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Further characterization of a putative serine protease contributing to the γ-secretase cleavage of β-amyloid precursor protein

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ABSTRACT

The 3-alkoxy-7-amino-4-chloro-isocoumarins JLK-6 and JLK-2 have been shown to markedly reduce the production of Amyloid β-peptide (Aβ) by Amyloid-β Precursor Protein (APP) expressing HEK293 cells by affecting the γ-secretase cleavage of APP, with no effect on the cleavage of the Notch receptor. This suggested that these compounds do not directly inhibit the presenilin-dependent γ-secretase complex but more likely interfere with an upstream target involved in γ-secretase-associated pathway. The mechanism of action of these compounds is unknown and there are high fundamental and therapeutical interests to unravel their target. Isocoumarin compounds were previously shown to behave as potent mechanism-based irreversible inhibitors of serine proteases, suggesting that the JLK-directed target could belong to such enzyme family. To get further insight into structure–activity relationships and to develop more potent isocoumarin derivatives, we have synthesized and evaluated a series of isocoumarin analogues with modifications at positions 3, 4 and 7. In particular, the 7-amino group was substituted with either acyl, urethane, alkyl or aryl groups, which could represent additional interaction sites. Altogether, the results highlighted the essential integrity of the 3-alkoxy-7-amino-4-chloro-isocoumarin scaffold for Aβ-lowering activity and supported the involvement of a serine protease, or may be more generally, a serine hydrolase. The newly reported 7-*N*-alkyl series produced the most active compounds with an IC_{50} between 10 and 30 μM. Finally, we also explored peptide boronates, a series of reversible serine protease inhibitors, previously shown to also lower cellular Aβ production. The presented data suggested they could act on the same target or interfere with the same pathway as isocoumarins derivatives.

1. Introduction

The Amyloid β-peptide (Aβ) is very likely a key element in the pathogenesis of Alzheimer's disease.[1,2] Aβ is proteolytically produced from Amyloid-β Precursor Protein (APP) by the sequential contribution of two aspartyl-proteases. BACE-1 is responsible of the β-secretase cleavage of APP, releasing C99 that begins at the N-terminal end of Aβ.[3–6] The γ-secretase cleavage of C99, which occurs in the transmembrane domain of APP, yields Aβ-40/42 and is mainly associated to a complex of at least four different proteins including a presenilin (PS) as the catalytic subunit.[7–9] Additional cleavage of APP by the so-called ε-secretase occurs on C99, 7–10 residues downstream to the γ-site and close to the cytoplasmic membrane boundary and produces a fragment called APP IntraCellular Domain (AICD).[10,11] It has been proposed that both γ- and ε-secretases depend on presenilin, but that they are differentially regulated.[12] Accumulation of Aβ and in particular of the most aggregation-prone Aβ-42 species first leads to the formation

Abbreviations: Aβ, Amyloid β-peptide; AICD, APP intracellular domain; APP, Amyloid-β Precursor Protein; BACE, beta-site APP cleaving enzyme; DCM, dichloromethane; DMSO, dimethylsulfoxide; ESI, electrospray ionization; EtOAc, ethyl acetate; FBS, fetal bovine serum; GSAP, γ-secretase activating protein; GSI, γ-secretase inhibitor; GSM, γ-secretase modulator; HEK, Human Embryonic Kidney; Hex, hexane; HRMS, high resolution mass spectrometry; LC/MS, liquid chromatography coupled to mass spectrometry; LDH, lactate dehydrogenase; NICD, Notch intracellular domain; PBS, phosphate buffered saline; PS, presenilin; RIPA, radioimmunoprecipitation assay; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; THF, tetrahydrofurane.

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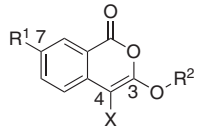
of soluble oligomers that likely account for the Aβ-related toxic potential.[13,14] β- and γ-Secretases early appeared as potentially relevant targets of therapeutic strategies aimed at slowing-down or blocking the Aβ-dependent neurodegenerative processes. Considerable efforts have been made over the last years to develop Aβ-reducing drugs, but with very disappointing results so far.[15–17] Although BACE-1 was initially considered as an ideal target, none of the inhibitors able to potently block BACE-1 in vitro or in animal models have yet proved beneficial in clinical trials. The main reasons are the existence of other important substrates like neuregulin-1,[18] which is involved in myelination, a wide substrate-binding domain and the necessity to cross the blood–brain barrier. More recently, Rajapaksha et al. suggested that BACE-1 could be involved in axonal guidance and that BACE-1 inhibitors could potentially display deleterious effects on sensory neurons.[19] Targeting PS-dependent γ-secretase complex is even more complicated. This generic term indeed comprises at least six distinct sub-complexes with specific protein composition involved in the intra-membrane cleavage of more than 60 proteins identified to date.[20] Among other vital functions related to their catalytic properties, PS-dependent γ-secretase is involved in the essential maturation of the Notch receptor leading to the production of NICD (Notch IntraCellular Domain).[21] NICD is involved in cell differentiation and embryonic development, the blockade of which leads to severe abnormalities.[17] This is probably one of the main reasons of premature interruption of two large phase III clinical trials of semagacestat, a non-Notch-sparing γ-secretase inhibitor (GSI), which not only worsened the cognition state of the patients, but also produced detrimental neoplastic effects related to Notch signalling interruption.[17] Notch-sparing GSI have been developed.[17,22] However, recent studies challenged the claimed selectivity of some of these compounds like begacestat or avagacestat, the latter being currently tested in phase II clinical trial.[23,24] Several classes of compounds were found to selectively modulate the γ-cleavage of APP (γ-secretase modulators, GSM), sparing Notch proteolysis.[17,22] A subset of non-steroidal anti-inflammatory drugs were found to modulate the γ-cleavage of APP, decreasing Aβ-40 and Aβ-42 production with increased generation of the poorly amyloidogenic Aβ-38 fragment.[25] These compounds also modulate the scissile bond position of Notch receptor but do not prevent NICD production.[26] Two large phase 3 clinical studies with one of these compounds, that is, tarenflurbil, were also completely negative,[27] due to low potency and brain penetration. However, they still represent the most promising Notch-sparing and Aβ-40/42 lowering approach.[28] Several modulator-based chemical probes carrying photoaffinity and biotin moieties were developed to identify their molecular target. Probably because the utilized agents belonged to different structural classes, these studies raised conflicting results. Whereas PS is the likely target of these compounds,[29] the Aβ region of APP was also labelled by one of these probes based on tarenflurbil.[30] Imatinib (Gleevec®), a tyrosine kinase inhibitor, was also found to potently reduce Aβ production without blocking NICD generation.[31] Photoactivatable and biotinylated probes of Imatinib targeted GSAP (γ-secretase activating protein), a newly discovered partner of PS.[32] Imatinib inhibits the binding of APP to GSAP, which does not interact with Notch, decreasing γ-cleavage of APP and increasing its ε-cleavage, thereby enhancing the production of AICD.

