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Synthesis of New 3-Alkoxy-7-amino-4-chloro-isocoumarin Derivatives as New β-Amyloid Peptide Production Inhibitors and Their Activities on Various Classes of Protease

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Abstract—A series of new 7-substituted-4-chloro-3-alkoxy isocoumarin derivatives were synthesized and evaluated as inhibitors of representative classes of proteases: serine protease (α-chymotrypsin, trypsin), cysteine protease (Caspase-3), and aspartyl protease (HIV-protease), 20S proteasome and also as inhibitors of amyloid peptide γ-secretase-mediated production. Protease inhibition selectivity is directly related to the structure of the substituent at the 7-position of the isocoumarin nucleus. 7-Nitro-isocoumarin derivatives (**4c**, **4d**, **4f**) are potent α-chymotrypsin inhibitors but slightly active or inactive on HIV-protease, as well as on cysteine protease. In contrast, only derivatives bearing a free amino (**5d**, **5f**) or a substituted amino group (**6f**) at the 7-position of the isocoumarin nucleus, were found weakly active or inactive on α-chymotrypsin, trypsin, Caspase-3 and HIV-protease, but prevent γ-secretase-mediated production of Aβ 40/42 amyloid peptides, which is known to be involved in Alzheimer's disease. Moreover, the most active compounds on β-amyloid peptide production [JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**)] show only weak or moderate inhibitory activity on the 20S proteasome. The obtained results suggest that the described new isocoumarin analogues could be of interest, since compounds like JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) can be considered as possible hits for the development of new agents directed towards Alzheimer's disease.

Introduction

Structure-based design of peptidomimetic peptidase inhibitors has proven to be highly successful for developing new drugs as illustrated by the development of HIV protease inhibitors.[1] Recent advances have led to protease inhibitors of reduced peptidic nature, which generally exhibit improved pharmacokinetic properties (oral bioavailability, excretion) and constitute often the main obstacles to drug development.[2] Among these inhibitors, the non-peptidic scaffolds coumarin and isocoumarin which occur naturally in many food sources, including citrus fruits, herbs, vegetables or isolated from bacteria or marine species have demonstrated promising protease inhibitory properties.[3–5] Schematically, protease inactivation by these coumarin or isocoumarin nuclei seems to result from a nucleophilic attack on coumarin or isocoumarin lactone carbonyl group by the active serine, or cysteine residues constitutive of the enzyme active sites.[6] In the case of aspartyl protease, inactivation could be due to the binding of the coumarin lactone carbonyl group to the protease aspartate residues. Depending on the nature of the substituents borne by the coumarinic and isocoumarinic rings, the resulting derivatives may

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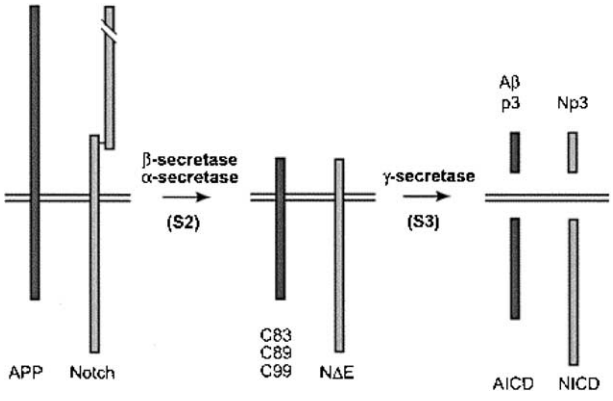
act as general or specific protease inhibitors. Considerable number of papers dealing with discrimination between different classes of proteases (α-chymotrypsin, human leukocyte elastase, ...) by coumarin or by isocoumarin derivatives have been published.[6–11]

We have recently reported new promising isocoumarin derivatives JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) as potential inhibitors of amyloid peptide production, which is the major component of the senile plaques associated with the progression of Alzheimer's neurodegenerative disease.[12] Indeed, isocoumarin compounds JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) appear to inhibit the Amyloid Protein Precursor (APP) cleavage mediated by the γ-secretase, enzyme considered as a member of aspartyl protease family.[13] Moreover these compounds represent the first non-peptidic inhibitors able to prevent γ-secretase cleavage of β-APP without affecting processing of mΔEnotch or endoproteolysis of presenilins as shown in Scheme 1.[14] Furthermore, Esler et al. have shown that while the isocoumarin analogues lower Aβ production in cell culture, these compounds do not inhibit isolated γ-secretase activity in a solubilized membrane preparation.[15] All these results seem to indicate that the isocoumarin analogues are not γ-secretase inhibitors but we do not know if the compounds may be upstream of γ-secretase as enzyme inhibitors or by another yet undefined mechanism.

It was therefore of interest to investigate the inhibitory properties of new isocoumarin compounds including JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) on representative classes of proteases: aspartyl proteases (HIV-protease); serine proteases (α-chymotrypsin and trypsin); cysteine protease (Caspase-3). The selectivity of these new isocoumarin derivatives in their inhibitory activity was further tested on the proteasome in vivo. The 20S proteasome is an intracellular barrel shape proteinase complex involved in cytosolic proteolysis and displaying three main catalytic activities defined as chymotrypsin-like, trypsin-like and peptidyl–glutamyl–peptide hydrolyzing enzyme (PGPH). The detection of an inhibitory activity of the isocoumarin compounds on central cellular proteases such as the proteasome, would impair significantly the clinical development of these drugs as *anti*-neurodegenerative agents.

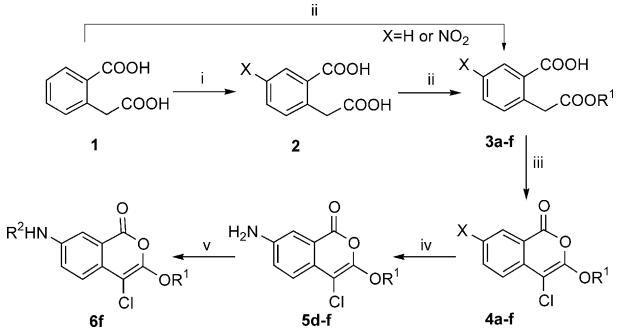


Scheme 1. APP and Notch secretase proteolysis, according to Wolfe et al.[14]

Chemistry

All the compounds described are based on a 4-chloro-isocoumarin scaffold. These compounds were synthesized as described in Scheme 2. 4-Nitrohomophthalic acid **2** was obtained by regioselective nitration of commercially available homophthalic acid in 88% yield. The monoesterification of **2** was achieved by a kinetic approach involving the use of a primary alcohol both reagent and solvent in the presence of a catalytic amount of concentrated sulphuric acid, leading to 2-alkoxy-carbonylmethylbenzoic acid **3a–f**. The mixture including compound **2** and the selected alcohol was heated until complete disappearance of the diacid. The reaction was then quenched before the formation of the corresponding diester (TLC analysis control) by addition of a large amount of bicarbonate solution. The esterification rate was dependent on the nature of the substituent at the 4-position of the aromatic ring, as the 4-nitro group decreased the esterification rate. Isolation and purification of intermediates **3a–f** required a careful work up described in the experimental part, the yields ranging from 70 to 98%.

The cyclization of derivatives **3a–f** was achieved by using PCl_5 refluxed in toluene,[16] leading to 3-alkoxy-4-chloro-isocoumarin derivatives **4a–f** in yields ranging from 30 to 70%. Quantitative reduction of 7-nitro-isocoumarin **4d–f** into the corresponding amino **5a–f** was performed using a catalytic amount of palladium on activated carbon under atmospheric pressure of hydrogen in THF. Compound **6f** resulted from the condensation of 1-adamantylfluoroformate on the corresponding 7-amino-isocoumarin derivative **5f** in the presence of Et_3N in THF. Due to the low nucleophilicity of the aniline function, this condensation required 3 days at room temperature (increased temperature led to product decomposition) in 50% yield. Alternatively, the use of 1-adamantylchloroformate or the use of 1-adamantol in the presence of phosgene gave the expected compound in lower yields. It should be also underlined the high chemical reactivity of the lactone function in compounds which incorporated at the 7-position an unsubstituted amino group. Indeed when a solution of **5f** in DMSO, not completely anhydrous, is left standing at room temperature for few days, we observed the hydrolysis of lactone group, as shown by the loss of UV



Scheme 2. (i) Fuming nitric acid, 2 h, rt; (ii) R^1OH, $[H_2SO_4]$, heat; (iii) PCl_5, toluene, reflux, 15 h; (iv) H_2/Pd/C, THF, rt; (v) adamantyl fluoroformate, TEA, THF, rt, 3 days.

absorption at 360 nm, which is a characteristic absorption for the isocoumarin nucleus. The proposed mechanism for the opening of the isocoumarin nucleus is the formation of an α-chloro-ester which led to an unstable quinonimide methide compound. Due to the highly electrophilicity of the carbon in α-position of the ester group, the quinonimide methide compound gave, in presence of water, the α-ketoester **8** characterized by LC-MS (Scheme 3).[17] In contrast, the stability of analogue **6f** in which the 7-amino group is substituted by the adamantyl group through a carbamate function, is increased because the electron doublet of the nitrogen atom is less engaged in the isocoumarin nucleus conjugation (Scheme 4).

