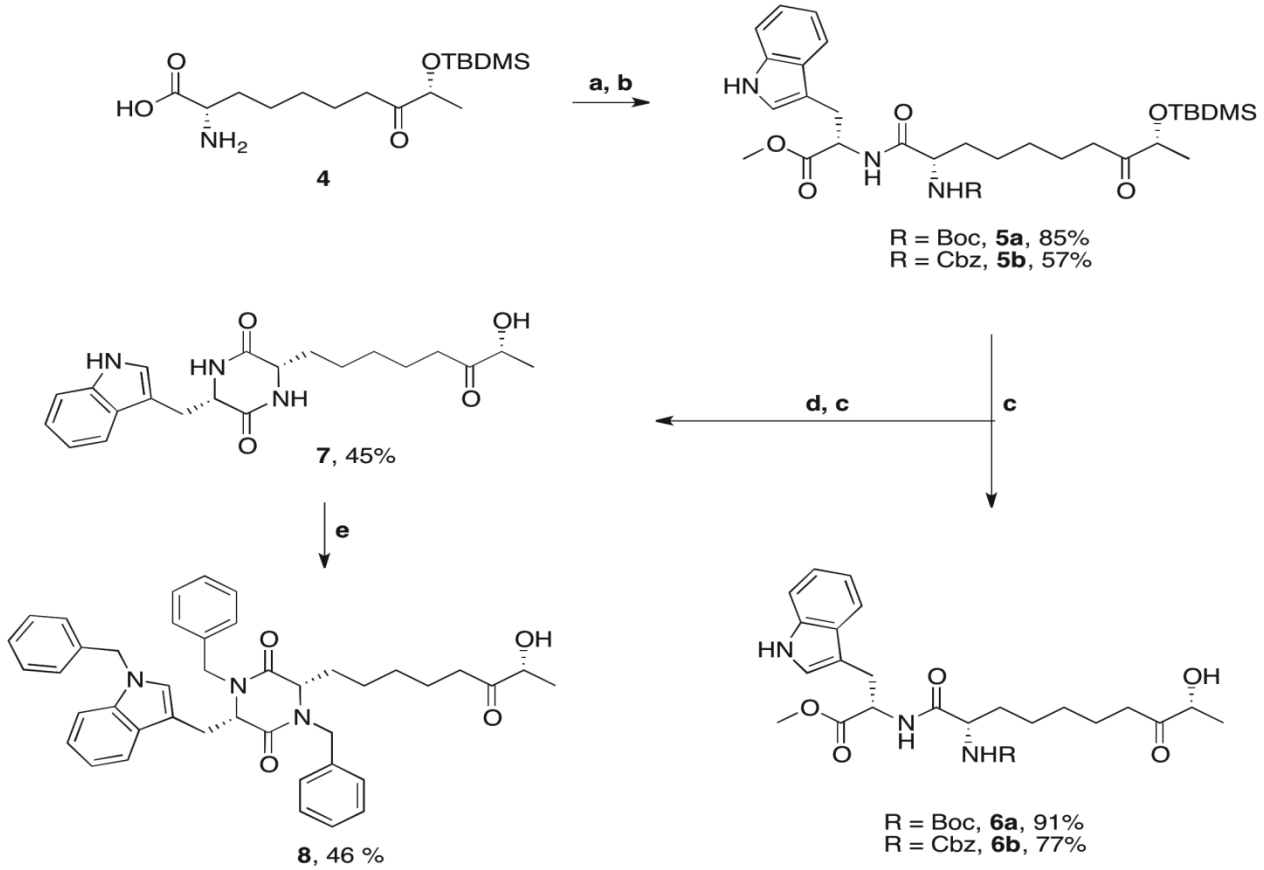


Figure 3. Molecular simplification of FR235222 by tryptophan insertion into linear (dipeptides) and cyclic (diketopiperazines) compounds.

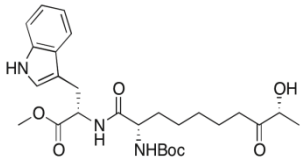
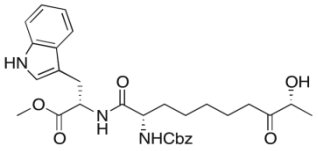
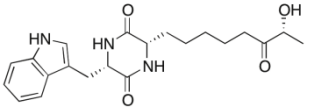
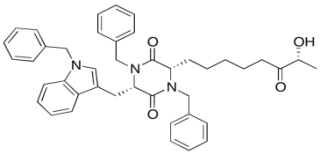


Scheme 1. Synthesis of dipeptides and diketopiperazines.



Reagents and conditions: a) $(Boc)_2O$, Et_3N, THF, r.t., 6h, **5a**; or CbzCl, NaOH, THF, r.t., 2h, **5b**; **b)** L-TrpOMe, DMTMM, NMM, THF, r.t., 12h; **c)** TBAF, THF, r.t., 4h; **d)** $HCOONH_4$, 2-Propanol, Pd/C, MW, 150 °C, 2 min; **e)** BnBr, Cs_2CO_3, DMF, MW, 120 °C, 20 min.

Table 1. Antiproliferative and deacetylation activities in U937 cells of linear **6a-b** and cyclic dipeptides (diketopiperazines **7** and **8**).

Molecular structure	**H4 acetylation** (at 5 mM)	**U937, (IC_{50} μM)** [a]
 **6a**	No acetylation	53
 **6b**	No acetylation	44
 **7**	No acetylation	>100
 **8**	No acetylation	>100