Another important class of Notch-sparing GSI is a series of isocoumarins that were shown to markedly reduce recovery of both Aβ-40 and Aβ-42 in a cell-based assay without affecting NICD generation.[33] The most efficient isocoumarins have been called JLK-6 and JLK-2 (Fig. 1). These compounds also do not alter Notch signalling in vivo and do not interfere with the processing of two other PS-dependent γ-secretase substrates, namely cadherins and CD44, nor with α- and β-secretases and GSK3β kinase.[34] JLK-2 also does not inhibit purified 20S proteasome,[34] while it was shown to interfere with the ubiquitin–proteasome pathway in a cell assay according to an unknown mechanism.[35] Finally, they are devoid of non-specific effects on the secretory machinery and do not display cell toxicity at 100 μM on various cell lines.[34,36,37] Their mechanism of action remains elusive, but they are believed to act upstream of the PS-dependent γ-secretase as they do not physically interact with PS.[36,38] Therefore, they act differently from other claimed Notch-sparing GSI like begacestat or avagacestat, which interact with PS. JLK-6 and JLK-2 belong to the family of 3-alkoxy-7-amino-4-chloro-isocoumarins, which were originally described as mechanism-based irreversible inhibitors of serine proteases by James C. Powers.[39,40] They were first reported as suicide inhibitors of elastases and chymotrypsin.[39,41] JLK-6 was also found to inhibit some other enzymes such as rhomboids,[42,43] which are intra-membrane cleaving proteases of the serine catalytic type, the mitochondrial pro-apoptotic serine protease HtrA2/Omi [Sévalle, J. et al., unpublished] and cholesterol esterase,[44] which is another kind of serine hydrolase. All these data support the hypothesis that JLK isocoumarins target a putative serine protease, or more generally a serine hydrolase acting upstream the γ-cleavage of APP. It is therefore important to identify this (these) putative cellular target(s) in order to better understand how JLK isocoumarins affect γ-secretase activity. This might help to develop new therapeutic strategies aimed at lowering Aβ content. The advantage of these isocoumarins is that they can form highly stable and even irreversible covalent complexes with their serine protease targets, according to a mechanism proposed by James C. Powers and shown in Figure 1S (Supplementary data, S1).[45] Successful isolation of serine proteases was already achieved by

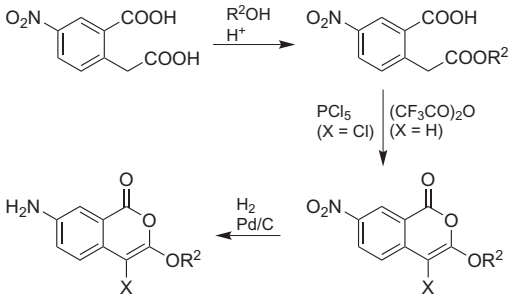
Figure 1. Upper panel, structures of JLK-6 and JLK-2. Lower panel, SDS–PAGE analysis of the concentration-dependent effect of JLK-6 on Aβ production by Swedish-mutated APP-transfected HEK293 cells.

Table 1
Structure of compounds **1**–**12** and their effect on Aβ production by transfected HEK293 cells



Compound	R^1	R^2	X	Act[a]	Compound	R^1	R^2	X	Act[a]
1	NO_2	Ethyl	Cl	–	**7**	NO_2	Methyl	H	+/–
2	NH_2	Ethyl	Cl	+	**8**	NH_2	Methyl	H	–
3	NO_2	Butyl	Cl	–	**9**	NO_2	Methoxyethyl	H	+
4	NH_2	Butyl	Cl	T	**10**	NH_2	Methoxyethyl	H	–
5	NO_2	Isobutyl	Cl	–	**11**	NO_2	2-Bromoethyl	H	+
6	NH_2	Isobutyl	Cl	T	**12**	NH_2	2-Bromoethyl	H	–

[a] Inhibitory activity of Aβ production by HEK293 cells overexpressing swedish mutant βAPP. Compounds **1**–**12** have been tested at 60 μM concentration. + indicated an inhibition >50% at the maximal concentration tested; – indicated inactive; T, significant cell toxicity. Toxic compounds were inactive at 10 μM.



Scheme 1. Synthesis of 3-alkoxy-isocoumarins **1**–**12**.

the introduction of a biotin label to isocoumarin derivatives and therefore, such strategy could also be envisioned for JLK-6 target.[46,47] However, the low potencies of JLK-6/2 in cell assay (IC_{50} of about 30–60 μM) and their low selectivity require prior development of more potent analogues.

We have synthesized several series of JLK-6-derived isocoumarins and evaluated their biological activity to further characterize the structural requirements driving the interaction with their target. Furthermore, we explored a series of peptide–boronate compounds, another class of serine protease inhibitors, which were found to possess similar Aβ-lowering activity in cell culture.[48]

2. Results and discussion

2.1. 3-Alkoxy-isocoumarin derivatives

To get further insight into the structural requirements of the JLK target for developing more potent compounds, we synthesized several series of 3-alkoxy-isocoumarins modified at the 3, 4 and 7 positions. Several crystal structures of elastase–inhibitor complexes have been resolved. Various binding modes such as interaction of the 3-alkoxy or the 7-substituent in S1 subsite or a positioning of the 3-alkoxy group in the S3 subsite or away from the enzyme have been reported, indicating the impossibility to establish a canonical binding mode for these isocoumarin derivatives.[40,41,49] Therefore, it is important to vary the 3-alkoxy group and to explore the substitution of the 7-amino group. The latter modification also adds potential interaction sites. Finally, the presence of the mechanistically important chlorine at position 4 was evaluated.

2.1.1. Synthesis

The 3-alkoxy-4-chloro/H-7-nitro/amino-isocoumarins **1**–**12** (Table 1) were obtained according to published procedures (Scheme 1).[50,51,35]

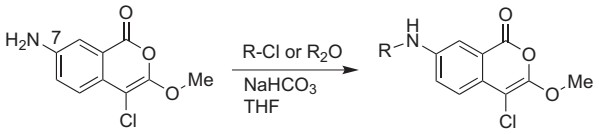
Briefly, 4-nitro-homophthalic acid was mono esterified by treatment with the corresponding alcohol as solvent in the presence of catalytic amounts of sulfuric acid. The resulting alkyl 2-carboxy-4-nitrophenylacetate derivatives were cyclized by treatment with PCl_5 or trifluoroacetic anhydride to yield the 3-alkoxy-4-chloro-7-nitroisocoumarins **1**, **3**, **5** or the 3-alkoxy-7-nitro-isocoumarins **7**, **9**, **11**, respectively. Catalytic reduction of the nitro group by hydrogenolysis gave 7-amino analogues **2**, **4**, **6**, **8**, **10**, **12** (Table 1). Compounds **8** and **10** are the chlorine lacking analogues of JLK-6 and JLK-2, respectively.