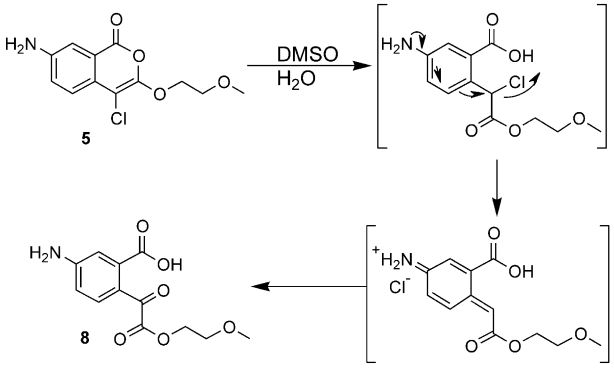
Results and Discussion

At first, activity of the isocoumarin derivatives was evaluated for their ability to affect ß-amyloid peptide production in HEK293 cells overexpressing wild-type βAPP. We have also assessed the potential of such pharmacological agents to influence presenilin endoproteolysis and Notch cleavage. In addition, we have evaluated the activity and the selectivity of the newly synthesized isocoumarin derivatives on representative proteases (serine, aspartyl or cysteine). Isocoumarin derivatives were tested with the serine proteases, α-chymotrypsin and trypsin, the HIV aspartyl protease and a cysteine protease, Caspase-3. In several experiments, enzymes were exposed to the novel isocoumarins using the preincubation method, in which the enzyme and the inhibitors are pre-incubated prior determination of the remaining enzyme activity at intervals of time. Inactivation potencies versus proteases were obtained and expressed by the determination of second order rate constants $k_{obs}/[I]\ M^{-1}\ min^{-1}$, as depicted in Table 1.

Since coumarin nucleus and analogues are inhibitors of various protease families, coumarin derivative **7** (Fig. 1) was also tested on the different enzyme models for comparison with the isocoumarin nucleus.[18]

Aβ-Peptide production measurements

Total Aβ secreted from HEK293 cells overexpressing wild-type βAPP (962 fmol mL^{-1} in 35-mm wells) was markedly reduced by JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) (Fig. 2a), whereas **4f** (data not shown) were apparently ineffective. A 100 μM concentration of inhibitors achieved 70–80% inhibition of total Aβ recovered (Fig. 2b). Interestingly, JLK6 (**5d**) and JLK2 (**5f**), but not **4f**,



Scheme 3.

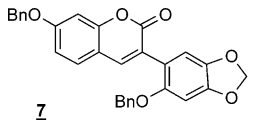
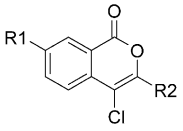


Figure 1. Structure of the coumarin analogue.

Table 1. Inhibitory activities of isocoumarin and coumarin compounds versus various proteases



Compd	R1	R2	Chymotrypsin[a]		Trypsin[b]		HIV-protease[c]			Caspase-3[d]	
			[I] (μM)	$k_{obs}/[I]$ ($M^{-1}\ s^{-1}$)	[I] (μM)	$k_{obs}/[I]$ ($M^{-1}\ s^{-1}$)	% inhibition				
							10 μM	50 μM	100 μM	50 μM	100 μM
4e	NO_2	OCH_2CH_2Br	0.5	260,000		Inactive	—	—	47	—	32
4f	NO_2	$OCH_2CH_2OCH_3$	1	1,400,000		Inactive	55	79	100	17	77
4c	NO_2	OCH_2CH_2CN	1	307,000		Inactive	—	—	51	—	48
4d	NO_2	OCH_3	1	247,000		Inactive	—	—	57	—	51
5e	NH_2	OCH_2CH_2Br	10	33,500		Inactive	—	21	77	—	24
5f	NH_2	$OCH_2CH_2OCH_3$	10	37,800		Inactive	—	—	57	Inactive	
5d	NH_2	OCH_3	100	2100		Inactive	—	32	70	23	68
4a	H	OCH_2CH_2I	100	16,300	100	2650	—	—	20	26	89
4b	H	OCH_2CH_2CN	100	9200	100	3280	—	58	84	—	25
6f	Adamantyl-CO–NH	$OCH_2CH_2OCH_3$	100	830		Inactive	35	85	100	—	20
7	Coumarin analogue		50% (10 μM)		50% (10 μM)		50	—	—	Inactive	

—, not determinated; rate constants were obtained as described in Materials and methods.
[a]Conditions were as followed: 0.1 M Hepes, 0.5 M NaCl, pH 6 at 25 °C.
[b]0.1 M phosphate, pH 8 at 25 °C.
[c]0.05 M NaOAc, 0.2 M NaCl, 0.005 M DTT, pH 4.9, 10% (v/v) glycerol at 37 °C.
[d]0.02 M Hepes, pH 7.4, 0.1% CHAPS, 0.005 M DTT, 0.002 M EDTA at 37 °C.

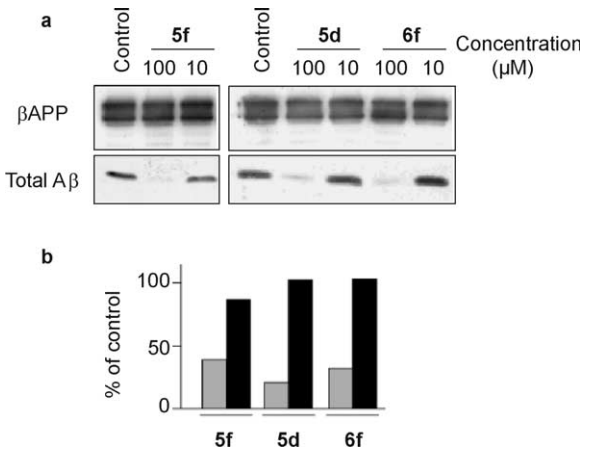


Figure 2. Effect of JLK inhibitors on βAPP maturation in HEK293 cells expressing wild-type βAPP. (a) Stably transfected HEK293 cells overexpressing wild-type βAPP (see Methods) were incubated for 7 h with 1% dimethylsulfoxide (control) or the indicated JLK inhibitors at 100 or 10 μM. Total Aβ was immunoprecipitated from conditioned media using FCA18 antibody and analysed on a 16.5% Tris–tricine gel. (b) Histograms show quantitative densitometric analyses of total Aβ levels secreted by HEK293 cells expressing wild-type βAPP; data are expressed as percentages of control secretion in the absence of JLK inhibitors. As far as the diagrams result directly from SDS-Page, standard-deviations (SD) do not appear on the diagrams.

potentiated the recovery of two products with relative molecular masses of ~12,000–14,000 (M_r 12–14 K; Fig. 3a). Previous immunological characterization of such fragments indicated that the upper-M_r band was labelled with FCA18,[19] a specific antibody against the Asp1 residue of Aβ, whereas the lower-M_r fragment was not.[20] The presence of these two products, which probably correspond to the β- and α-secretase-derived C-terminal stubs, respectively, was increased by active isocoumarin inhibitors (Fig. 3a). It should be noted that most intracellular Aβ-related species were not detected by FCA18, and therefore probably correspond to N-terminally truncated fragments of Aβ (data not shown).

