Inhibition of proprotein convertases-1, -7 and furin by diterpines of *Andrographis paniculata* and their succinoyl esters

Ajoy BASAK^{*1,2}, Sam COOPER[†], André G. ROBERGE[†], Upen K. BANIK[‡], Michel CHRÉTIEN^{§1} and Nabil G. SEIDAH^{||}

^{*}Laboratories of Structure and Metabolism of Neuropeptides, Clinical Research Institute of Montréal (Affiliated to the University of Montréal), 110 Pine Avenue West, Montréal, Qué H2W 1R7, Canada, [†]INRS-Santé, 245 boul. Hymus, Pointe Claire, Qué H9R 1G6, Canada, [‡]Bioscan Continental Inc, Ste-Eustache, Qué J7R 5R4, Canada, [§]Molecular Neuroendocrinology, Clinical Research Institute of Montréal (Affiliated to the University of Montréal), 110 Pine Avenue West, Montréal, Qué H2W 1R7, Canada, and ^{||}Biochemical Neuroendocrinology, Clinical Research Institute of Montréal (Affiliated to the University of Montréal), 110 Pine Avenue West, Montréal, Qué H2W 1R7, Canada

Studies were performed to investigate the prohormone/protein convertase (PC)-inhibitory properties of chemical constituents of the medicinally active plant *Andrographis paniculata* (AP; from the family Acanthaceae), also known as 'King of Bitters'. Among the individual components tested against the clinically important convertases, furin and PC1, neoandrographolide (a C₃ O-glucoside derivative of the major constituent andrographolide) exhibited the highest inhibitory action with an IC₅₀ of 53.5 µM against furin. The data further revealed that although andrographolide, the major bitter principle of AP, exhibited a relatively small enzyme inhibition (IC₅₀ = 1.0 mM and K_i = 200 µM against furin), upon succinoylation, its inhibitory action against the above convertases was enhanced significantly with a K_i in the low micromolar range (< 30 µM), suggesting that a specific structural modification of the andro-

grapholide skeleton may be exploited to develop a new class of non-peptide inhibitors of PCs. When tested against PC7, these succinoylated derivatives of andrographolide also displayed strong inhibitory action, with K_i values again in the low micromolar range. This potentially interesting observation may be attributed to the reported anti-HIV property of 14-dehydroandrographolide succinic acid monoester (DASM). It is suggested here that DASM, by virtue of this protease inhibitory property, possibly acts by suppressing the proteolytic cleavage of envelope glycoprotein gp160 of HIV, which is known to be PC-mediated, particularly by furin and PC7.

Key words: antiviral property, inhibition constant, labdane diterpines, protease inhibitor.

INTRODUCTION

Intracellular endoproteolytic processing at specific sites (usually C-terminal of a pair of basic amino acids, such as Lys-Arg or Arg-Arg) is a common post-translational modification of membrane and secretory proteins, which are synthesized initially as inactive polypeptides or proproteins. These proteins include a wide range of hormones, neuropeptides, growth factors, coagulation factors, cell-surface receptor proteins and adhesion molecules, as well as the surface viral glycoproteins [1]. The long quest for the processing enzyme(s) of these proproteins culminated recently in the discovery and molecular characterization of a family of bacterial subtilisin/yeast kexin-like enzymes, collectively known as proprotein/prohormone convertases (PCs; reviewed in [1–3]). To date, seven members of this family have been identified, namely PC1/PC3, PC2, furin, paired-amino-acid-cleaving enzyme (PACE)⁴, PC4, PC5/PC6 and PC7. On the basis of the distribution among tissues, co-localization and studies *in vitro* and *in vivo*, a number of these convertases are implicated in many disease states and vital functions [4–6]; for example, PC5 is involved in atherosclerosis [6], PC1 in obesity [6–8], furin in viral infection [6] and embryogenesis [9], PC4 in fertilization [10], PACE4 and PC7 in breast cancer [4,6,11], and

PC7, PC5 and furin in Alzheimer's disease, hypertension and neoplasia [6]. These findings suggest that PCs are important therapeutic targets. Many studies *in vitro* have already indicated that furin-specific inhibitors, such as α₁-PDX (a double arginine mutant of α₁-antitrypsin Pittsburgh [12,13] at positions 355 and 358), are able to block significantly the processing of viral glycoprotein gp160 of HIV mediated by furin [14–16] and, consequently, its membrane fusogenic properties [12].