The acyl derivatives of JLK-6 **13**–**17** (Table 2) were obtained in good to moderate yields (35–85%) by reaction of JLK-6 with the corresponding acyl chlorides or anhydrides (see Section 4) (Scheme 2). The urethane derivatives of JLK-6 **18**–**24** (Table 2) were similarly obtained in good to moderate yields (30–85%) by reaction of JLK-6 with the corresponding halogenoformates or anhydrides.

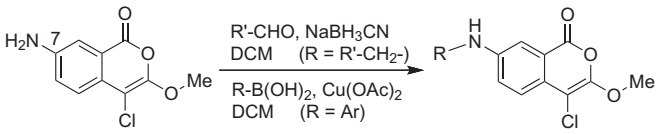
Table 2
Structure of compounds **13**–**24** and their effect on Aβ production by transfected HEK293 cells

Compound	R	Act[a]	Compound	R	Act[a]
13	Acetyl	–	**19**	Adamantyloxycarbonyl	–
14	Glutaryl	–	**20**	Fluorenylmethyloxycarbonyl	–
15	Pentafluoropropanoyl	–	**21**	Ethyloxycarbonyl	–
16	Octanoyl	–	**22**	*t*-Butyloxycarbonyl	–
17	Pivaloyl	+/–	**23**	Trichloroethoxycarbonyl	–
18	Benzyloxycarbonyl	–	**24**	Allyloxycarbonyl	–

[a] Inhibitory activity of Aβ production by HEK293 cells overexpressing swedish mutant βAPP. Compounds **13**–**24** have been tested at 60 μM concentration. + indicated an inhibition >50% at the maximal concentration tested; – indicated inactive.



Scheme 2. Synthesis of *N*-acylated (**13**–**17**) and urethane (**18**–**24**) derivatives of JLK-6.



Scheme 3. Synthesis of 7-*N*-alkylated (**25**–**42**) or arylated (**43**, **44**) analogues of JLK-6.

The synthesis of the *N*-alkylated/arylated derivatives of JLK-6 required careful attention according to the instability of the 3-alkoxy-isocoumarin in alcoholic, nucleophilic or acidic medium (Table 3, Scheme 3).

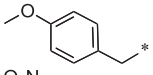
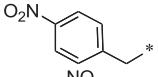
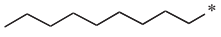
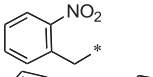
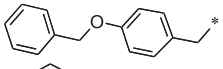
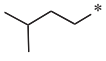
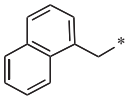
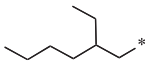
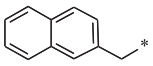
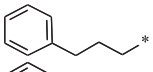
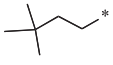
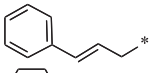
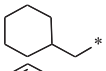
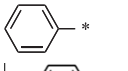
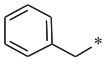
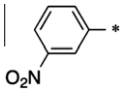
The *N*-alkylated derivatives **25**–**42** were prepared by reductive amination of JLK-6 with various alkyl- or aryl-aldehydes. Their synthesis was first attempted following two classical protocols using $NaB(OAc)_3H$/dichloroethane in the presence of acetic acid or $NaBH_3CN$/MeOH. The isocoumarin was not enough stable in the presence of acetic acid or in methanol, leading to complex mixtures. Conditions using $NaBH_3CN$ were varied and dichloromethane was finally chosen as the most convenient solvent. Compounds were obtained in low to moderate yields (10–50%) but in good purity. N-arylation was performed by Suzuki coupling of arylboronic acids in anhydrous conditions in the presence of copper acetate.[52]

2.1.2. Biological evaluation

All compounds were evaluated in a cell-based assay and the Aβ production by APPswe overexpressing HEK293 cells[53] was assessed after 8 h incubation. In this context, it is important to highlight the limited stability and lifetime of the isocoumarin derivatives in aqueous medium and in the presence of various nucleophilic components including thiols and amines present in small molecules or proteins.[45,51,54] In a cell assay, the effect of substituents and/or structural modifications on cell entrance could be a questionable parameter in the interpretation of activity data. However, most introduced substituents were hydrophobic and structural modifications were limited. Then, these molecules remained sufficiently small. For these reasons, we assumed that the efficiency of the modified compounds to reach their target was not significantly influenced by structural variations.

2.1.2.1. Modifications of JLK-6 at positions 3 and 4 (Table 1). All 3-alkoxy-4-chloro-7-nitro-isocoumarins (**1**, **3**, **5**) were found non toxic but inactive in reducing Aβ production at a 60 μM concentration, in accordance with previous results obtained with the 7-nitro analogues of JLK-6 and JLK-2.[33] Although the nitro derivatives are usually more reactive inhibitors of serine proteases than the amino analogues, they decompose rapidly in aqueous medium (half-lives of less than 20 min in buffers) and the acylated species formed by reaction with the catalytic serine hydrolyses spontaneously with rapid reactivation of the enzyme and concomitant release of henceforth inactive opened isocoumarin-derived product.[45] In the test conditions, the inhibition by these compounds would therefore be too transitory (until total

Table 3
Structure of compounds **25**–**44** and their effect on Aβ production by transfected HEK293 cells

Compound	R	Act[a]	Compound	R	Act[a]
25		T	**35**		–
26		+	**36**[b]		+
27		+/–	**37**		–
28[b]		+	**38**		–
29		+	**39**		+
30		T	**40**		–
31		T	**41**		–
32		T	**42**		–
33		T	**43**		T
34		–	**44**		–

[a] Inhibitory activity of Aβ production by HEK293 cells overexpressing Swedish mutant βAPP. Compounds **25**, **26**, **29**, **31**, **34**, **35**, **39**, **41**–**44** have been tested at 60 μM. All others, compounds **27**, **28**, **30**, **32**, **33**, **36**–**38**, **40** have been tested at 10 and 30 μM concentration. + indicated an inhibition >50% at the maximal concentration tested; – indicated inactive; T, significant cell toxicity.

[b] IC_{50} between 10 and 30 μM.

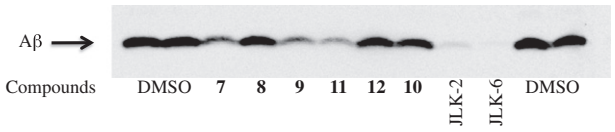


Figure 2. Effect of compounds **7**–**12** (60 μM) on the production of Aβ by βAPPswe over-expressing HEK293 cells (DMSO and JLK-2/6 60 μM as controls).

destruction of the inhibitor) to observe any Aβ-lowering effect in the time of the assay. The 7-amino analogue **2** with an ethoxy group at position 3 behaved like JLK-6 (3-methoxy group) whereas the two other 3-alkoxy-7-amino-4-chloro-isocoumarins (**4**, **6** with a butoxy and an isobutoxy group, respectively) were toxic at 60 μM and inactive at 10 μM concentration (not shown). The activity of **2** and JLK-6 in this cell assay would have two reasons. First, only the 3-alkoxy-7-amino-4-chloro analogues could form irreversible covalent complexes with their putative serine protease target, the activity of which being recovered only after a new biosynthesis. Second, these analogues are more stable to hydrolysis than the nitro derivatives in buffers (about 3.5 h in Hepes buffer[45,55]). The toxicity of compounds **4** and **6** could be explained by the targeting of other protease(s), the function of which being essential for cell survival. Therefore, it appeared difficult to further explore the structural requirements at position 3.