Inhibitors did not affect the expression of wild-type βAPP (Fig. 2a). Moreover, APPα, the α-secretase-derived βAPP catabolite, was not affected by any of the isocoumarin inhibitors (Fig. 3b). This indicates that

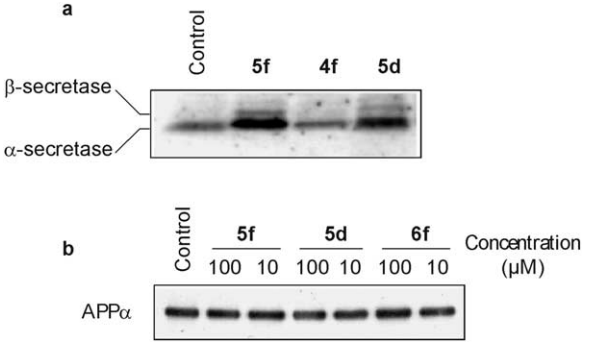
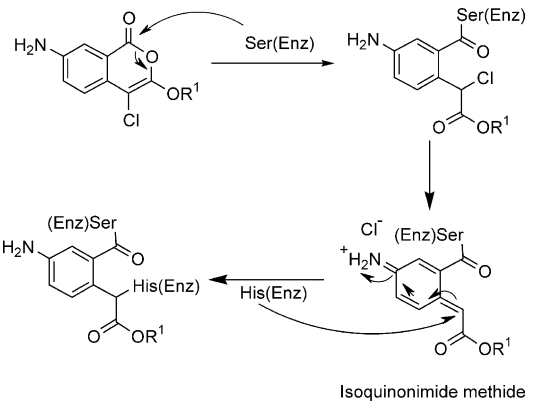


Figure 3. Effect of JLK inhibitors on β-, α-secretase products and APPα. α- and β-secretase-derived C-terminal fragments (a) were revealed using BR188 antibody. Secreted APPα was immunoprecipitated with 207 antibody and was detected by western blotting with 10D5C antibody (b) (see Methods).



Scheme 4.

active isocoumarin inhibitors do not affect α-secretase. Although the possibility that isocoumarin inhibitors can affect secretory processes cannot be definitely ruled out, these results indicate that isocoumarin agents may directly target γ-secretase cleavage of βAPP.

As previously reported, HEK293 cells revealed the expected endogenous N-terminal (NPS1; Fig. 4a) and C-terminal (CPS1; Fig. 4b) fragments of presenilin 1

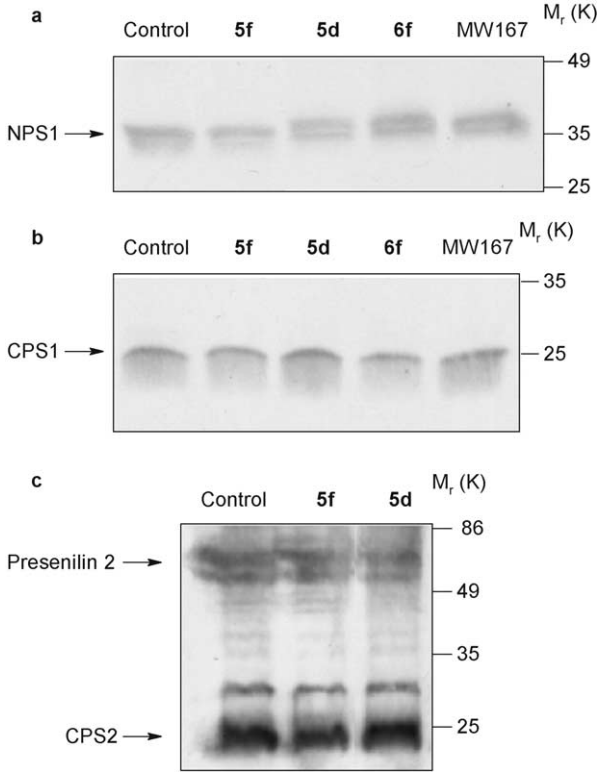


Figure 4. Effect of JLK inhibitors and DFK167 on endoproteolysis of endogenous or overexpressed presenilins. a, b, HEK293 cells were incubated for 7 h without inhibitor (control) or with the indicated inhibitor (JLK at 100 μM; MW167 at 200 μM). Endoproteolysis of endogenous presenilin 1 was monitored by western blotting for N-terminal (NPS1) (a) and C-terminal (CPS1) (b) fragments using Ab444 and Ab111 antibodies, respectively (see Methods). No endogenous holoprotein was detected. (c) Stably transfected HEK293 cells overexpressing wild-type presenilin 2 were incubated with the indicated JLK inhibitors as in (a) and (b). Overexpressed presenilin 2 holoprotein (M_r ~56 K) and CPS2 (M_r ~22 K) were detected by western blotting with Ab333 antibody.

without holoprotein-like immunoreactivity (data not shown). Figure 4a and b clearly shows that isocoumarin inhibitor did not affect proteolytic cleavage of endogenous presenilin 1. MW167, an inhibitor of Aβ production did not influence endoproteolysis of endogenous presenilin 1 (Fig. 4a and b).[21] We also examined the putative effect of inhibitors JLK6 (**5d**) and JLK2 (**5f**) on the hydrolysis of presenilin 2 in stably transfected cells. As expected, a strong immunoreactivity corresponding to CPS2 was observed, together with PS2 holoprotein-like immunoreactivity (Fig. 4c). Again, isocoumarin agents were unable to affect presenilin 2 proteolysis (Fig. 4c).

As stated above, some lines of evidence indicate that presenilins may contribute either directly or indirectly to proteolysis of Notch, as well as of βAPP. Particularly, invalidation of presenilin genes leads to full abolishment of the generation of NICD.[22,23] We have established stably transfected cells lines overexpressing Myc-tagged mΔEnotch-1. As previously described,[24] these cells produce three bands that exhibit Myc immunoreactivity (Fig. 5a). The intermediate is derived from initiation at methionine 1727 and was unaffected by any of our cell treatments. The low-M_r protein migrated as overexpressed control NICD (Fig. 5a). Pretreatment of these cells with MW167 effectively prevented NICD production (by ~80%, Fig. 5b), and its mΔENotch precursor was thereby strongly protected (Fig. 5a). However, none of the isocoumarin inhibitors that affected Aβ secretion had an effect on NICD generation (Fig. 5a and b).

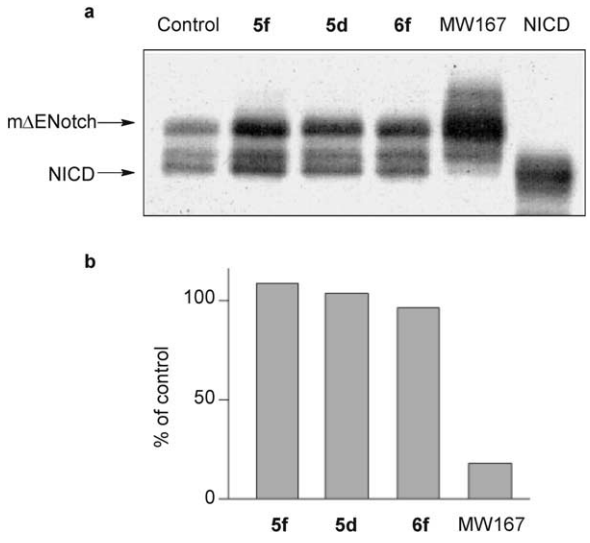


Figure 5. Effect of JLK inhibitors and MW167 on NICD generation by HEK293 cells overexpressing Myc-tagged mΔENotch. (a) Cells overexpressing Myc-tagged mΔENotch were incubated without inhibitor (control) or with JLK inhibitors or MW167 as described in Figure 4. Intact mΔENotch and NICD were detected by western blotting with 9E10 antibody. The rightmost lane corresponds to HEK293 cells transiently transfected with 2 μg of NICD cDNA and analysed 72 h after transfection for their intracellular NICD content by western blotting with 9E10. (b) Histograms showing quantitative densitometric analyses of NICD content; data are expressed as percentages of control values. As far as the diagrams result directly from SDS–PAGE, standard-deviations (SD) do not appear on the diagrams.