In the light of these studies, we became interested in the development of specific inhibitors of PCs. Our efforts had already yielded several peptide-based inhibitors [17–22] for PC1 and furin. During the development of these peptide inhibitors, we realized their limitations, owing to their proteolytic instability and poor membrane permeance. We then started looking for non-peptide inhibitors from natural sources, particularly from the medicinal plants, which in the past had provided a large number of therapeutic agents. One plant, *Andrographis paniculata* (AP, from the family Acanthaceae; also known as 'King of Bitters' or 'Rice Bitters') [23], has especially attracted our attention because of its widespread use in south-east Asian and east Indian countries in a variety of human illnesses. These range from acute hepatitis, bacillary dysentery, digestive ailments with infectious conditions, such as bacterial diarrhoea, meningitis,

Abbreviations used: AP, *Andrographis paniculata*; DASM, dehydroandrographolide succinic acid monoester; PACE, paired-amino-acid-cleaving enzyme; PC, prohormone/protein convertase; pGlu-Arg-Thr-Lys-Arg-MCA, L-pyroglutamyl-L-arginyl-L-threonyl-L-lysyl-L-arginyl-4-methyl-7-coumarinamide; RP, reverse phase; R_t, retention time; SEA, succinoyl ester of andrographolide.

¹ Present address: Loeb Health Research Institute, Ottawa Civic Hospital, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4K9

² To whom correspondence should be addressed (e-mail Basaka@ircm.umontreal.ca).

choriocarcinoma (a form of cancer), dyspepsia, influenza, malaria, filaria, and many other acute inflammatory conditions [24–34]. Previous studies have indicated the presence of andrographolide (the major bitter principle) [35], a diterpine of the labdane family, along with its 19-O-glucoside derivative, called andrographiside [35]. A number of related diterpines and some flavonoids were also isolated from AP as minor constituents [36–42]. In 1988, Chang and Yeung [43] and, later, Yao et al. [44] reported *in vitro* anti-viral properties of a crude extract of AP towards HIV. It was further revealed that dehydroandrographolide succinic acid monoester (DASM), prepared from andrographolide, exhibited vastly improved anti-HIV properties [45], at concentrations ranging from 50–200 µg/ml. DASM interfered with the development of HIV by a mechanism other than induction with interferon. It was noted that it did not inactivate cellular HIV or inhibit reverse transcriptase activity, but it partly interfered with both the HIV-induced cell fusion and binding of HIV to the T-helper-cell line (H9), and in phytohaemagglutinin-activated blood mononuclear cells from HIV-seronegative persons. Furthermore, it also partly inhibited HIV replication after the virus had become attached to cell receptors [45]. Overall, DASM exerts its suppressive effect on HIV growth by interference with both the binding of virions to cells and a step in the viral replication cycle subsequent to virus–cell binding. However, its effect on the processing of viral glycoprotein gp160 was never investigated. We envisage that DASM might interfere in this processing, which is known to be mediated by PCs such as furin and/or PC7 [12,15,46]. This prompted us to examine the possibility of inherent PC-inhibitory properties in DASM and andrographolide-related substances from AP. In the present study, we present data which demonstrate that andrographolide-related labdane diterpines possess PC1-, PC7- and furin-inhibitory properties that are enhanced significantly upon the selective formation of derivatives, such as succinoylated ones.

MATERIALS AND METHODS

Materials

A number of chemical constituents of AP used in the present study, namely andrographolide, 14-deoxy-11,12-didehydroandrographolide, neoandrographolide and 14-deoxyandrographolide, were generously given by Professor J. D. Connolly (Department of Chemistry, University of Glasgow, Scotland, U.K.). Andrographolide was also purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.), whereas andrographolide triacetate, phlogantholide (2 α -hydroxy-3-deoxyandrographolide-19-galactoside), its triacetate and 2-O-galactoside derivatives were gifts from Professor A. K. Barua (Bose Institute, Calcutta, India).

The fluorogenic substrate L-pyroglutamyl-L-arginyl-L-threonyl-L-lysyl-L-arginyl-4-methyl-7-coumarinamide (pGlu-Arg-Thr-Lys-Arg-MCA) was purchased from Peptides International (Louisville, KY, U.S.A.). Reverse-phase (RP)-HPLC was performed using a CSC-Exsil analytical C₁₈ column (25 cm $\times$ 0.46 cm; Chromatography Specialty Corp., St-Laurent, PQ, Canada). The buffer system consisted of an aq. 0.1% (v/v) trifluoroacetic acid (TFA) solution and an organic phase of CH₃CN, also containing 0.1% (v/v) TFA. The elution was performed by using a linear gradient from 5–60% organic phase in 60 min, following a 5-min isocratic-gradient step at 5% organic phase; the flow was adjusted to 1 ml/min. The separation was monitored by measuring UV absorbance at 225 nm. ¹H-NMR spectra (in deuterated DMSO-D₆) and mass spectra were recorded on Varian Associate Bruker 400 MHz and MS-50 HMTCTA (chemical-ionization mode) instruments respectively.

Sources of convertases

The endoproteases used in the present study, human (h)PC1 and hFurin, were obtained from the medium of the GH4C1 cell line, following infection with respective recombinant vaccinia virus [17,18], whereas recombinant hPC7 was obtained by following a procedure reported previously [47]. Enzymically active mouse (m)PC1 was recovered from the medium of baculovirus-infected Sf9 insect cell lines (A. Boudreault, N. G. Seidah, M. Chrétien and C. Lazure, unpublished work). The enzymic activity of PC1, PC7 and furin was measured by fluorimetric assay, as described previously [18,47].