The results obtained with compounds **7**–**12** lacking the 4-chlorine were quite contradictory to those obtained in the 4-chloro series since the 7-amino derivatives **8**, **10**, **12** were inactive, while the 7-nitro analogues **7**, **9**, **11** showed Aβ-lowering activity (Fig. 2). As a matter of fact, the inactivity of compounds **8**, **10** and **12** was in good agreement with a previous study in which compound **8** was shown to inactivate serine proteases quite slowly or not at all, indicating the significance of the chlorine atom. In the same study the nitro analogue **7** was found much more active and the enzymes inactivated by **7** were shown to undergo only transient inactivation and to rapidly regain full activity.[45] However the observed Aβ-lowering activity of the nitro analogues **7**, **9**, **11** could be explained by a higher stability of the resulting acyl enzyme complex formed with their target, as it is known that this stability both depends on the structure of the 3-substituent and on the involved enzyme.[41,45] Overall, **7**, **9**, **11** were found less active than JLK-6 and no modification could be easily considered in this series to increase their potency.

2.1.2.2. Substitution of the 7-amino group of JLK-6. The 7-amino group was the most evident position to substitute with various groups to add possible interaction sites and therefore increase the affinity for the target. This was shown for several *N*-acyl, urethane and ureido derivatives of 7-amino-4-chloro-3-methoxy-isocoumarin (JLK-6), which were better elastase inhibitors compared to the unsubstituted compound.[45,49,55]

A series of acylated derivatives of JLK-6 was prepared (compounds **13**–**17**, Table 2). With exception of compound **17**, all were found inactive at 60 μM concentration. This might be due to unfavourable interactions of the substituents with the target. Another reason might be their inability to form irreversible complexes as it was reported for most *N*-acyl-JLK-6 elastase inhibitors of the above mentioned series,[49] indicating that acylation of the 7-amino group decreases the ability of the inhibitor to alkylate serine proteases by lowering the strong electron-releasing properties of the free amino group. As a result, these acylated compounds might be slow 'enzyme alkylators'.[55] With this view, compounds **13**–**16** could form reversible acyl enzyme complexes, and then undergo rapid hydrolysis with concomitant enzyme reactivation. A similar negative result was previously obtained with JLK-8, the 7-*N*-phenylalanyl derivative of JLK-6.[33] The observed activity of **17** with a pivaloyl, nevertheless less efficient than JLK-6, could be the result of a highly stable complex between **17** and its target as stated above and as it has also been observed for a few *N*-acyl-JLK-6 elastase inhibitors.[49]

None of the compounds **18**–**24** (Table 2) of the carbamate series showed Aβ-lowering activity at the same 60 μM concentration. Again, these negative results could result from unfavourable binding caused by the substituents. It can also be hypothesized that the more electro-attracting oxycarbonyl group, compared to an acyl group, could result in a further decrease of the 7-nitrogen lone pair mobility toward the phenyl ring, disfavouring the irreversible alkylation of an enzyme active site (Fig. 1S). It remains that the negative result obtained with compound **19** with a 7-Adoc substituent is contradictory to that reported for the equivalent analogue of JLK-2 (JLK-7[33]), which showed Aβ-lowering activity in the cell assay. Again, such results could be due to the unpredictable stability of a particular complex, even for close compounds, when no irreversible link between the inhibitor and the putative enzyme is expected.

In these *N*-acyl and carbamate series, it was difficult to establish structure–activity relationships, which could help in choosing further modifications of the isocoumarin derivatives and the formation of a sufficiently stable complex appeared highly unpredictable. Therefore, we developed a last series of JLK-6 analogues with an alkylated (**25**–**42**) or arylated (**43**, **44**) 7-amino group, in which the 7-nitrogen lone-pair was supposedly more prone to electron delocalization (Table 3). All substituents were hydrophobic and classified as linear (**25**–**27**) or branched (**28**–**33**) alkyl chains, arylalkyl (**34**–**42**) and aryl groups (**43**–**44**). Several compounds (**30**–**33**, **43**) showed significant cytotoxicity (measured by an in vitro lactate dehydrogenase (LDH) release assay) at the maximal concentration tested (30 or 60 μM, see note of Table 3). It could be noticed that compounds **30**–**33** shared a branched and bulky alkyl chain. On the contrary, with the exception of compound **25** with an ethyl substituent, all other compounds with a linear (**26**, **27**) or a small branched (**28**, **29**) alkyl chain showed significant Aβ-lowering activity in the cell assay (Table 3). 7-Arylalkyl derivatives were devoid of toxicity at the tested concentrations but most of them did not show any significant activity, with the exception of compounds **36** and **39** with a 4-nitrophenylmethyl

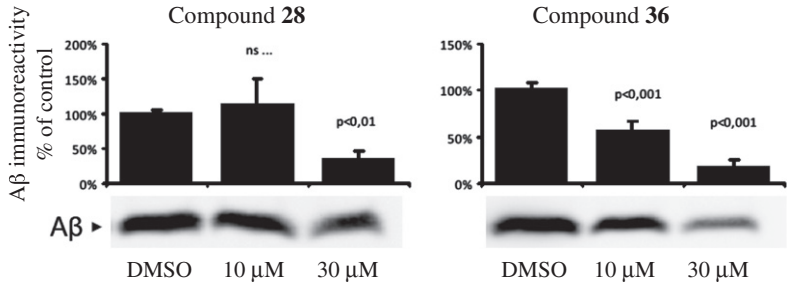


Figure 3. Inhibition of Aβ production of βAPPswe expressing HEK293 cells by compounds **28** and **36**. ns, Non significant.

and 1-naphthalen-1-ylmethyl substituent, respectively. A limited dose–response study was performed for the two most active compounds, **28** and **36**, which were shown to possess similar and slightly higher potency compared to JLK-6, respectively (Fig. 3).

The significant Aβ-lowering activity of several N-alkylated JLK-6 analogues and the cytotoxicity of some others were consistent with an expected higher ability of these compounds to form an irreversible complex with cellular serine proteases compared to acylated and urethane analogues. Therefore, they represented the most interesting series of JLK-6 analogues to further develop to try to increase affinity.