α-Chymotrypsin (α-CT)

It is noteworthy that the presence of a nitro group as substituent at the 7-position of the isocoumarin nucleus greatly enhances the inactivation rate constant magnitude comparatively to other groups like NH_2, *N*-adamantyloxycarbonyl or H, whatever the nature of the substituent at the 3-position of the isocoumarin ring. For example, compound **4f** the most potent inhibitor of α-CT inhibitor ($k_{obs}/[I] = 1{,}400{,}000$) is about 40 times more potent than its homologue JLK2 (**5f**) ($k_{obs}/[I] = 37{,}800$) and more than three orders of magnitude than its analogue JLK7 (**6f**). It can be also observed that inside the series of nitro analogues, inactivation rates also depend on both nature and length of the side arm located at the 3-position of the isocoumarin nucleus; for example, analogue **4e** bearing a 3-(2-bromoethoxy) substituent is 5 times less efficient than its corresponding analogue **4f** bearing a 3-(2-methoxyethoxy) substituent and as effective as analogue **4d** bearing a 3-methoxy substituent. These results suggest that the substitution at the 7-position of the isocoumarin nucleus appears to be determinative for these compounds to be processed by α-chymotrypsin. Compounds bearing electron-withdrawing groups at the 7-position such as NO_2 are highly preferentially processed compared to the corresponding analogues bearing electron-donating substituents at the 7-position like NH_2. All these structural observations are in agreement with the known primary specificity of α-CT related to the presence of an hydrophobic pocket,[8] in which the 7-nitro and 3-(2-methoxyethoxy) substituents of the compound **4f** give the best fit with the S1 site of α-chymotrypsin. Interestingly, it should be noted that compound JLK7 (**6f**) bearing a *N*-adamantyloxycarbonyl amino substituent at the 7-position of the nucleus, which has been reported as inhibitor of the production of β-amyloid peptides, displays only a very weak inhibitory activity on α-CT ($k_{obs}/[I] = 830$).

Trypsin

The most potent α-CT inhibitors (**4c–f**) failed to efficiently inhibit trypsin. Only compounds (**4a**, **4b**) which are not substituted at the 7-position display a weak or moderate inhibitory potency on trypsin. Second-order kinetic rate ($k_{obs}/[I]$) for these two compounds were respectively 2600 and 3200. These values are 3 and 8 times weaker than the values observed in the case of α-CT for these analogues. It can be also underlined that the three Aβ secretion inhibitors JLK6 (**5d**), JLK2 (**5f**) and JLK 7 (**6f**) were devoided of inhibitory efficiency on this enzyme.

Comparatively to the isocoumarin nucleus, coumarin analogue **7** tested on both serine protease enzyme models (α-CT and trypsin), failed to demonstrate significant inhibitory activity and did not displayed any selectivity between the two serine proteases.

HIV-aspartate protease

As already reported, 4-hydroxycoumarin rings have been used in the design of non-peptidic HIV protease inhibitors.[25] Indeed it has been shown that salient

hydrogen bonding interactions occurring on the one hand between the lactone oxygen atoms of the coumarin with the NH groups of the flap region isoleucine residues 50/50′ of the HIV protease and on the other hand between the 4-hydroxy group of the coumarin ring and the catalytic aspartate residues 25/25′ of the enzyme to which the 4-hydroxy group is pseudosymmetrically bonded. Some of these leads display anti-HIV protease activity at submicromolar concentrations. As illustrated in Table 1, the newly synthesized isocoumarin derivatives display weak inhibitory activities on recombinant HIV protease, IC_{50} ranging from 10 to 100 μM. Probably, the presence of the lipophilic chlorine at the 4-position of the isocoumarin ring is not favourable for the binding to the catalytic aspartate residues 25/25′ comparatively to the 4-hydroxyl substituent of the coumarin nucleus. The most active compounds of this new series was derivative JLK7 (**6f**) (IC_{50} ~10 μM). Similar inhibitory activity was also obtained with the coumarin derivative **7** (IC_{50} = 10 μM). Besides no significant difference in inhibitory activity was found whatever the nature of the substituents incorporated at the 3- or 7-positions on the isocoumarin ring. These low inhibitory anti-HIV protease potencies clearly indicate that the fit of these isocoumarin rings onto the surface of the active site of the HIV-protease is not optimised.

Caspase-3

The activity of isocoumarin compounds was also evaluated on the enzyme Caspase-3, which belongs to the cysteine protease family.[26] As shown in Table 1, all compounds inhibited only moderately Caspase-3. Their inhibitory potencies were around one order magnitude less active than the standard Caspase-3 peptidic inhibitor (Acetyl-Asp-Glu-Val-Asp-CHO; IC_{50} = 1 μM). Compound JLK7 (**6f**) is a very weak Caspase-3 inhibitor, only 20% inhibition observed at 100 μM and JLK2 (**5f**) was devoided of inhibitory efficiency on this enzyme. This result is interesting because it has been first reported that Caspases were involved in the proteolytic cascade events leading to β-amyloid secretion,[27] but recently it has been demonstrated that β-amyloid peptide resulting from the cleavage pathway of its precursor APP is independent of its cleavage by Caspases,[28] our results seem in agreement with this last report. Coumarin derivative **7** was found inactive on Caspase-3.

Proteasome

Inhibition of the proteasome by inhibitors such as epoxomicin or lactacystin greatly enhances the cytosolic accumulation of poly-ubiquitinated proteins which are the natural substrates of the proteasome.[29] We therefore evaluated the effect of different isocoumarin inhibitors on the total protein ubiquitination levels in HeLa cells by confocal microscopy using the FK2 monoclonal antibody. FK2 is specific for mono- or poly-ubiquitinated proteins and, importantly, does not react with free ubiquitin (Ub).[30] Interference of the isocoumarin derivatives with proteasome activity lead to an increase in FK2 staining and important re-localization together with the proteasome in the perinuclear area (Fig. 6).[31] Individual compounds were incubated with cells for 12 h prior visualization. Interestingly, the high *anti*-chymotrypsin activity of compounds **4c** and **4e** did not correlate with proteasome inhibition, while the related product, **4d**, displayed a strong proteasome inhibition equivalent to the one induced by epoxomicin. Two of the inhibitors, **5e** and JLK2 (**5f**), were slightly cytotoxic for HeLa cells at the concentration of 10 μM and were therefore tested at 1 μM. At this low concentration, JLK2 (**5f**) had a weak inhibitory effect on the ubiquitin–proteasome pathway (not shown). The other two isocoumarin derivatives, JLK6 (**5d**) and JLK7 (**6f**), able to block Aβ secretion, were also found to have some proteasome inhibitory effect. The inhibition was stronger (proteasome re-localization) for JLK6 (**5d**) than for JLK7 (**6f**), although the magnitude of these effects was much weaker than the one induced by the specific proteasome inhibitor epoxomicin or even by compound **4d**.

General *anti*-protease isocoumarin derivatives activity

We have described a new generation of non-peptidic inhibitors that have distinct effects on Aβ secretion and Notch cleavage. Furthermore, the fact that an identical protective effect was observed on the C-terminal βAPP fragments C99 and C83, which are generated by β- and α-secretase, respectively, indicates that these inhibitors [JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**)] probably target γ-secretase-mediated cleavage. However, these inhibitors do not affect production of NICD in identical cells, although we confirmed that this cleavage can be blocked by MW167.[32] The ability of isocoumarin inhibitors to discriminate between Aβ production and Notch cleavage was recently confirmed by Esler et al., which have proved that isocoumarin analogues JLK 2 (**5f**) and JLK6 (**5d**) do not block Aβ production by directly inhibiting the γ-secretase enzyme but their observations strongly suggest that these inhibitors reduce Aβ released from cells by interacting with an unknown target upstream of γ-secretase.[15]

However, other processes such as destabilization of the reported protein complex associated with the γ-secretase cannot be ruled out.[33] Since we have shown the unexpected properties of JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) compounds on the inhibition of the β-amyloid peptide secretion, and their possible use as new tools in the understanding of Alzheimer's disease, it was of interest to explore the selectivity of this series of isocoumarin derivatives on representative families of proteolytic enzymes. First of all, it can be observed differential behavior of isocoumarin derivatives towards the representative proteolytic enzymes. Formation of the acyl-enzyme intermediate which results from the attack of the active serine residue of α-CT or trypsin on the lactone carbonyl group isocoumarin ring, and consequently the nature of substituents at 3- and 7-position induced the inhibition selectivity between the two serine proteases. Second-order kinetic rate constants ($k_{obs}/[I]$) determined for α-CT and trypsin inactivation clearly support these findings. The observed differences in inhibition potencies between α-CT and trypsin give rise by these derivatives can be explained by the structure of