Preparation of succinoyl ester of andrographolide (SEA)

The conditions used were essentially the same as those described previously for the preparation of DASM [45]. Briefly, andrographolide (500 mg; 1.43 mmol) was dissolved in pyridine (5 ml). Succinic anhydride (450 mg; 4.5 mmol) was added and the mixture was stirred at ambient temperature for 20 h. Solvent was removed under pump and the residue obtained was washed extensively with ether. The ether-insoluble material was mixed twice with water (50 ml fractions) and filtered. The filtrate was then freeze-dried to yield crude SEA as a powder (568 mg; 70% yield).

Purification of crude SEA

Crude SEA (20 mg) was dissolved in DMSO (1 ml) and then diluted with water to a final concentration DMSO of 10%. RP-HPLC was performed under the conditions described above, except that a flow rate of 2 ml/min with a semi-preparative C₁₈ column (25 cm $\times$ 1.0 cm; Chromatography Specialty Corp.) was used. Peaks (five in all, including the unreacted andrographolide) were collected, freeze-dried and characterized by both MS and ¹H-NMR spectral analyses.

Analysis of fractions

Note that the absolute configurations given below at the C-14 position in SEA-1 and SEA-2 could not be fully established and are therefore tentatively assigned as 14 α and 14 β for fractions 1 and 2 respectively. The value for the retention time (*R*_t) representative of the C₁₈ semi-preparative column is given within parentheses, whereas the other value refers to time on a C₁₈ analytical column. The peak intensities of the signals (relative to each other) in mass spectral data are characterized as follows: vw, very weak; w, weak; ms, medium; s, strong; vs, very strong.

Fraction-1 (SEA-1)

For andrographolide 3 α ,14 α ,19-*O*-trisuccinate, *R*_t = 40.16 (33.9) min. MS analysis: *m/z* 651 (vw; *M* + *H*)⁺, 633 (s; *M* + *H* – H₂O)⁺, 615 (ms; *M* + *H* – 2H₂O)⁺, 519 (ms; *M* + *H* – CH₂OCOCH₂CH₂COOH)⁺. For ¹H-NMR analysis, (i) CH₃ groups: δ 0.76 (3 H) 1.18 (3 H); (ii) ring CH/CH₂ groups: δ 1.20–1.75 (7 H); (iii) allylic H atoms: δ 1.98 (1 H), 2.01 (1 H), 2.46 (1 H), 2.52 (1 H) and 2.59 (1 H); (iv) lactonyl CH₂ groups: 3.96 (1 H) and 3.36 (1 H); (v) carbinol H atoms: δ 4.14 (1 H), 4.51 (2 H); (vi) succinoyl CH₂ groups: δ 1.80–2.35 (12 H); (vii) olefinic H atoms: δ 4.70 (1 H), 4.90 (1 H) and 6.74 (1 H), 4.9 (1 H) and 4.7 (1 H).

Fraction-2 (SEA-2)

For andrographolide 3 α ,14 β ,19-*O*-trisuccinate, *R*_t = 41.59 (34.95) min. MS analysis: *m/z* 651 (vw; *M* + *H*)⁺, 633 (vs;

$M+H-H_2O)^+$, 615 (ms; $M+H-2H_2O)^+$, 519 (w, ms; $M+H-CH_2OCOCH_2CH_2COOH)^+$. 1H -NMR data are inconsistent with this structure.

Fraction-3 (SEA-3)

For unreacted andrographolide, $R_t = 42.06$ (36.0) min. MS analysis: m/z 351 ($M+H$) $^+$. 1H -NMR data are in agreement with its structure.

Fraction-4 (SEA-4)

For monopyridinium salt of andrographolide $3\alpha,14\alpha/\beta,19-O$ -trisuccinate, $R_t = 42.8$ (36.6) min, MS analysis: m/z 731 (vw; $M+H$) $^+$, 637 (vs; $M+H-Py-CH_3$) $^+$, 622 ($M+H-Py-2CH_3$) $^+$. 1H -NMR data are in agreement with its structure.

Fraction-5 (SEA-5)

For DASM, $R_t = 50.99$ (44.0) min. MS analysis: m/z 533 (vs, $M+H$) $^+$. 1H -NMR data are inconsistent with its structure.

Enzyme-inhibition studies

Determination of kinetic parameters, K_i and IC_{50} values

The kinetic parameters K_i and IC_{50} were determined according to the procedure described previously [17,18]. For determination of IC_{50} values, inhibitor concentrations were varied over a range wide enough to generate a residual protease activity of 20–80 % of the original. K_i values were derived from Dixon and Cornish-Bowden plots, using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-MCA at 15 μM and 45 μM concentrations, or calculated from the IC_{50} values by using the equation $K_i = IC_{50}/(1 + [S]/K_m)$, applicable for reversible competitive inhibition [24], where K_m is the Michaelis–Menten constant.

