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SHORT COMMUNICATION

Inhibition of Cathepsin D by Tripeptides Containing Statine Analogs

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ABSTRACT. Various analogs of statine, a remarkable amino acid component of the protease inhibitor pepstatine, were synthesized and evaluated as tripeptide derivatives for their activity against cathepsin D and HIV-1 protease. BIOCHEM PHARMACOL **58**;2:329–333, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. statine; inhibitor; cathepsin D; tripeptide; protease; cell proliferation

Inoperable invasive slow-growing tumors are a major challenge in cancer therapy. The proteases produced by cancer cells and involved in extracellular matrix digestion play a well-established and important role in the tumor invasion mechanism [1, 2]. Matrix protease inhibitors can therefore be considered as potential antimetastatic and antitumor therapeutic agents [3]. Thus, some inhibitors of collagenase type metalloproteases are currently under clinical investigation and have yielded promising results [4]. Collagenases are involved in extracellular fiber degradation, but other proteases could well be involved in the degradation of attachment proteins that link the cells to the matrix.

It has been shown, for instance, that the level of the aspartyl protease cathepsin D is consistently more important in some breast invasive tumors than in normal tissues. This overexpression has been correlated to the risk of metastasis in several clinical studies [5–7]. The deregulated overexpression of cathepsin D by transfection in rat tumor cells increases their metastatic potential via a mechanism probably requiring cathepsin D catalytic activity [8]. Moreover, cathepsin D is mitogenic in different cell types, and different substrates which could be maturated by the protease, such as growth inhibitors or precursors of growth factors, are proposed to mediate this activity [9]. Some studies also pointed to the mitogenic activity of the secreted procathepsin D that can be internalized in cancer cells via different membrane receptors [10–12]. This suggests the implication of this protease in the metastatic progression of invasive tumors.

Pepstatine is a natural pentapeptide isolated from various species of *Actinomyces* [13]. It is a good inhibitor of most aspartyl proteases, including cathepsin D, with a K_i in the nanomolar range [14, 15]. Thus, pepstatine (Fig. 1) or its analogs could be good candidates for antiproliferative activity. One of the major problems, however, remains the poor solubility and membrane permeation of pepstatine, as well as its lack of selectivity among aspartyl proteases, which precludes its use as a therapeutic agent. A tremendous amount of work has been done on the design and synthesis of new analogs, with the general aim of improving the bioavailability and selectivity of these compounds. For our part, we have described a new route to statine [16, 17], one of the most interesting amino acid components of pepstatine, which allows the synthesis of a wide variety of analogs. We present in this paper the biological results obtained with a representative family of tripeptides containing statine analogs.

MATERIALS AND METHODS

Synthesis of Statine Analogs and their Incorporation into Tripeptides

The synthetic methods and characterizations described briefly here have been detailed elsewhere [17]. We prepared a series of statine analogs (Fig. 2) with various C-terminal substituents and side chains and with different configurations, starting from isovaleraldehyde or hexanal. The corresponding allylic alcohols obtained via a Grignard reaction were then enantioselectively epoxidized [18, 19]. As it has been described that the hydroxyl function is essential for the activity [20], a blank compound, deprived of this hydroxyl, was prepared by hydrogenation of the allylic alcohol and amination of the resulting saturated alcohol. The epoxy–alcohols were subsequently transformed into the epoxy–azides with inverted configurations [21]. The compounds without the C-carboxyl group were obtained by lithium aluminum hydride (LAH) reduction of the epoxy–azides to the corresponding hydroxy–amines. The cyano compounds were obtained by nucleophilic addition of

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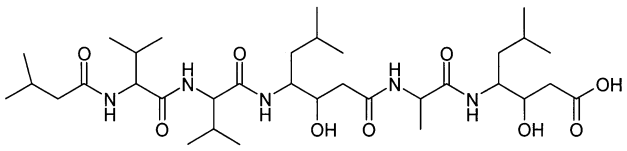

FIG. 1. Structure of pepstatine.

cyanide anion to the corresponding epoxides. The resulting nitriles were then hydrolyzed to the acids and esterified with 2-(trimethylsilyl)ethanol and trimethylchlorosilane. The structures and enantiomeric purity of the compounds were determined by high resolution NMR.