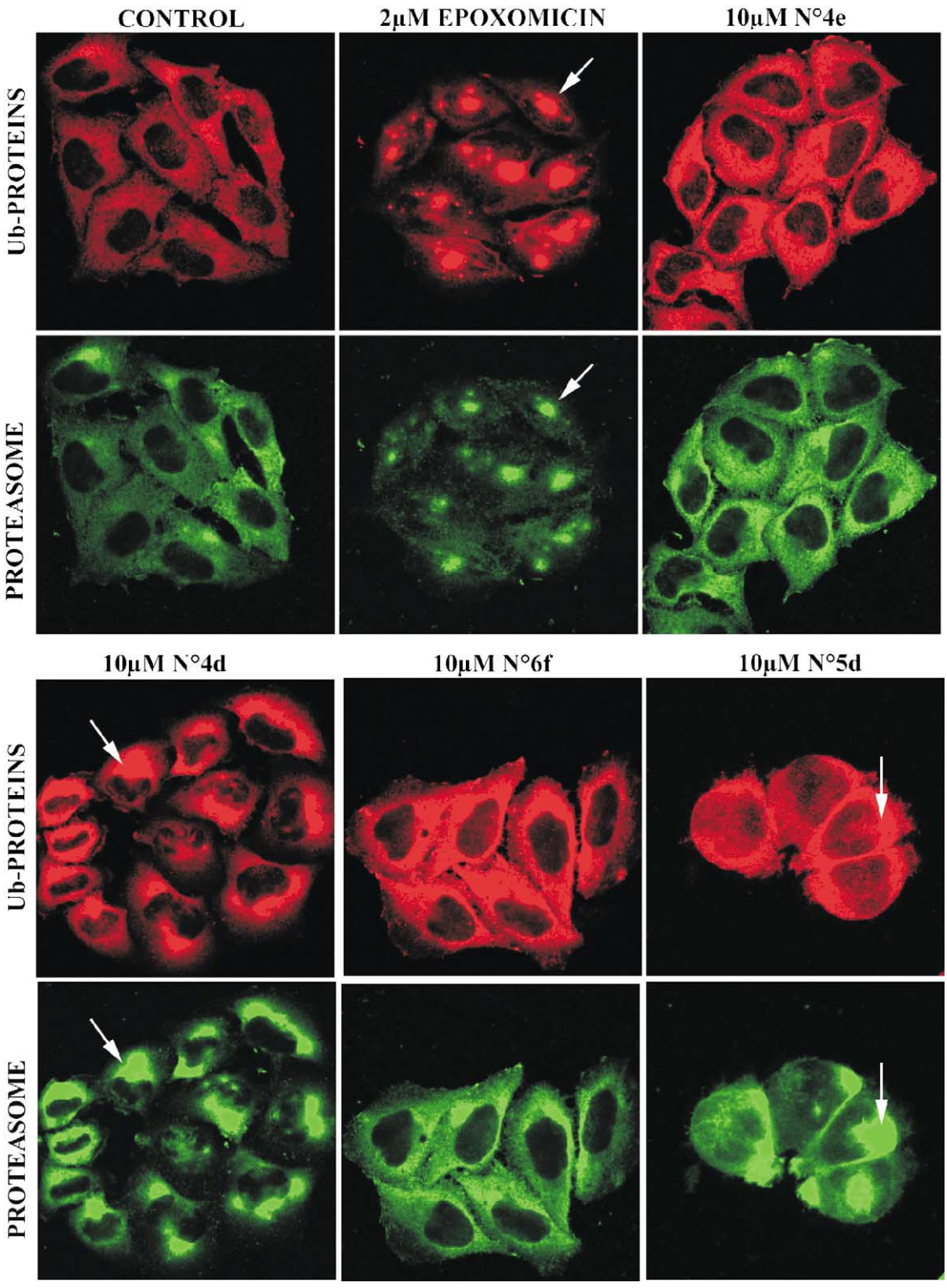


Figure 6. Effect of different isocoumarin inhibitors on proteasomal activity. HeLa cells were treated for 12 h without (control) or with the different inhibitors and as a positive control with epoxomicin, a selective inhibitor of proteasomal activity. Cells were fixed, permeabilized and stained for proteasome with a rabbit polyclonal antibody (green) and for poly-ubiquitinated proteins (Ub-protein) using the FK2 monoclonal antibody (red). Effects of the different inhibitors were visualized on a confocal microscope by an increase in FK2 staining and an important re-localization together with the proteasome in the perinuclear area (arrows).

their hydrophobic S1 site (nomenclature of Schechter and Berger).[34] In trypsin, the base of S1 contains the negatively charged carboxylate side chain of Asp-189 which is not the case for the S1 α-CT hydrophobic pocket. This structural S1 difference accounts for the specificity of these two enzymes. In contrast, in the case of the other classes of protease (HIV-aspartyl protease or cysteine protease Caspase-3), no significant inhibitory selectivity was obtained with this series of compounds. Interestingly, high chymotrypsin inhibition

potency was not correlated with proteasome inhibition in vivo, suggesting a different enzymatic selectivity on this proteolytic complex. The weak proteasome inhibition observed with derivatives JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**) cannot be precisely quantified in our relatively crude assay, however this effect could have a profound significance for ß-amyloid peptide secretion. A correlation of the inhibition of the chymotrypsin-like activity of the proteasome with suppression of ß-amyloid peptide secretion has been described,[35] however the mechanism by which the proteasome exert this effect remain unclear.

Nevertheless, it should be pointed out that in the case of the inactivation of proteases involved in the secretion of amyloid peptide, the presence of an amino group at the 7-position of the isocoumarin ring fulfilled the criteria for suicide-type inactivation at least for compounds **5d** and **5f**. Indeed after the nucleophilic attack of the carbonyl group leading to the acyl-enzyme, the presence of the electron doublet of the amino group at the 7-position allows a new possible enzyme alkylation reaction, consequently after the departure of the chlorine at the position 4 of the isocoumarin ring (Scheme 3). Nevertheless, this mechanism cannot be validated for different reasons: isocoumarin target is not identified, purified and structurally resolved, and also in compound **6f** delocalization of the amino lone pair electron is excluded.

The present results bring to light that isocoumarin compounds such as JLK6 (**5d**), JLK2 (**5f**) and JLK7 (**6f**), which have demonstrated potent *anti*-secretive activity of ß-amyloid peptide through the inactivation of protease, show moderate or weak inhibitory activities on the whole different tested protease models, including the 20S proteasome. This specificity could be of interest in the case of some of these isocoumarin derivatives which are considered as possible hits for the development of new *anti*-neurodegenerative drugs.

Experimental

Synthesis

All common chemicals and solvents were reagent grade or better. The purity of each compound was checked by ^{1}H and ^{13}C NMR spectroscopy, mass spectroscopy, IR spectroscopy, thin-layer chromatography, melting point, and elemental analysis and results are consistent with the proposed structures. The ^{1}H NMR spectra were recorded on either a Bruker AC-250 MHz instrument, and ^{13}C NMR spectra, on a Bruker AC-60 MHz instrument. Fast atom bombardment mass spectra (FAB) were recorded on a Jeol DX-100 spectrometer using a caesium ion source and glycerol/thioglycerol (GT) (1:1) or *m*-nitrobenzylalcohol (NOBA) as matrix. Elemental analysis were performed by Service Central d'Analyses du CNRS (Venaison, France) and were within 0.4% of the theoretical values. Thin-layer chromatographic analysis (TLC) were conducted on silica gel plates 0.2 mm thick ($60F_{254}$ Merck) with various mixtures as eluent. Preparative flash column chromatographies were carried out on silica gel (230–240 mesh, G60 Merck). Analytical purity was assessed by LC-MS using a Waters 2790 system. The stationary phase was a Waters Symmetry C-18 column (2.1 × 50 mm). The mobile phase employed 0.1% formic acid with acetonitrile as the organic modifier and a flow rate of 0.3 mL/min. Low resolution mass spectra were recorded on a Micromass ZMD mass spectrometer. Mass spectra were acquired in either the positive or negative ion mode under electrospray ionization (ESI).