RP-HPLC of some AP constituents and their derivatives

For RP-HPLC analysis of all materials, including several components of AP, such as andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographolide, 14-deoxyandrographolide, phlogantholide, phlogantholide triacetate and phlogantholide glucoside, the samples were dissolved in DMSO and then diluted with an appropriate volume of water to give a final concentration of 1.0 $\mu g/\mu l$ in 10 % (v/v) DMSO. HPLC was performed on 20 μl of each stock solution, using the conditions as described above.

RESULTS

Effect of individual components of AP towards furin, PC1 and PC7 activity

A number of individual components of AP, including andrographolide (the major constituent), were examined for any potential inhibitory response towards the proteolytic activities of clinically important PCs, i.e. furin, PC1 and, in some cases, PC7. Some of these compounds, which share a common diterpine labdane skeleton [35,36], showed a weak-to-moderate inhibition of these convertases when measured against pGlu-Arg-Thr-Lys-MCA substrate, with IC_{50} values ranging from the low micromolar to the millimolar level (Table 1). Among the compounds tested, neoandrographolide was found to be the most potent furin inhibitor, with a measured IC_{50} value of 53.5 μM . This is nearly 20-fold more potent than andrographolide ($IC_{50} = 1.0$ mM and $K_i = 200$ μM against furin). However, andrographolide inhibits hPC1 ($IC_{50} = 750$ μM) more strongly than

Table 1 Comparison of the ability of various labdane diterpines isolated from AP to inactivate PC1 and furin

IC_{50} values, shown here as averages, were determined either in duplicate or in triplicate according to the procedure described in the Materials and methods section using pGlu-Arg-Thr-Lys-Arg-MCA at a final concentration of 100 μM . a, b and c denote 11%, 10% and 18% inhibition respectively at 400 μM concentration of the inhibitor.

List of compounds	IC_{50}	
	Furin	PC1
Andrographolide	1.0 mM	750 μM
Andrographolide triacetate	> 2.0 mM	a
Neoandrographolide (14-deoxyandrographiside)	53.5 μM	—
Dehydroandrographolide	> 4.0 mM	—
14-Deoxyandrographolide	634 μM	205 μM
Phlogantholide	368 μM	b
Phlogantholide galactoside acetate	521 μM	c

Figure 1 Chemical structures of andrographolide, DASM and various SEA fractions

Structure **V** shown here for SEA-4 is one out of several possibilities, and the configurations of the 14-succinoyl group in SEA-1 (**III**) and SEA-2 (**IV**) are only tentatively assigned.

furin (Table 1). To date, to our knowledge it represents the first non-peptide inhibitor of PCs, and could be considered as an important precursor structure that might require further structural modification for enhancement of its enzyme-inactivation

Figure 2 Stop-time assay showing the gradual decrease in the proteolytic activity of hPC1 upon prior incubation with increasing amounts of crude SEA

The assay was performed using the fluorogenic peptide, pGlu-Arg-Thr-Lys-Arg-MCA (100 μ M) in 50 mM NaOAc/5 mM CaCl₂, pH 6.0 (see the Materials and methods section).

property. It should be emphasized that, unlike most other constituents of AP, neoandrographolide possesses a unique C₃-*O*-glucosyl moiety, that perhaps could explain its increased level of furin inactivation. This observation indicates that the specific derivative formation of the C₃-OH group, such as glycosylation in this case, might lead to a molecule with a greater capacity to inhibit furin. In fact, as demonstrated below, succinylation of this group also improves its PC1- and furin-inhibitory properties to a significant extent. In comparison, PC7 is inhibited relatively weakly by the components of AP, including neoandrographolide (IC₅₀ ranging from 500 μ M to 2 mM; results not shown).

Succinylation of andrographolide

In 1991, Chang et al. [45] reported that, upon treatment with succinic anhydride, andrographolide yielded DASM, which showed potent anti-viral properties against HIV-1 and HIV-2. It was assumed that DASM was a mixture of two isomeric esters (structures **IIa** and **IIb**), as shown in Figure 1. However, no attempts were made to purify and characterize the individual components of DASM. When we repeated the reaction (see the Materials and methods section), we obtained a crude material called SEA. Its mass spectrum indicated the presence of a mixture of mainly trisuccinoyl ester of andrographolide (M_r = 651), in addition to small amounts of monosuccinate (M_r = 451), disuccinate (M_r = 551) and 14-dehydrodisuccinate (M_r = 533). RP-HPLC of crude SEA (results not shown) yielded four major fractions (SEA-1, SEA-2, SEA-4 and SEA-5), in addition to the unreacted andrographolide (SEA-3), as confirmed by HPLC and MS with an authentic sample. The four new fractions, SEA-1, SEA-2, SEA-4 and SEA-5, were identified by MS and ¹H-NMR spectroscopic analyses as being respectively: andrographolide-3,14 α ,19-trisuccinate [**III**, R_t = 40.16 min, m/z 651 ($M+H$)⁺]; andrographolide-3,14 β ,19-trisuccinate [**IV**, R_t = 41.59 min, m/z 651 ($M+H$)⁺], monopyridinium salt of andrographolide-3,14 α -

Figure 3 Progress curve showing the dose-dependent inactivation of mPC1 by crude SEA

For further details, see the Materials and methods section.