2.2. Peptide boronates

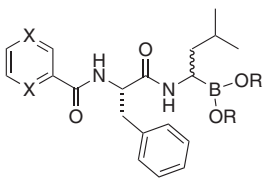
It was shown that small peptide-boronates efficiently block Aβ formation by APP-transfected IMR-32 neuronal cells through inhibition of γ-secretase cleavage.[48] Known as proteasome inhibitors, it was first suggested that the multicatalytic complex proteasome could be directly or indirectly responsible for Aβ production. However, such activity has not been confirmed so far. Another hypothesis is that these compounds act through an unknown target. Peptide–boronates have also been described as potent and reversible serine–protease inhibitors.[56,57] For instance, it was recently reported that Bortezomib belonging to this family efficiently inhibits cathepsin G, chymase and, in particular a mitochondrial protease involved in neuronal cell survival, HtrA2/Omi[58] as do isocoumarins [Ref. 45 and Sévalle J. et al. unpublished results]. That peptide–boronates and isocoumarins share a common target could therefore be envisioned.

To further characterize these compounds, we synthesized a series of 8 peptide boronates (**45**–**52**) differing by the stereochemistry of the amino-boronate residue, the nature of the N-terminal substituent (phenyl or pyrazine) and the boronate state (free boronic acid or pinanediol ester) (Table 4). The peptide boronates were prepared by the general method of Kettner and Shenvi[57] and Matteson et al.[56] (Supplementary data, S2, Scheme 1S). All compounds were tested on βAPPswe overexpressing HEK293 cells at a 60 μM concentration (Fig. 2S). As already noticed,[57,59] the pinanediol esters showed similar behaviours as their free boronic counterparts, consistent with ester hydrolysis occurring in the culture medium leading to free boronic acids. The most active compound was the pinanediol ester **47** with a benzoyl N-terminal residue and a (*R*) stereochemistry. The corresponding boronic acid **45**, which is the most active compound in Christie's study (IC_{50} of about 9 nM),[48] was found less potent. This might be due to a lower cell penetration of the compound related to a lower hydrophobicity compared to the pinanediol ester. An IC_{50} ranging between 10 and 30 μM was measured for **47**, indicating a lower potency than that reported in Christie's study for the free boronic acid. This discrepancy could come from different cell type or assay conditions. A striking result was that none of the pyrazine-containing compounds was found active at 60 μM concentration. This included Bortezomib (compound **46**), a well-known proteasome inhibitor approved for the treatment of multiple myeloma.[60] In Christie's study, Bortezomib was found active but with a fivefold reduction in potency compared to the phenyl-containing counterpart (the two compounds have similar K_i values on proteasome). According to the low activity of **47** and **45** in our assay, a similar fivefold lower activity of Bortezomib **46** (compared to **45**) or its pinanediol ester **48** (compared to **47**) might not allow to observe any activity for the pyrazinyl-containing compounds at an only 60 μM concentration. At least, these results indicated that the peptide boronates target involved in Aβ production was distinct from the proteasome and would possess a high specificity at the level of the P3 substituent (phenyl vs pyrazinyl). It is noteworthy that isocoumarin JLK-6 does not inhibit proteasome.[34] A second striking result was that the (*S*) isomers of **45** and **47** (compounds **49** and **51**, respectively) also inhibited Aβ production, although with lower potency compared to the (*R*) isomers (IC_{50} of compound **51** between 30 and 60 μM). Again, the boronic acid **49** was found less active than the pinanediol ester **51**. The (*S*) stereochemistry corresponds to a D-amino acid. This observation was somewhat unexpected regarding 'normal' interaction of a '*L*' P1 residue with a protease subsite. However, as this residue is the C-terminal one, it is not impossible that the differently oriented boronic moiety can still be accomodated by a protease for covalent linking with the catalytic serine residue, albeit with a lower efficiency. Similar results were reported for close inhibitors showing only three to fivefold differences in IC_{50} between the two isomers toward chymotrypsin and proteasome, respectively.[57,59]

None of the peptide boronates was able to inhibit Aβ production from recombinant C100 substrate present in membrane preparations (Fig. 3S).[61] This result indicates that like isocoumarins, the peptide–boronates did not directly interact with the presenilin-dependent γ-secretase complex.

Altogether, although there is still no direct proof, the similar behaviour of isocoumarins and peptide–boronates observed in our assays suggest they could share an identical target.

Table 4
Structure of compounds **45**–**52** and their effect on Aβ production by transfected HEK293 cells



Compound	X	Stereo-chemistry	R	Act[a]	Compound	X	Stereo-chemistry	R	Act[a]
45	CH	R	H	+	**49**	CH	S	H	+
46	N	R	H	–	**50**	N	S	H	–
47[b]	CH	R	Pinane-diol ester	+	**51**	CH	S	Pinane-diol ester	+
48	N	R	Pinane-diol ester	–	**52**	N	S	Pinane-diol ester	–

[a] Inhibitory activity of Aβ production by HEK293 cells overexpressing swedish mutant βAPP. Compounds **45**–**52** have been tested at 60 μM concentration. + indicated an inhibition >50% at the maximal concentration tested; – indicated inactive.
[b] IC_{50} between 10 and 30 μM.

Peptide–boronates are reversible serine protease inhibitors. They might be modified with a reactive (photoreactive for instance) moiety and used as probes to fish the target.

3. Conclusions

According to the expected limited stability of the isocoumarins in culture medium, an irreversible inactivation of the target should occur in order to observe the inhibition of cellular Aβ production after 8-hours incubation. Supporting this irreversible inactivation was the observation that 1 h treatment of APPswe overexpressing HEK293 cells with 60 μM JLK-6, followed by 7 h incubation in JLK-6-free medium, resulted in Aβ-lowering at a level close to that observed after a 8 h standard treatment (Sévalle et al., unpublished). These results suggested that the isocoumarins performed most of their effect at the beginning of the assay and that this effect is durable, supporting an irreversible target blockade. In the case of peptide–boronates, which are reversible inhibitors, their activity could account for a higher stability, which warrants their presence in the medium for all duration of the assay. The most interesting results were obtained with analogues of the 7-*N*-alkyl-isocoumarin series, which showed Aβ-lowering activity comparable to that of JLK-6. These results together with the inactivity of the acyl and urethane derivatives of JLK-6, except for compound **17**, strongly suggested the significance of the nitrogen electron pair mobility, an essential feature for the irreversible inhibition of serine proteases. These results therefore reinforced the idea that a serine protease, or maybe more generally a serine hydrolase, contributes to a metabolic pathway leading to APP γ-secretase cleavage.

Although a few N-alkylated analogues showed slightly higher potency than JLK-6, this was not sufficient to design efficient molecular probes based on these compounds. We nevertheless synthesized two biotinylated probes of JLK-6, one with a biotinyl–acyl moiety[46] and the other with a biotinyl–aminoalkyl moiety (Supplementary Data, S4; Scheme 2S). The first one was inactive in the cell assay as expected and it was also the case for the second one, indicating that there are still efforts to elaborate isocoumarin-derived efficient probes. The development of isocoumarins substituted at unexplored positions is now considered.

4. Experimental

4.1. Chemistry

Reagents and solvents were obtained from Aldrich, Acros Organics or Alfa Aesar and used without further purification.