4-Nitrohomophthalic acid (2). To a solution of fuming nitric acid (20 mL) at 0 °C was added homophthalic acid (5 g, 31 mmol). The mixture was stirred at room temperature for 6 h. The solution was poured onto ice and filtered. The resulting solid was dried under reduced pressure to give 4-nitrohomophthalic acid **2** (3.88 g, 88% yield). R_f (methanol/methylene chloride [1:1]) 0.60; mp 217 °C; ^{1}H NMR (250 MHz, DMSO) δ 8.43 (d, 1H, $J = 2.4$ Hz), 8.18 (dd, 1H, $J = 2.4$, 8.4 Hz), 7.48 (d, 1H, $J = 8.4$ Hz), 3.93 (s, 2H); FAB-MS (GT/FAB+) $(M+H)^+$ 226.

2-(2-Iodo-ethoxycarbonylmethyl)-benzoic acid (3a). Homophthalic acid (1 g, 5.55 mmol) was dissolved in 2-iodoethanol (2.5 mL) and sulphuric acid (95%, 100 μL, 1.84 mmol) was added to the solution. The mixture was stirred at 70 °C for 7 min. EtOAc (10 mL) was added to the mixture and the solution was extracted with a $NaHCO_3$ solution (5%, 3 × 10 mL). The aqueous layer was acidified by a citric solution up to pH 3. This solution was extracted with EtOAc (3 × 10 mL), dried over $MgSO_4$ and the solvent was removed in vacuum to give 2-(2-iodo-ethoxycarbonylmethyl)-benzoic acid **3a** (1.32 g, 71% yield). R_f (methanol/methylene chloride [1:9]) 0.70; mp 87 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.09 (d, 1H, $J = 6.7$ Hz), 7.49 (t, 1H, $J = 6.7$ Hz), 7.35 (t, 1H, $J = 6.6$ Hz), 7.23 (d, 1H, $J = 7.3$ Hz), 4.30 (t, 2H, $J = 6.9$ Hz) 4.00 (s, 2H), 3.23 (t, 2H, $J = 6.9$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 172.91, 171.28, 137.20, 134.69, 133.70, 131.86, 130.11, 128.21, 70.76, 41.63, −0.28; FAB-MS (NOBA/FAB+) $(M+H)^+$ 335. The following compounds **3b–f** were prepared as described previously.

2-(2-Cyano-ethoxycarbonylmethyl)-benzoic acid (3b). (70% yield) R_f (methanol/methylene chloride [1:9]) 0.58; mp 94 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.10 (dd, 1H, $J = 1.3$, 7.7 Hz), 7.50 (td, 1H, $J = 1.4$, 7.5 Hz), 7.37 (td, 1H, $J = 1.1$, 7.5 Hz), 7.23 (d, 1H, $J = 7.5$ Hz), 4.25 (t, 2H, $J = 6.3$ Hz) 4.02 (s, 2H), 2.65 (t, 2H, $J = 6.3$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 172.81, 171.88, 136.96, 134.34, 133.39, 132.86, 129.11, 128.71, 117.73, 59.76, 41.46, 18.69; FAB-MS (GT/FAB-) (M−H) 232.

2-(2-Cyano-ethoxycarbonylmethyl)-5-nitro-benzoic acid (3c). (98% yield) R_f (methanol/methylene chloride [1:9]) 0.65; mp 142 °C; ^{1}H NMR (250 MHz, CD_3OD) δ 8.99 (d, 1H, $J = 2.5$ Hz), 8.52 (dd, 1H, $J = 2.5$, 8.4 Hz), 7.77 (d, 1H, $J = 8.4$ Hz), 4.38 (s, 2H), 3.91 (t, 2H, $J = 6.1$ Hz), 2.78 (t, 2H, $J = 6.1$ Hz); ^{13}C NMR (60 MHz, CD_3OD) δ 173.90, 168.41, 149.57, 145.90, 135.11,

133.26, 128.17, 127.85, 117.79, 59.26, 41.12, 18.19; FAB-MS (NOBA/FAB+) $(M+H)^+$ 277.

2-Methoxycarbonylmethyl-5-nitro-benzoic acid (3d). (76% yield) R_f (ethyl acetate/acetic acid [14.5:0.5]) 0.57; mp 158 °C; ^{1}H NMR (250 MHz, CD_3OD) δ 8.71 (d, 1H, $J=2.4$ Hz), 8.24 (dd, 1H, $J=2.4$, 8.4 Hz), 7.48 (d, 1H, $J=8.4$ Hz), 4.07 (s, 2H), 3.57 (s, 3H); ^{13}C NMR (60 MHz, CD_3OD) δ 173.80, 168.91, 149.27, 145.50, 135.91, 133.86, 128.27, 127.65, 53.43, 41.82; FAB-MS (GT/FAB+) $(M+H)^+$ 240.

2-(2-Bromo-ethoxycarbonylmethyl)-5-nitro-benzoic acid (3e). (73% yield) R_f (methanol/methylene chloride [2:8]) 0.69; mp 90 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.78 (d, 1H, $J=2.6$ Hz), 8.21 (dd, 1H, $J=2.6$, 8.4 Hz), 7.39 (d, 1H, $J=8.4$ Hz), 4.11 (s, 2H), 4.31 (t, 2H, $J=6.0$ Hz), 3.39 (t, 2H, $J=6.0$ Hz); ^{13}C NMR (60 MHz, CD_3OD) δ 173.20, 168.11, 149.77, 145.20, 135.71, 133.16, 127.97, 127.15, 70.31, 41.22, 28.99; FAB-MS (GT/FAB+) $(M+H^+)$ 332.

2-(2-Methoxy-ethoxycarbonylmethyl)-5-nitro-benzoic acid (3f). (87% yield) R_f (methanol/methylene chloride [2:14]) 0.70; mp 55 °C; ^{1}H NMR (250 MHz, CD_3OD) δ 8.99 (d, 1H, $J=2.5$ Hz), 8.52 (dd, 1H, $J=2.5$, 8.4 Hz), 7.77 (d, 1H, $J=8.4$ Hz), 4.38 (s, 2H), 4.38 (t, 2H, $J=4.6$ Hz), 3.76 (t, 2H, $J=4.6$ Hz), 3.52 (s, 3H); ^{13}C NMR (60 MHz, CD_3OD) δ 174.10, 168.31, 149.67, 145.10, 135.81, 133.19, 127.37, 127.23, 70.10, 69.80, 59.87, 41.42; FAB-MS (NOBA/FAB+) $(M+H)^+$ 284.

4-Chloro-3-(2-iodo-ethoxy)-isocoumarin (4a). **3a** (1.23 g, 3.68 mmol) and phosphorus pentachloride (1.7 g, 8.3 mmol) were added to toluene (20 mL). The mixture was refluxed for 15 h. The solvent was removed under reduced pressure and ice was poured onto the residue. The solution was refluxed for 1 h and the mixture was extracted with EtOAc (20 mL) and the organic layer was washed with brine (3 × 10 mL), dried over $MgSO_4$, and the solvent was removed in vacuum. The residue was recrystallized from methanol to give 4-chloro-3-(2-iodo-ethoxy)-isocoumarin **4a** (380 mg, 30% yield). R_f (ethyl acetate/cyclohexane [6:12]) 0.68; mp 67 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.32 (d, 1H, $J=7.9$ Hz), 7.86 (m, 2H), 7.54 (td, 1H, $J=1.6$, 8.1 Hz), 4.72 (t, 2H, $J=7.0$ Hz), 3.56 (t, 2H, $J=7.0$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 159.32, 152.17, 137.52, 135.86, 130.25, 126.76, 122.64, 117.62, 92.17, 70.33, −0.34; FAB-MS (NOBA/FAB+) $(M+H)^+$ 347; elemental analysis calcd (%) for $C_{11}H_8ClIO_3$: C 37.69, H 2.30; found C 37.68, H 2.30. The following compounds **4b–f** were prepared according to the same procedure.

3-(4-Chloro-1-oxo-1H-isochromen-3-yloxy)-propionitrile (4b). (67% yield) R_f (ethyl acetate/cyclohexane [7:16]) 0.36 ; mp 115 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.20 (d, 1H, $J=7.5$ Hz), 7.75 (m, 2H), 7.44 (dd, 1H, $J=1.4$, 8.1 Hz), 4.57 (t, 2H, $J=6.3$ Hz), 2.90 (t, 2H, $J=6.3$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 159.93, 152.46, 138.01, 136.73, 131.03, 127.82, 123.59, 118.44, 116.93, 93.55, 65.29, 19.57; FAB-MS (NOBA/FAB+) $(M+H)^+$ 250; elemental analysis calcd (%) for $C_{12}H_8ClNO_3$: C 57.73, H 3.23, N 5.61; found C 57.75, H 3.22, N 5.64.