/ β -19-*O*-trisuccinate [shown as **V**, out of several possible structures; R_t = 42.8 min, m/z 731 ($M+H$)⁺] and 14-dehydroandrographolide 3,19-disuccinate [**VI**, R_t = 50.99 min, m/z 533 ($M+H$)⁺], where the configurations at the C-14 position of structures **III** and **IV** (α and β respectively) are only tentatively assigned. Therefore, in the present study, the major product of succinylation of andrographolide was 3,14,19-tri-*O*-succinate, whereas the β -elimination of the 14-hydroxy group occurred only as a minor side reaction, in contrast with that reported by Chang et al. [45], in which a complete elimination of this hydroxy group of DASM was observed.

Inhibition of PC1, PC7 and furin by crude SEA

The amidolytic activity of h/mPC1, hPC7 and hfurin, as measured with the fluorogenic substrate, pGlu-Arg-Thr-Lys-Arg-MCA, were all inhibited relatively strongly by crude SEA in a concentration-dependent manner, as shown in Figure 2 for hPC1 using a stop-time assay and in Figure 3 for mPC1, using an initial-rate assay. SEA inhibited PC1 (IC₅₀ = 150 μ M for mPC1 and 50 μ M for hPC1) much more strongly than andrographolide itself (IC₅₀ = 750 μ M with hPC1) under identical conditions. This observation reinforced the notion that the formation of hydroxy group derivatives of andrographolide enhances its inhibitory potency towards PCs. As shown in Figure 3, purified baculovirus-derived mPC1 was gradually inhibited by treatment with increasing amounts of crude SEA. The observed 3-fold difference in the degree of inhibition of hPC1 and mPC1 by SEA might be explained by the fact that there are varying levels of 85 and 66 kDa forms of PC1 in the two enzyme preparations, which were derived from two different cell lines. It should be pointed out that the 66 kDa form is enzymically more active than the 85 kDa form [48], and therefore the IC₅₀ value is dependent upon the relative amounts of the two forms present.

Table 2 Comparison of the ability of various fractions of SEAs to inactivate PC1 and furin

The fraction SEA-3 was identified as unreacted andrographolide, and hence was not included in this table. K_i values were computed by Dixon plotting using two concentrations of pGlu-Arg-Thr-Lys-Arg-MCA substrate, namely 15 and 45 μM (see the Materials and methods section). Each study was performed in duplicate, and the results were averaged. ND, not determined.

Compound	K_i (μM)		
	hPC1	hfurin	hPC7
Crude SEA	50.0 $\pm$ 1.5	42 $\pm$ 2.2	38.6 $\pm$ 2.6
SEA-1 (andrographolide 3 α ,14 α ,19- <i>O</i> -trisuccinate)	18.4 $\pm$ 0.9	64.7 $\pm$ 3.3	ND
SEA-2 (andrographolide 3 α ,14 β ,19- <i>O</i> -trisuccinate)	10.1 $\pm$ 0.6	36.3 $\pm$ 2.1	36 $\pm$ 1.0
SEA-4 (monopyridinium andrographolide 3 α ,14 α / β ,19- <i>O</i> -trisuccinate)	21.9 $\pm$ 1.1	2.6 $\pm$ 0.1	26 $\pm$ 1.9
SEA-5 (14-dehydroandrographolide 3,19- <i>O</i> -disuccinoyl monoester)	23.4 $\pm$ 1.5	20.0 $\pm$ 1.5	30.4 $\pm$ 1.2

Figure 4 Dixon plot showing the inactivation of proteolytic activity of hPC1 by various isolated fractions of SEA

Upper left panel: SEA-1; upper right: SEA-2; bottom left: SEA-4; bottom right: SEA-5. The activity of PC1 was followed at two different substrate (pGlu-Arg-Thr-Lys-Arg-MCA) concentrations for each inhibition study: namely, 15 μM (S1) and 45 μM (S2).

Further studies by us have indicated that crude SEA is also capable of inhibiting PC7, another clinically important convertase. In fact, we found that crude SEA inhibited hPC7 with a measured K_i of 38 μM compared with 50 μM and 42 μM for hPC1 and hfurin respectively (Table 2). Thus it seems that, as an inhibitor, SEA is not very selective towards any particular member of the PC family.