^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker instrument using $CDCl_3$ or DMSO-d_6 solutions. Splitting patterns of ^{1}H spectra are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Chemical shifts are reported in ppm relative to solvant signal ($CDCl_3$: δ = 7.26 ppm (^{1}H); 77.16 (^{13}C); DMSO-d_6: δ = 2.50 ppm (^{1}H); 39.52 (^{13}C)).

Thin-layer chromatographies were performed on aluminium backed sheets of silica gel F_{254} (0.2 mm, Fluka), and were visualised under 254 nm light and by spraying with a 2% EtOH solution of ninhydrin followed by heating, or by charring with an aqueous solution of ammonium sulfate and sulfuric acid [200 g $(NH_4)_2SO_4$ and 40 mL concentrated sulfuric acid in 1 L of water]. Column chromatography separations were performed using Merck silica gel 60 of particle size 40–63 μm. Reverse phase HPLC analysis was performed on a Chromolith SpeedRod C18 column (0.46 × 5 cm) by means of a linear gradient (0–100%) of acetonitrile in 0.1% aqueous TFA over 5 min (flow rate: 3 mL/min). Preparative reverse phase HPLC purification was performed on a Waters Delta Pak C18 column (40 × 100 mm, 15 μm, 100 Å) by means of a linear gradient (1%/min) of 0.1% TFA/acetonitrile in 0.1% aqueous TFA over 30 min (flow rate: 28 mL/min). Mass spectrometry: samples were prepared in acetonitrile/water (50/50 v/v) mixture. The LC/MS system consisted of a Waters Alliance 2690 HPLC, coupled to a Waters-Micromass ZQ spectrometer (electrospray ionization mode, ESI+). All analyses were carried out using a RP C18 monolithic Onyx Phenomenex column (25 × 4.6 mm) by means of a linear gradient (0–100%) of eluent B in A over 3 min (flow rate: 3 mL/min). Eluent A: 0.1% aqueous formic acid; eluent B: acetonitrile/0.1% formic acid. Positive ion electrospray mass spectra were acquired at a solvent flow rate of 100–500 μL/min. Nitrogen was used as both the nebulizing and drying gas. The data were obtained in a scan mode in 0.1 s intervals; 10 scans were summed up to get the final spectrum. HRMS were recorded on Q-TOF spectrophotometer using ESI positive mode.

Compounds **1**–**6** were synthesized as previously described.[50,55] Their physico-chemical characteristics are reported in the Supplementary data.

4.1.1. General procedure for the preparation of 4*H* analogues of JLK6 (7–12) from alkyl 2-carboxy-4-nitrophenylacetate

To a solution of alkyl 2-carboxy-4-nitrophenylacetate in anhydrous DCM was added 1.1 equiv of trifluoroacetic acid. After stirring for 1 night at room temperature, the mixture was washed with water and saturated aqueous $NaHCO_3$. The organic phase was then dried on Na_2SO_4, filtered and evaporated under vacuum. The 3-alkoxy-7-nitro-isocoumarin was precipitated in propan-2-ol.

A solution of the preceding compound in THF was treated with hydrogen in the presence of a catalytic amount of 10% Pd/C. After 5 h stirring at room temperature, the mixture was filtered through celite and the celite was washed with THF. The filtrate was evaporated to yield the 3-alkoxy-7-amino-isocoumarins.

4.1.1.1. 3-Methoxy-7-nitro-isocoumarin [3-methoxy-7-nitro-1*H*-2-benzopyran-1-one] (7)[39,45]. Pale yellow solid. Yield, 68% from 500 mg (2.1 mmol) of methyl 2-carboxy-4-nitrobenzeneacetate.[50] HPLC t_R = 2.24 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.67 (d, J = 2.3 Hz, 1H), 8.47 (dd, J = 8.8, 2.3 Hz, 1H), 7.71 (d, J = 8.8 Hz, 1H), 6.23 (s, 1H), 4.01 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 163.2, 160.2, 146.8, 145.3, 130.3, 127.5, 125.9, 117.7, 80.1, 58.2. m/z (ES+) 222.1 ($M+H^+$). HRMS, calcd for $C_{10}H_8NO_5$ ($M+H^+$) 222.0402, found 222.0408.

4.1.1.2. 7-Amino-3-methoxy-isocoumarin [7-amino-3-methoxy-1*H*-2-benzopyran-1-one] (8)[39,45]. Pale yellow solid. Yield, 50% from **7** after HPLC purification. HPLC t_R = 1.09 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.38 (d, J = 2.2 Hz, 1H), 7.32 (d, J = 8.4 Hz, 1H), 7.19 (dd, J = 8.4, 2.2 Hz, 1H), 5.83 (s, 1H), 3.83 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.5, 157.6, 143.0, 131.1, 126.1, 125.0, 117.7, 113.3, 78.4, 56.2. m/z (ES+) 192.1 ($M+H^+$). HRMS, calcd for $C_{10}H_{10}NO_3$ ($M+H^+$) 192.0661, found 192.0677.

4.1.1.3. 3-(2-Methoxyethoxy)-7-nitro-isocoumarin [3-(2-methoxy ethoxy)-7-nitro-1*H*-2-benzopyran-1-one] (9). Yellow solid. Yield, 28% from 300 mg (1.06 mmol) of 2-methoxyethyl 2-carboxy-4-nitrobenzeneacetate.[35] HPLC t_R = 2.30 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.73 (d, J = 2.2 Hz, 1H), 8.51 (dd, J = 8.8, 2.2 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H), 6.28 (s, 1H), 4.40 (t, J = 4.3 Hz, 2H), 3.75 (t, J = 4.3 Hz, 2H), 3.37 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.9, 160.0, 146.5, 145.1, 130.1, 127.3, 125.7, 117.5, 80.7, 70.2, 70.1, 59.1. m/z (ES+) 266.1 ($M+H^+$).

4.1.1.4. 7-Amino-3-(2-methoxyethoxy)-isocoumarin [7-amino-3-(2-methoxyethoxy)-1*H*-2-benzopyran-1-one] (10). Yellow solid. Yield, 70% from **9** after HPLC purification. HPLC t_R = 1.24 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.36 (d, J = 1.8 Hz, 1H), 7.32 (d,

J = 8.4 Hz, 1H), 7.18 (dd, J = 8.4, 1.8 Hz, 1H), 5.88 (s, 1H), 4.22 (t, J = 4.3, 2H), 3.70 (t, J = 4.3 Hz, 2H), 3.35 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 161.5, 157.1, 145.4, 134.1, 126.9, 125.3, 118.7, 113.1, 80.5, 70.6, 69.2, 59.1. m/z (ES+) 236.1 (M+H$^+$).