3-(4-Chloro-7-nitro-1-oxo-1H-isochromen-3-yloxy)-propionitrile (4c). (36% yield) R_f (ethyl acetate/cyclohexane [4:12]) 0.92; mp 150 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.99 (d, 1H, $J=2.3$ Hz), 8.52 (dd, 1H, $J=2.3$, 8.9 Hz), 7.84 (d, 1H, $J=8.9$ Hz), 4.63 (t, 2H, $J=6.1$ Hz), 2.91 (t, 2H, $J=6.1$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.43, 155.81, 149.93, 143.37, 129.61, 126.93, 123.72, 117.29, 116.33, 93.35, 65.21, 19.17; FAB-MS (NOBA/FAB+) $(M+H)^+$ 295; elemental analysis calcd (%) for $C_{12}H_7ClN_2O_5$: C 48.92, H 2.39, N 9.51; found C 48.89, H 2.37, N 9.58.

4-Chloro-3-methoxy-7-nitro-isocoumarin (4d). (70% yield) R_f (ethyl acetate/hexane [3:12]) 0.57; mp 115 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.20 (d, 1H, $J=2.3$ Hz), 8.69 (dd, 1H, $J=2.3$, 8.9 Hz), 7.99 (d, 1H, $J=8.9$ Hz), 4.33 (s, 3H); ^{13}C NMR (60 MHz, CD_3OD) δ 157.42, 156.19, 145.12, 143.16, 129.89, 126.41, 123.53, 116.59, 89.65, 57.41; FAB-MS (NOBA/FAB+) $(M+H)^+$ 256; elemental analysis calcd (%) for $C_{10}H_6ClNO_5$: C 46.99, H 2.37, N 5.48; found C 46.96, H 2.38, N 5.45.

3-(2-Bromo-ethoxy)-4-chloro-7-nitro-isocoumarin (4e). (44% yield) R_f (ethyl acetate/cyclohexane [5:12]) 0.31; mp 120 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.97 (d, 1H, $J=2.3$ Hz), 8.48 (dd, 1H, $J=2.4$, 8.9 Hz), 7.80 (d, 1H, $J=8.9$ Hz), 4.69 (t, 2H, $J=6.1$ Hz), 3.63 (t, 2H, $J=6.0$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.57, 145.94, 143.20, 130.29, 127.12, 124.28, 118.07, 117.60, 91.26, 70.01, 28.09; FAB-MS (NOBA/FAB+) $(M+H)^+$ 349; elemental analysis calcd (%) for $C_{11}H_7BrClNO_5$: C 37.91, H 2.02, N 4.02; found C 37.94, H 2.04, N 3.99.

4-Chloro-3-(2-methoxy-ethoxy)-7-nitro-isocoumarin (4f). (69% yield) R_f (ethyl acetate/methylene chloride/hexane [7:3:12]) 0.37; mp 127 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.95 (d, 1H, $J=2.3$ Hz), 8.45 (dd, 1H, $J=2.4$, 8.9 Hz), 7.78 (d, 1H, $J=8.9$ Hz), 4.54 (t, 2H, $J=4.4$ Hz), 3.71 (t, 2H, $J=4.4$ Hz), 3.37 (s, 3H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.51, 155.82, 149.98, 143.14, 129.80, 126.43, 123.76, 117.03, 90.45, 70.51, 70.09, 59.31; FAB-MS (NOBA/FAB+) $(M+H)^+$ 300; elemental analysis calcd (%) for $C_{12}H_{10}ClNO_6$: C 48.10, H 3.36, N 4.67; found C 48.20, H 3.35, N 4.66.

7-Amino-4-chloro-3-methoxy-isocoumarin (5d). **4d** (2 g, 7 mmol) was dissolved in THF (30 mL) and palladium on activated carbon (10% Pd, 50 mg) was added to the solution, and the reaction mixture was stirred under atmospheric pressure of hydrogen at room temperature for 6 h, filtered and concentrated in vacuo to give a residue which was recrystallized from ethanol, to give 7-amino-4-chloro-3-methoxy-isocoumarin **5d** (1.6 g, 7 mmol, quantitative yield) as a yellow powder. R_f (ethyl acetate/hexane [7:12]) 0.56; mp 115 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.10 (d, 1H, $J=2.3$ Hz), 8.69 (dd, 1H, $J=2.3$, 8.9 Hz), 7.99 (d, 1H, $J=8.9$ Hz), 4.33 (s, 3H), 3.57 (s, 2H); ^{13}C NMR (60 MHz, CD_3OD) δ 157.43, 156.12, 145.17, 141.31, 129.85, 126.48, 123.56,

116.58, 89.66, 57.41; FAB-MS (NOBA/FAB+) $(M+H)^+$ 226; elemental analysis calcd (%) for $C_{10}H_8ClNO_3$: C 53.23, H 3.57, N 6.21; found C 53.26, H 3.54, N 6.24. The following compounds **5e–f** were prepared according to the same procedure.

7-Amino-4-chloro-3-(2-bromo-ethoxy)-isocoumarin (5e). (Quantitative yield) R_f (ethyl acetate/hexane [4:6]) 0.35; mp 110 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.18 (d, 1H, $J=8.3$ Hz), 7.89 (d, 1H, $J=2.7$ Hz), 7.25 (dd, 1H, $J=2.8$, 8.3 Hz), 4.68 (t, 2H, $J=4.3$ Hz), 3.67 (t, 2H, $J=4.4$ Hz); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.42, 155.91, 149.89, 141.28, 129.97, 126.13, 123.64, 117.60, 91.26, 70.01, 28.09; FAB-MS (NOBA/FAB+) $(M+H)^+$ 318; elemental analysis calcd (%) for $C_{11}H_9BrClNO_3$: C 41.47, H 2.85, N 4.40; found C 41.50, H 2.82, N 4.46.

7-Amino-4-chloro-3-(2-methoxy-ethoxy)-isocoumarin (5f). (Quantitative yield) R_f (ethyl acetate/hexane [9:12]) 0.39; mp 127 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.48 (d, 1H, $J=8.6$ Hz), 7.38 (d, 1H, $J=2.4$ Hz), 7.05 (dd, 1H, $J=2.4$, 8.6 Hz), 4.39 (t, 2H, $J=4.5$ Hz), 3.68 (t, 2H, $J=4.5$ Hz), 3.38 (s, 3H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.51, 155.83, 149.92, 141.37, 129.94, 126.33, 123.77, 117.04, 90.48, 70.53, 70.04, 59.31; FAB-MS (NOBA/FAB+) $(M+H)^+$ 270; elemental analysis calcd (%) for $C_{12}H_{12}ClNO_4$: C 53.44, H 4.49, N 5.19; found C 53.45, H 4.48, N 5.18.

[4-Chloro-3-(2-methoxy-ethoxy)-1-oxo-1H-isochromen-7-yl]-carbamic acid adamantan-1-yl ester (6f). **5f** (50 mg, 0.22 mmol) was dissolved in THF (1.25 mL) and 1-adamantyl fluoroformate (225 mg, 2.1 mmol) and triethylamine (140 μL, 1 mmol) were added to this solution. The reaction mixture was stirred at room temperature for 3 days. The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (10 mL). The organic layer was washed with a $NaHCO_3$ solution (5%, 2 × 10 mL), brine (2 × 10 mL), and a citric acid solution (5%, 2 × 10 mL), dried over $MgSO_4$ and the solvent was removed in vacuo to give a residue which was purified by flash chromatography on silica gel, using EtOAc–cyclohexane [6:12], to give [4-chloro-3-(2-methoxy-ethoxy)-1-oxo-1H-isochromen-7-yl]-carbamic acid adamantan-1-yl ester **6f** (50 mg, 50% yield) as a yellow powder. R_f (ethyl acetate/hexane [6:12]) 0.59; mp 140 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.93 (d, 1H, $J=2.2$ Hz), 7.73 (dd, 1H, $J=8.7$ Hz), 7.47 (d, 1H, $J=8.7$ Hz), 6.59 (s, 1H), 4.31 (t, 2H, $J=4.5$ Hz), 3.58 (t, 2H, $J=4.5$ Hz), 3.27 (s, 3H), 2.00 (bs, 9H), 1.51 (bs, 6H); ^{13}C NMR (60 MHz, $CDCl_3$) δ 157.52, 155.87, 154.85, 149.91, 139.39, 129.94, 126.36, 123.78, 117.00, 90.48, 70.51, 70.00, 65.86, 59.34, 40.82, 37.82, 22.61, 21.65; FAB-MS (NOBA/FAB+) $(M+H)^+$ 449; elemental analysis calcd (%) for $C_{23}H_{26}ClNO_6$: C 61.67, H 5.85, N 3.13; found C 61.69, H 5.86, N 3.12.