It has been shown previously that tripeptides corresponding to the initial sequence of pepstatine, although less active than the original pentapeptide, could advantageously be used for the preliminary evaluation of new statine derivatives [22]. Therefore, we condensed three different dipeptides with the synthesized amines to give the corresponding tripeptides using PyBOP (benzotriazol-1-yloxy tripyrrolidinophosphonium hexafluoro phosphate) as the activator in a well-established route. The products were then purified by reversed phase HPLC (Fig. 3).

Enzymatic and Biochemical Procedures

Cathepsin D inhibition was evaluated in two ways: 1) The radioactivity of the acid-soluble supernatant of the digestion products of [^{14}C] methemoglobin was measured in the presence of different concentrations of inhibitors [23]; and 2) The difference in optical density observed at 300 nm, after cleavage of the Glu-Phe(NO_2) bond of the 0.1 mM substrate Pro-Thr-Glu-Phe(NO_2)-Arg-Leu by a 47 nM cathepsin D, compared to a blank experiment, was correlated to the percent of inhibition at different concentrations of inhibitors. The same assay method was used with a 100 nM HIV-1 protease which cleaves between Phe(NO_2) and Pro, the 0.4 mM substrate Val-Ser-Gln-Asn-Phe(NO_2)-Pro-Ile-Val, with an optical density increase at 300 nm. The effectiveness of the compounds on cell proliferation was determined on a metastatic and high cathepsin D-expressing cell line MDA-MB-231 after 6 days in 5% fetal calf serum by measuring DNA synthesis as previously described [9]. Detection of the effects of the cathepsin D inhibitors was made possible by using the lowest serum concentration supporting maximal proliferation.

RESULTS AND DISCUSSION

The analogs of statine were condensed with the C-terminal side of three different *N*-carbobenzyloxydipeptides: *N*-CBZ-Val-Val (**8**, **9**, **10**, **15**, **18**, **21**); *N*-CBZ-Val-Phe (**11**, **12**, **16**, **19**); and *N*-CBZ-Val-Trp (**13**, **14**, **17**, **20**). The resulting tripeptides therefore included three distinctive types of derivatives:

—A blank compound (**8**), lacking the hydroxyl function on the statine analog;

—Analogs with a linear alkyl side chain, a deoxy termination, and of different stereochemistry **9**, **11**, **13**, (*S*, *S*) and **10**, **12**, **14** (*R*, *R*);

—Analogs bearing the isopropyl side chain of statine with different functions on the terminal side: **15**, **16**, **17** (deoxy termination); **18**, **19**, **20** (cyano terminal group); and **21** (ester group).

The first results of inhibition on cathepsin D were obtained for compounds **8**, **9**, **15**, and **21** (Fig. 4). [^{14}C] Methemoglobin was used as a substrate with different concentrations of inhibitors. As expected, compound **8** was not active and thus could be used as a blank in the following experiments. Three different analogs of statine incorporated into CBZ-Val-Val dipeptides **9**, **15**, and **21** gave the same activity. Then, the antiproliferative properties of compounds **8**, **9**, **15**, and **18** were tested on the growth of MDA/MB231 cancer cells. Again compound **8** was inactive, while compounds **9** and **18** showed significant activity (Fig. 5). Compound **15** appeared inactive whereas its inhibitory

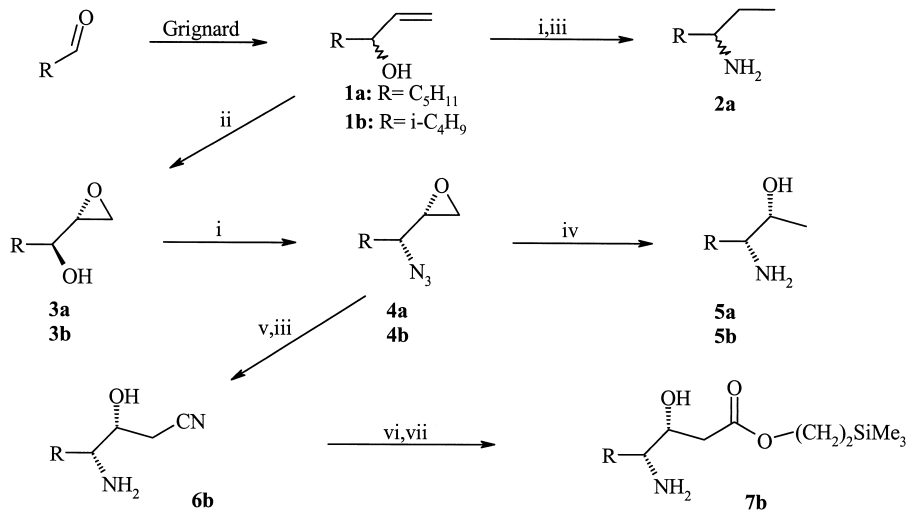

FIG. 2. (i) N_3H, DEAD, Ph_3P; (ii) Ti(IV)OiPr$_4$, D- or L-diisopropyl tartrate, tBHP; (iii) H_2, Pd/C; (iv) Li-AlH$_4$, diethyl ether; (v) KCN, EtOH; (vi) Na_2O, tBuOH, H_2O; (vii) 2-(trimethylsilyl) ethanol, trimethylchlorosilane.