4.1.1.5. 3-(2-Bromoethoxy)-7-nitro-isocoumarin [3-(2-bromoethoxy)-7-nitro-1*H*-2-benzopyran-1-one] (11)[44,62]. Yellow solid. Yield, 45% from 200 mg (0.6 mmol) of 2-bromoethyl 2-carboxy-4-nitrobenzeneacetate.[35,62] HPLC t_R = 2.71 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.60 (d, J = 2.4 Hz, 1H), 8.44 (dd, J = 8.8, 2.4 Hz, 1H), 7.66 (d, J = 8.8 Hz, 1H), 6.26 (s, 1H), 4.54 (t, J = 5.3 Hz, 2H), 3.84 (t, J = 5.3 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.8, 159.4, 145.8, 144.8, 129.7, 126.9, 125.2, 117.3, 80.7, 69.9, 30.7. m/z (ES+) 314.0 and 316.0 (M+H$^+$).

4.1.1.6. 7-Amino-3-(2-bromoethoxy)-isocoumarin [7-amino-3-(2-bromoethoxy)-1*H*-2-benzopyran-1-one] (12)[44,62]. Yellow solid. Yield, 63% from **11** after HPLC purification. HPLC t_R = 1.55 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.28–7.36 (m, 2H), 7.17 (dd, J = 8.4, 2.1 Hz, 1H), 5.94 (s, 1H), 4.45 (t, J = 5.5, 2H), 3.87 (t, J = 5.5 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 162.5, 155.7, 145.7, 129.9, 126.5, 124.6, 118.4, 112.2, 80.8, 69.3, 30.8. m/z (ES+) 284.0 and 286.0 (M+H$^+$).

4.1.2. General procedure for the preparation of *N*-acyl analogues of JLK-6 (13–17)

Amides **13**–**17** were obtained by acylation of JLK-6 with the appropriate acid chlorides or anhydrides. To a solution of JLK-6 (100 mg, 0.44 mmol) in DCM (10 mL) placed at 0 °C were added dropwise 5 equiv of acid chloride or anhydride in DCM solution (5 mL). After 2 h stirring at room temperature, the solution was diluted in DCM. The organic phase was washed with a 5% aqueous $NaHCO_3$ solution and brine, and dried over Na_2SO_4, filtered and evaporated to dryness. The final pure isocoumarin was crystallized from petroleum ether.

4.1.2.1. 7-(Acetylamino)-4-chloro-3-methoxyisocoumarin [*N*-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-acetamide] (13)[49,50]. Obtained from acetic anhydride. Yellow solid. Yield, 85%. mp 220 °C (Lit. >210 °C). HPLC t_R = 2.24 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.50 (d, J = 1.8 Hz, 1H), 8.00 (dd, J = 8.7, 2.1 Hz, 1H), 7.6 (d, J = 8.7 Hz, 1H), 4.00 (s, 3H), 2.05 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.1, 159.1, 153.0, 138.2, 132.4, 127.5, 122.8, 117.7, 117.2, 57.7, 30.8, 17.5. m/z (ES+) 268.0 and 270.0 (M+H$^+$). HRMS, calcd for $C_{12}H_{10}ClNO_4$ (M+H$^+$) 268.0377, found 268.0374.

4.1.2.2. 7-(4-Carboxybutyramido)-4-chloro-3-methoxyisocoumarin [5-[(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-amino]-5-oxo-pentanoic acid] (14)[49]. Obtained from glutaric anhydride. Yellow solid. Yield, 40%. mp 194 °C (Lit. 194 °C). HPLC t_R = 2.25 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.50 (d, J = 1.8 Hz, 1H), 8.00 (dd, J = 8.7, 2.0 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 3.95 (s, 3H), 2.72 (m, 2H), 2.41 (m, 1H), 2.27 (m, 1H), 1.82 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 174.6, 174.6, 159.2, 153.1, 138.2, 132.5, 127.7, 122.9, 118.6, 117.8, 89.9, 57.8, 35.8, 33.2, 20.8. m/z (ES+) 340.0 and 342.0 (M+H$^+$). HRMS, calcd for $C_{15}H_{15}ClNO_6$ (M+H$^+$) 340.0588, found 340.0596.

4.1.2.3. 7-(Pentafluoropropionylamino)-4-chloro-3-methoxyisocoumarin [*N*-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-2,2,3,3-pentafluoro-propionamide] (15). Obtained from pentafluoropropionic anhydride. Orange powder. Yield, 47%. mp 184 °C. HPLC t_R = 3.06 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.45 (d, J = 1.8 Hz, 1H), 8.15 (dd, J = 8.7, 2.0 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 4.05 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 158.8, 155.9, 153.9, 135.1, 134.8, 132.8, 129.4, 123.1, 121.3, 117.7, 106.5, 89.3, 57.7; ^{19}F NMR (not decoupled, DMSO-d_6): 122 (s, CF_2), 82 (s, CF_3). m/z (ES+) 372.0 and 374.0 (M+H$^+$). HRMS, calcd for $C_{13}H_8ClF_5NO_4$ (M+H$^+$) 372.0062, found 372.0066.

4.1.2.4. 7-(Octanoylamino)-4-chloro-3-methoxyisocoumarin [*N*-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-octanamide] (16). Obtained from octanoyl chloride. Pale yellow solid. Yield, 35%. mp 148 °C. HPLC t_R = 3.46 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.50 (d, J = 2.1 Hz, 1H), 8.05 (dd, J = 8.7, 2.2 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 3.95 (s, 3H), 2.14 (m, 2H), 1.44 (m, 2H), 1.21 (m, 8H), 0.82 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 171.8, 158.7, 152.6, 137.9, 132.1, 127.2, 122.5, 118.1, 117.4, 89.6, 57.4, 36.5, 31.2, 28.6, 28.5, 25.0, 22.1, 13.9. m/z (ES+) 352.0 and 354.0 (M+H$^+$). HRMS, calcd for $C_{18}H_{23}ClNO_4$ (M+H$^+$) 352.1316, found 352.1328.

4.1.2.5. 7-(Pivaloylamino)-4-chloro-3-methoxyisocoumarin [*N*-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-pivalamide] (17). Obtained from pivaloyl chloride. Pale yellow solid. Yield, 65%. mp 187 °C. HPLC t_R = 2.80 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.60 (s, 1H), 8.50 (d, J = 2.0 Hz, 1H), 8.15 (dd, J = 8.7, 2.0 Hz, 1H), 7.60 (d, J = 8.7 Hz, 1H), 4.05 (s, 3H), 1.25 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.4, 159.2, 153.1, 138.4, 137.1, 132.6, 128.7, 122.6, 119.8, 117.6, 89.8, 57.7, 27.5. m/z (ES+) 310.0 and 312.0 (M+H$^+$). HRMS, calcd for $C_{15}H_{17}ClNO_4$ (M+H$^+$) 310.0846, found 310.0832.