K_i values of various purified fractions of SEA against convertases

All four succinoyl derivatives of andrographolide (SEA-1, -2, -4 and -5) were capable of inhibiting PC1, PC7 and furin activity,

as measured with pGlu-Arg-Thr-Lys-Arg-MCA as the substrate. This is clearly demonstrated by the Dixon plots in Figure 4, showing the inactivation of hPC1 by various purified SEA fractions using two different substrate concentrations, namely 15 μM (S1) and 45 μM (S2). K_i values for these derivatives were calculated to range from 10 to 24 μM . Among them, SEA-2 (IV, andrographolide 3,14 β ,19-trisuccinate), possessed the greatest inhibitory action, with a K_i of 10.1 μM against hPC1. The Dixon plots also confirmed the reversible competitive nature of this inhibition. As shown in Table 2, the individual fractions of SEA displayed a much greater selectivity for inhibition of furin, as compared with PC1 and PC7. In particular, SEA-4 (V; as a representative structure, monopyridinium salt of andrographolide 3 α ,14 α / β ,19-*O*-trisuccinate), demonstrated a high degree of potency towards furin inhibition, with $K_i = 2.6$ μM . In fact, SEA-4 is a 9–10-fold more potent inhibitor of hfurin than either hPC1 or hPC7. Seemingly, the presence of a positively charged pyridine ring in one of the three succinic acid functions (3 α , 14 α / β or 19) of SEA-4 contributes significantly towards the enhancement of the furin-inhibitory property. It should be mentioned that, at this stage, the precise location of this pyridinium ring in SEA-4 is not known. With the exception of SEA-4, all other fractions of SEA, as well as crude SEA material, display hardly any significant specificity, in terms of enzyme inhibition towards any of the three convertases tested to date.

DISCUSSION

Our studies revealed that, among the various compounds of AP tested, neoandrographolide, a C_3 -diterpine glucoside, possesses the highest inhibitory action against PCs, particularly furin. This is the first report of inactivation of PCs by diterpines although, previously, a limited number of polycyclic triterpines (C_{30} atoms), such as ursolic acid, was shown to inhibit the proteolytic activity of human leucocyte elastase [49]. Our data further revealed that although andrographolide itself, the major constituent of AP, exhibited only a relatively weak inhibitory action towards PC1 and furin, this property was significantly enhanced upon the formation of the hydroxy group derivatives via succinoylation. This observation provided the first scientific rationale for the reported anti-viral properties of crude extract of AP and, in particular, DASM, a crude mixture of succinoylated derivatives of dehydroandrographolide, towards HIV-1 and HIV-2 [43–45]. As explained previously [12,14], viral infection requires interaction of viral-coat glycoproteins, such as gp160 in the case of HIV, with proteins at the plasma membrane of susceptible cells. Some of these glycoproteins need to be processed proteolytically in order to be able to mediate viral infectivity. There is now

sufficient experimental evidence to suggest that this processing is mediated by cellular proteases at sites (Arg-Glu-Lys-Arg) for gp160, compatible with the cleavage specificity of furin [9,12] and PC7 [47]. This post-translational cleavage is one of several events that determine the viral infectivity and virulence. Previously, it was demonstrated by studies both *in vivo* and *in vitro* that furin- and PC7-directed inhibitors were able to block significantly the proteolytic processing of gp160 [12–14], and therefore these displayed anti-viral properties towards HIV. Likewise, it may be argued that the SEAs, succinoylated derivatives of andrographolide, which display powerful inhibitory properties towards PCs, particularly furin, are equally capable of preventing the furin-mediated processing of gp160 and therefore display anti-HIV properties. In fact, preliminary studies *in vitro* performed in our laboratory seem to suggest that administration of crude SEA is not only capable of partially preventing the formation of gp120 and gp41 from gp160 (E. Decroly, A. Basak and N. G. Seidah, unpublished work), but might also have effects on the processing of other protein precursors.

Our observation that formation of the free hydroxy group derivatives of andrographolide significantly enhanced its ability to inhibit convertase activity (compare SEAs versus andrographolide) has now provided an opportunity for the development of a new class of PC inhibitory substances on the basis of andrographolide's structure. This could be accomplished by various means of modification of hydroxy groups, such as palmitoylation, farnesylation, decanoylation, silylation, alkylation, etc. Some of these modifications are also feasible within the cell membrane via the action of appropriate enzymes. The actual mechanism by which these diterpines exert their inhibitory action against convertases is not currently understood; however, it should be emphasized that these molecules contain a highly reactive 5-membered lactone ring, like the β -lactam antibiotics, which are well-known inhibitors of serine proteases of the elastase family [50]. Studies are now in progress to determine both the molecular modelling structure of diterpines of the labdane family and their possible role(s) in terms of binding to convertases.

Conclusion

In conclusion, this study has revealed that diterpines of the labdane family could represent an interesting class of non-peptide inhibitors of PCs, namely PC1, PC7 and furin. To our knowledge, this is the first report of protease-inhibitory properties of diterpines (C_{20} compounds). Though relatively modest in their action, it might be possible to enhance their inhibitory properties by further structural modification. This study has now provided a scientific rationale for the reported anti-viral properties of AP constituents, and particularly some of the synthetic derivatives. Therefore natural products with or without chemical modification might offer a great potential for the future development of new lead compounds as PC inhibitors.