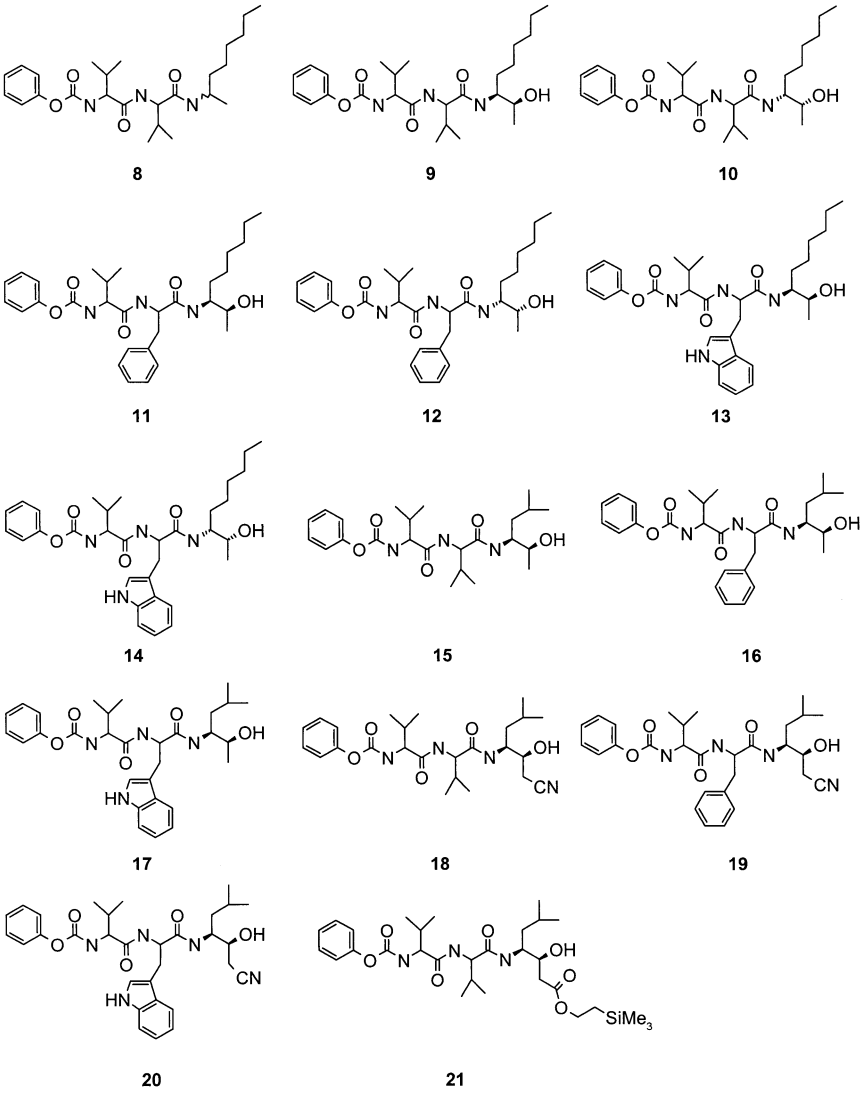

FIG. 3. Structure of the different peptides prepared for the evaluation of the synthesized statine derivatives.

activity on methemoglobin was similar to that of compounds **9** and **18**. This suggests differences in the bioavailability of these compounds. The fact that pepstatine was inactive at these concentrations (data not shown) also confirmed its well-known difficulty in penetrating into intact cells.