7-Benzyloxy-3-(6-benzyloxy-benzo[1,3]dioxol-5-yl)-chromen-2-one (7). Synthesis of coumarin derivative **7** has already been reported.[18]

Inhibitor treatment of cells and Aβ immunoprecipitation. Stably transfected HEK293 cells overexpressing wild-type βAPP were incubated for 7 h in the presence of various inhibitor concentrations. Media were collected, diluted in 1/10 RIPA 10× buffer and incubated overnight with a 200-fold dilution of FCA18 antibody for total Aβ. After incubation for a further 3 h with protein A-sepharose (Zymed) and subsequent centrifugation, pelleted proteins were submitted to 16.5% Tris–tricine gels and western blotted for 45 min. Nitrocellulose membranes were heated in boiling PBS for 5 min and capped with 5% skimmed milk in PBS containing 0.05% Tween 20 for 1 h. Membranes were then incubated overnight with WO2 antibody and blots were visualized by enhanced chemiluminescence (ECL; Amersham).

Immunoprecipitation of sAPPα. Conditioned media from HEK293 cells overexpressing wild-type βAPP751 were incubated with a 700-fold dilution of 207 antibody (against the N-termini of βAPP and sAPPα) in the presence of pansorbin (Calbiochem) as described.[20] After centrifugation, pellets were resuspended with loading buffer, subjected to 8% Tris–glycine electrophoresis and western blotted for 2 h. Nitrocellulose membranes were incubated overnight with a 500-fold dilution of 10D5C antibody and blots were visualized as described above.

Detection of βAPP and C-terminal fragments. Cells were lysed in a 50 mM Tris buffer containing 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and 0.5% deoxycholate, and 30 μg of proteins were resuspended in the loading buffer, electrophoresed on 8% SDS-polyacrylamide gel and western blotted for 2 h. Nitrocellulose sheets were incubated in skimmed milk and exposed overnight to a 2000-fold dilution of WO2 (βAPP); blots were visualized as described above. C83 and C99 fragments from the same lysates were analyzed by 12% SDS-polyacrylamide gels, western blotted and then incubated with BR188; blots were visualized using with horseradish peroxidase (HRP)-conjugated goat-*anti* rabbit antibody as described.[20]

Transfection of mΔEnotch-1 and analysis of clones. HEK293 cells were cotransfected by DAC30 (Eurogentec, Brussels, Belgium) with 2 μg of Myc-tagged mΔEnotch-1-pCS2 complementary DNA and 0.3 μg of pZeoSV2 cDNA. Zeocin-resistant clones were washed with PBS and lysed, and ~30 μg of proteins were electrophoresed on large 8% SDS–polyacrylamide gels and western blotted for 2 h. Stable transfectants were identified after exposure of membranes to a 2000-fold dilution of 9E10 (*anti*-Myc) antibody, *anti*-mouse immunoglobulin G coupled to HRP (1/5000), and ECL.

Transient transfection of NICD. Mock HEK293 cells were transiently transfected with 2 μg of cDNA encoding Myc-tagged NICD. After 2 days, NICD expression in cell lysates was detected as described above. This cellular extract was used as standard control to estimate NICD production in mΔEnotch-expressing cells by SDS–PAGE.

Detection of presenilins and their endoproteolytic fragments. HEK293 cells expressing endogenous presenilins or overexpressing wild-type presenilin 2 were lysed and

50 μg of proteins were subjected to 12% SDS–PAGE and western blotted for 1.5 h. Nitrocellulose sheets were incubated in skimmed milk and exposed overnight to 0.2–1.0 μg mL^{-1} Ab444 (for NPS1), Ab111 (for CPS1) or Ab333 (for CPS2).

Antibodies. The 207 antibody recognizes the N-termini of βAPP and sAPPα. WO2 interacts with a sequence of five to eight amino acids in Aβ. BR188 was raised against the 12 C-terminal residues of βAPP, and BR188 recognizes whole βAPP as well as its α- and β-secretase-derived fragments. 10D5C is directed against the C terminus of APPα. FCA18 specifically interacts with free Asp1 residues of all Aβ species, but does not interact with N-terminally truncated Aβ-related fragments.[21] FCA3340 and FCA3542 specifically label Aβ40 and Aβ42, respectively.[19]

Detection of poly-ubiquitinated proteins and proteasome

Immunofluorescence confocal microscopy with Leica TCS 4D was performed as described.[36] mAb FK2 and *anti*-proteasome were obtained from Affinity (Mamhead, UK). All secondary antibodies were from Immunotech (Marseille, France). For pharmacological treatments, HeLa cells grown in DMEM with 5% FCS were incubated respectively with 1 μM epoxomicin, or 10 μM of the tested compounds.

Enzyme inhibition kinetics—materials and methods

Chymotrypsin, was purchased from ICN Biomedicals, Ohio. Trypsin and Caspase-3 were purchased from Sigma Chemical Co., St Louis, MO. HIV-protease was purchased from Bachem, USA.

Enzyme inhibition. For the HIV-protease inhibition assay, the enzyme was preincubated for 10 min at 37 °C with inhibitor by adding an aliquot (10 μL) of the inhibitor in DMSO to the enzyme (20 μL of a 0.294 μM solution) in buffer (370 μL, 0.05 M NaOAc, pH 4.9, 0.2 M NaCl, 0.005 M DTT, 10% (v/v) glycerol). Percentage of inhibition was spectrophotometrically measured (λ = 300 nm) on a Uvikon 930 spectrophotometer by adding an aliquot of the substrate solution (20 μL of a 573 μM solution of H-His-Lys-Ala-Arg-Val-Leu-*p*NO_2Phe-Glu-Ala-Nle-Ser-NH_2) to the enzyme-inhibitor solution into a cuvette.

For the Caspase-3 inhibition assay, the enzyme was preincubated for 5 min at 37 °C with inhibitor by adding an aliquot (10 μL) of the inhibitor in DMSO to the enzyme (10 μL of a 2 μg/mL solution) in buffer (970 μL, 0.02 M Hepes, pH 7.4, 0.1% CHAPS, 0.005 M DTT, 0.002 M EDTA). Percentages of inhibition were spectrophotometrically measured (λ = 405 nm) by adding an aliquot of the substrate solution (10 μL of a 20 mM solution of Acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) to the enzyme-inhibitor solution into a cuvette.

Determination of inactivation rates in the presence of substrate. Progress curve method. For the chymotrypsin and trypsin inhibition assays, substrates are *N*-succinyl-Ala-Ala-Pro-Phe-*p*nitroanilide in Hepes buffer (0.1 M Hepes, 0.5 M NaCl, pH 6) and *N*-α-Benzoyl-L-Arginine-*p*nitroanilide in 0.1 M phosphate buffer (pH 8), respectively. $k_{obs}/[I]$ values were determined in the presence of substrate as described by Tian and Tsou.[37] For example, inactivation of chymotrypsin (0.147 μM) by **4f** (0,5 μM) in the presence of 52 μM *N*-succinyl-Ala-Ala-Pro-Phe-*p*nitroanilide was measured by addition of a 70 μL of the enzyme solution to a mixture of substrate and inhibitor containing 2.5% DMSO. The increase in absorbance was monitored (410 nm) until no further release of *p*-nitroanilide was observed. The $k_{obs}/[I]$ values were calculated from plots of log ($[P]-[P]_t$) versus time, where $[P]$ and $[P]_t$ are the concentrations of *p*-nitroanilide after total inactivation and at time *t*, respectively. The reaction rates were based on two determinations, and the standard errors are ±10% or less.

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