We thank Dr. M. Evans, Chemistry Department, Montréal University, Canada, and Dr. K. Carpenter, Clinical Research Institute of Montréal (IRCM), Canada, for recording the mass and NMR spectra respectively. Thanks are also due to D. Gauthier, D. Savaria and A. Lemieux, of the Clinical Research Institute for technical assistance. We also thank Dr. Jon Scott Munzer and Mei Zhong for the recombinant PC7. We acknowledge the support from Dr. C. Lazure, Director of the Laboratory of Structure and Metabolism of Neuropeptides, Clinical Research Institute. Finally, we thank the following persons for their interest in the work: Dr. J. Archambault, Université de Trois-Rivières and Université de Montréal, Dr. J. Langlais, Montréal General Hospital, Canada, and Dr. D. Groleau, Biotechnology Research Institute, Montreal, Canada. This research was partly supported by grants from J. A. de Séve Foundation and from Medical Research Council, Canada, as well as PENCE.

REFERENCES

- Seidah, N. G. and Chrétien, M. (1997) *Curr. Opin. Biotechnol.* **8**, 602–607
- Seidah, N. G., Chrétien, M. and Day, R. (1994) *Biochimie (Paris)* **76**, 197–209
- Van de Ven, W. J. W. and Roebroek, A. (1993) *Crit. Rev. Oncogene* **4**, 115–136
- Chrétien, M., Mbikay, M., Gaspar, L. and Seidah, N. G. (1995) *Proc. Assoc. Am. Physicians* **107**, 47–66
- Mbikay, M., Seidah, N. G. and Chrétien, M. (1993) *Ann. N. Y. Acad. Sci.* **680**, 13–19
- Seidah, N. G., Mbikay, M., Marcinkiewicz, M. and Chrétien, M. (1997) in *Proteolytic and Cellular Mechanisms in Prohormone and Neuropeptide Precursor Processing* (Hook, Y. H., ed.), Landes Bioscience Publishers, Springer Verlag, Heidelberg
- O'Rahilly, S., Gray, H., Humphreys, P. J., Krook, K., Polonsky, K. S., White, A., Gibson, S., Taylor, K. and Carr, C. (1995) *New Engl. J. Med.* **333**, 1386–1390
- Jackson, R. S., Creemers, J. W. M., Ohagi, S., Raffin-Sanson, M. L., Sanders, L., Montague, C. T., Hutton, J. C. and O'Rahilly, S. (1997) *Nat. Genet.* **16**, 303–306
- Roebroek, A. J. M., Pauli, I., van Leuven, F. and Van de Ven, W. J. M. (1997) *Keystone Symposium on Molecular and Cellular Biology-Processing of Peptide Hormones, Neurotransmitters, Growth Factors and Viral Proteins*
- Mbikay, M., Tadros, H. N., Ishida, N., Lerner, C. P., Lamirande, E. D., Chen, A., El-Alfy, M., Clermont, Y., Seidah, N. G., Chrétien, M. et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6842–6846
- Cheng, M., Watson, P. H., Paterson, J. A., Seidah, N. G., Chrétien, M. and Shiu, R. P. C. (1997) *Int. J. Cancer* **71**, 966–971
- Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. and Thomas, G. (1992) *J. Biol. Chem.* **267**, 16396–16402
- Jean, F., Stella, K., Thomas, L., Liu, Y., Xiang, Y., Reason, J. and Thomas, G. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7293–7298
- Hallenberger, S., Bosch, V., Anglikar, H., Shaw, E., Klenk, H. D. and Garten, W. (1992) *Nature (London)* **360**, 358–361
- Decroly, E., Vandenbranden, M., Ruysschaert, J. M., Jacob, G. S., Howard, S. C., Marshall, G., Kompelli, A., Basak, A., Jean, F., Lazure, C. et al. (1994) *J. Biol. Chem.* **269**, 12240–12247
- Vollenweider, F., Benjannet, S., Decroly, E., Savaria, D., Lazure, C., Thomas, G., Chrétien, M. and Seidah, N. G. (1996) *Biochem. J.* **314**, 521–532
- Basak, A., Jean, F., Seidah, N. G. and Lazure, C. (1994) *Int. J. Pept. Protein Res.* **44**, 253–261
- Basak, A., Schmidt, C., Ismail, A. A., Seidah, N. G., Chrétien, M. and Lazure, C. (1995) *Int. J. Pept. Protein Res.* **46**, 228–237
- Jean, F., Basak, A., Dugas, H. and Lazure, C. (1994) in *Peptides, Chemistry, Structure and Biology* (Hodges, R. S. and Smith, J. A., eds.), pp. 613–615, ESCOM, Leiden
- Jean, F., Basak, A., DiMaio, J., Seidah, N. G. and Lazure, C. (1995) *Biochem. J.* **307**, 689–695
- Basak, A. and Lazure, C. (1997) *Protein Pept. Lett.* **4**, 189–194
- Basak, A., Ernst, B., Brewer, D., Seidah, N. C., Lazure, C. and Lajoie, G. A. (1997) *J. Pept. Res.* **49**, 596–603
- Chopra, H. N., Nayar, S. L. and Chopra, I. C. (1956) in *Glossary of Indian Medicinal Plants*, p. 18, CSIR, New Delhi
- Gupta, S., Chowdhury, M. A., Yadav, J. N. S., Srivastava, V. and Tandon, J. S. (1990) *Int. J. Crude Drug Res.* **28**, 273–278
- Puri, A., Saxena, R., Saxena, R. P. and Saxena, K. C. (1993) *J. Nat. Prod.* **56**, 995–999
- Dutta, A. and Sukul, N. C. (1982) *J. Helminthol.* **56**, 81–84
- Shukla, B., Visen, P. K. S., Patnaik, G. K. and Dhawan, B. N. (1992) *Planta Medica* **58**, 146–149
- Zhang, C. Y. and Tan, B. K. (1996) *Clin. Exp. Pharmacol. Physiol.* **23**, 675–678
- Borhanuddin, M., Shamsuzzoha, M. and Hussain, A. H. (1994) *Bangladesh Med. Res. Council Bull.* **20**, 24–26
- Wang, D. W. and Zhao, H. Y. (1994) *Chinese Med. J. (Beijing, Engl. Ed.)* **107**, 464–470
- Kapil, A., Koul, I. B., Banerjee, S. K. and Gupta, B. D. (1993) *Biochem. Pharmacol.* **46**, 182–185
- Zhao, H. Y. and Fang, W. Y. (1991) *Chinese Med. J. (Beijing, Engl. Ed.)* **104**, 770–775
- Leelarasamee, A., Trakulsomboon, S. and Sittisomwong, N. (1990) *J. Med. Assoc. Thailand* **73**, 299–304
- Akbarsha, M. A., Manivannan, B., Hamid, K. S. and Vijayan, B. (1990) *Ind. J. Exp. Biol.* **28**, 421–426
- Cava, M. P., Chan, W. R., Stein, R. P. and Willis, C. R. (1965) *Tetrahedron* **21**, 2617–2632
- Fujita, T., Fujitani, Y., Takeda, Y., Takaishi, Y., Yamada, T., Kido, M. and Miura, I. (1984) *Chem. Pharm. Bull.* **32**, 2117–2122