A systematic evaluation of the inhibitory potencies of all the compounds synthesized was made on cathepsin D and

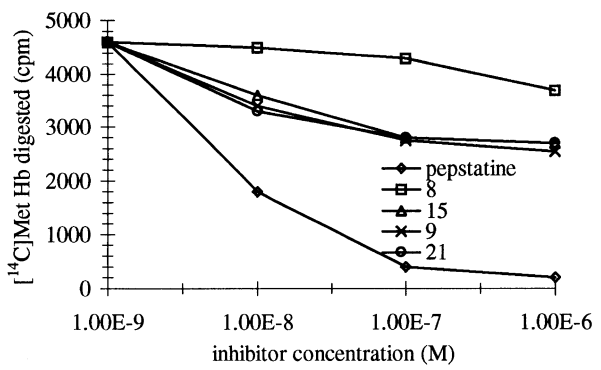

FIG. 4. Inhibition of cathepsin D-induced proteolysis of methemoglobin *in vitro* by statine derivatives.

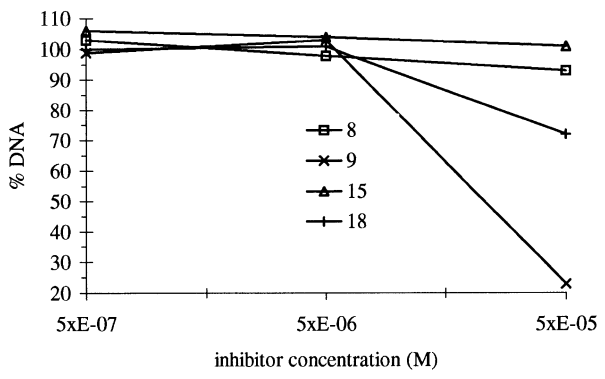

FIG. 5. Antiproliferative activities of statine derivatives on MDA-MB-231 breast cancer cells.

TABLE 1. Inhibition (%) of cathepsin D and HIV protease I by the tripeptides containing statine derivatives

Compound (mM)	Cathepsin D inhibition (%)	HIV protease inhibition (%)
V-V analogs		
8 10^{-5}	20	9
10^{-7}	ND	
9 10^{-5}	91	24
(*S-S*) 10^{-7}	25	
15 10^{-5}	99	22
(*S-S*) 10^{-7}	31	
18 10^{-5}	99	11
(*S-S*) 10^{-7}	32	
21 10^{-5}	99	8
(*S-S*) 10^{-7}	28	
V-F analogs		
11 10^{-5}	93	12
(*S-S*) 10^{-7}	13	
12 10^{-5}	<4	8
(*R-R*) 10^{-7}	ND	
16 10^{-5}	98	22
(*S-S*) 10^{-7}	17	
19 10^{-5}	98	17
(*S-S*) 10^{-7}	24	
V-W analogs		
13 10^{-5}	94	13
(*S-S*) 10^{-7}	6	
14 10^{-5}	20	13
(*R-R*) 10^{-7}	ND	
17 10^{-5}	97	11
(*S-S*) 10^{-7}	9	
20 10^{-5}	95	
(*S-S*) 10^{-7}	23	

Valine-valine V-V; valine-phenylalanine V-F; valine tryptophane V-W. ND, not determined.

HIV protease using the synthetic peptide as substrate. These results (Table 1) confirmed the stereoselectivity of the inhibition (compounds **11–13**, [*S-S*]; **12–14**, [*R-R*]) and showed the influence of the amino acid residue next to the statine derivative (**9-15-18**, Val; **11-16-19**, Phe; and **13-17-20**, Trp). The Val-Val sequence corresponding to pepstatine (**9-15-18-21**) was the most active of the three series synthesized with different statine analogs, exemplified by an IC_{50} of 480 nM for **9** (Val-Val containing tripeptide) compared to 2.8 μM for **17** (Val-Trp containing tripeptide). All the compounds tested were 100 times less inhibitory to HIV-1 protease than to cathepsin D, thus displaying interesting selectivity properties.

The tripeptide derivatives of the compounds described showed good inhibitory property and interesting selectivity with cathepsin D compared to another aspartyl protease, the HIV protease. Furthermore, significant effectiveness against cancer cell proliferation at relatively high concentrations (50 μM) was evidenced. These concentrations appeared at least 50-fold higher than those inhibiting cathepsin D-induced proteolysis *in vitro*. Although a non-specific effect cannot be excluded at such high concentrations, this could suggest that cellular uptake of these compounds remains a limiting factor in their action, and thus improvement in their membrane permeation should be considered. On the other hand, since tripeptides are actively absorbed through the transepithelial barrier of the gastrointestinal track, these new products could be promising as orally absorbed inhibitors of extracellular cathepsin D and as such for the therapy of some invasive tumors and metastases or for inflammation treatment.

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