- 37 Balmain, A. and Connolly, J. D. (1973) *J. Chem. Soc. Perkin Trans.* **1**, 1247–1252
- 38 Chan, W. R., Taylor, D. R., Willis, C. R., Boddien, R. L. and Fehlhaben, P. S. (1971) *Tetrahedron* **27**, 5081–5091
- 39 Govindachari, I. R., Parthasarathy, P. C., Pai, B. R. and Subramanian, P. S. (1965) *Tetrahedron* **21**, 3237–3242
- 40 Jalal, M. F., Overton, K. H. and Rycroft, D. S. (1979) *Phytochemistry* **18**, 149–156
- 41 Kuroyanagi, M., Sato, M., Ueno, A. and Nishi, K. (1987) *Chem. Pharm. Bull.* **35**, 4429–4435
- 42 Marsuda, T., Kuroyanagi, M., Sugiyama, S., Umehara, K., Aeno, A. and Nishi, K. (1994) *Chem. Pharm. Bull.* **42**, 1216–1225
- 43 Chang, R. S. and Yeung, H. W. (1988) *Antiviral Res.* **9**, 163–176
- 44 Yao, X. J., Wainberg, M. A. and Parniak, M. A. (1992) *Virology* **187**, 56–62
- 45 Chang, R. S., Ding, L., Gai-Qing, C., Qi-Chao, P., Ze-Lin, Z. and Smith, K. M. (1991) *Proc. Soc. Exp. Biol. Med.* **197**, 59–66
- 46 Decroly, E., Benjannet, S., Savaria, D. and Seidah, N. G. (1997) *FEBS Lett.* **405**, 68–72
- 47 Munzer, J. S., Basak, A., Zhong, M., Mamarbassi, A., Hamelin, J., Savaria, D., Lazure, C., Benjannet, S. and Seidah, N. G. (1997) *J. Biol. Chem.* **272**, 19672–19681
- 48 Anderson, E. D., Thomas, L., Hayflick, J. S. and Thomas, G. (1993) *J. Biol. Chem.* **268**, 24887–24891
- 49 Ying, Q. L., Rinchart, A. R., Simon, S. R. and Cheronis, J. C. (1991) *Biochem. J.* **277**, 521–526
- 50 Vey, M., Schafer, W., Berghofer, S., Klenk, H. D. and Garten, W. (1994) *J. Cell Biol.* **127**, 1829–1842

Received 24 June 1998/28 October 1998; accepted 24 November 1998