4.1.3. General procedure for the preparation of carbamoyl analogues of JLK6 (18–24)

Carbamates **18**–**24** were obtained by acylation of JLK-6 with the appropriate halogenoformates or anhydrides. In a typical experiment, 50 mg (0.22 mmol) of JLK-6 was suspended in dry THF (1 mL). After cooling at 0 °C, $NaHCO_3$ (41 mg, 0.49 mmol) was added, followed by R-CO_2X (X = Cl, F) or (R-CO)$_2$O (1.1 equiv, 0.24 mmol). After warming to room temperature, the reaction mixture was stirred until disappearance of the starting product (3–48 h). The reaction was then quenched with water and extracted with AcOEt. Organic phases were finally dried on Na_2SO_4, filtered and evaporated under vacuum.

4.1.3.1. 7-[((Benzyloxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [phenylmethyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (18)[49]. Obtained from benzyl chloroformate (42 mg, 35 μL). Yellow solid. Yield, 51%. HPLC t_R = 3.24 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.18 (s, 1H), 8.28 (s, 1H), 7.87 (dd, J = 8.7, 1.2 Hz, 1H), 7.59 (d, J = 8.7 Hz, 1H), 7.31–7.42 (m, 5H), 5.15 (s, 2H), 3.97 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.1, 158.9, 153.8, 138.2, 136.7, 132.0, 128.9, 128.6, 128.5, 126.9, 123.1, 118.0, 117.4, 92.9, 66.5, 57.8. m/z (ES+) 360.1 and 362.1 (M+H$^+$). HRMS, calcd for $C_{18}H_{15}ClNO_5$ (M+H$^+$) 360.0639 and 362.0609, found 360.0645 and 362.0650.

4.1.3.2. 7-[((Adamantyloxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [adamantyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (19). Obtained from adamantyl fluoroformate (48 mg). Yellow solid. Yield, 76%. HPLC t_R = 3.75 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.81 (s, 1H), 8.29 (s, 1H), 7.84 (d, J = 9.0 Hz, 1H), 7.54 (d, J = 8.7 Hz, 1H), 3.96 (s, 3H), 1.52–2.10 (m, 15H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.1, 152.8, 152.6, 138.6, 131.7, 126.9, 122.9, 117.9, 117.3, 90.0, 79.9, 57.8, 45.7, 41.6, 40.1, 36.3, 36.0, 35.5, 31.1, 30.8, 30.5. m/z (ES+) 404.2 and 406.2 (M+H$^+$). HRMS, calcd for $C_{21}H_{23}ClNO_5$ (M+H$^+$) 404.1265 and 406.1235, found 404.1268 and 406.1257.

4.1.3.3. 7-[((9-Fluorenylmethoxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [9-fluorenylmethyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (20)[49]. Obtained from 9-fluorenylmethyl chloroformate (62 mg), the solid was washed with MeCN. Pale yellow solid. Yield, 30%. HPLC t_R = 3.67 min. ^{1}H

NMR (300 MHz, DMSO-d_6): δ 10.09 (s, 1H), 8.27 (s, 1H), 7.87 (m, 3H), 7.71 (d, J = 6.9 Hz, 1H), 7.56 (d, J = 8.1 Hz, 1H), 7.29–7.41 (m, 4H), 4.50 (d, J = 5.4 Hz, 2H), 4.29 (t, J = 5.7 Hz, 1H), 3.96 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.0, 153.8, 152.9, 144.1, 141.2, 138.1, 132.1, 128.1, 127.5, 127.0, 125.5, 123.0, 120.6, 118.0, 117.6, 89.9, 66.2, 57.8, 47.0. m/z (ES+) 448.2 and 450.2 (M+H$^+$). HRMS, calcd for $C_{25}H_{19}ClNO_5$ (M+H$^+$) 448.0952 and 450.0922, found 448.0958 and 450.0801.

4.1.3.4. 7-[((Ethyloxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [ethyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (21)[49]. Obtained from ethyl chloroformate (26 mg, 23 μL). Yellow solid. Yield, 54%. HPLC t_R = 2.77 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.02 (s, 1H), 8.27 (s, 1H), 7.87 (dd, J = 8.7, 1.2 Hz, 1H), 7.58 (d, J = 8.7 Hz, 1H), 4.12 (q, J = 6.9 Hz, 2H), 3.97 (s, 3H), 1.23 (t, J = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.1, 153.9, 152.8, 138.4, 131.9, 126.9, 123.0, 118.0, 117.4, 89.9, 61.0, 57.8, 14.9. m/z (ES+) 298.1 and 300.1 (M+H$^+$). HRMS, calcd for $C_{13}H_{13}ClNO_5$ (M+H$^+$) 298.0482 and 300.0453, found 298.0490 and 300.0463.

4.1.3.5. 7-[((*t*Butyloxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [*t*butyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (22). Obtained from di-tertbutyldicarbonate (52 mg). Yellow–orange solid. Yield, 67%. HPLC t_R = 3.12 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.77 (s, 1H), 8.30 (s, 1H), 7.84 (dd, J = 8.7, 0.6 Hz, 1H), 7.55 (d, J = 8.7 Hz, 1H), 3.96 (s, 3H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.1, 153.1, 152.8, 138.7, 131.7, 126.8, 122.9, 117.9, 117.2, 90.0, 80.2, 57.8, 28.5. m/z (ES+) 326.1 and 328.1 (M+H$^+$). HRMS, calcd for $C_{15}H_{17}ClNO_5$ (M+H$^+$) 326.0795 and 328.0766, found 326.0789 and 328.0782.

4.1.3.6. 7-[((Trichloroethoxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [trichloroethyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (23). Obtained from trichloroethyl chloroformate (153 μL). Yellow solid. Yield, 85%. mp 203 °C. HPLC t_R = 3.30 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.35 (d, J = 1.8 Hz, 1H), 7.95 (dd, J = 8.8, 2.1 Hz, 1H), 7.65 (d, J = 8.8 Hz, 1H), 5.00 (s, 2H), 4.05 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.0, 153.1, 152.2, 137.4, 132.6, 128.9, 125.3, 123.1, 118.0, 96.1, 74.0, 57.7, 29.4. m/z (ES+) 400.0, 402.0 and 404.0 (M+H$^+$).

4.1.3.7. 7-[((Allyloxy)carbonyl)amino]-4-chloro-3-methoxyisocoumarin [allyl-(4-chloro-3-methoxy-1-oxo-1*H*-2-benzopyran-7-yl)-carbamate] (24). Obtained from allyl chloroformate (29 mg, 25 μL). Yellow solid. Yield, 60%. HPLC t_R = 2.89 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.14 (s, 1H), 8.27 (s, 1H), 7.88 (dd, J = 8.7, 0.6 Hz, 1H), 7.59 (d, J = 8.7 Hz, 1H), 5.95 (m, 1H), 5.35 (d, J = 17.7 Hz, 1H), 5.21 (d, J = 10.2 Hz, 1H), 4.60 (d, J = 4.8 Hz, 2H), 3.97 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.1, 153.6, 152.9, 138.2, 133.5, 132.1, 126.9, 123.1, 118.3, 118.0, 117.4, 89.9, 65.4, 57.8. m/z (ES+) 310.1 and 312.1 (M+H$^+$). HRMS, calcd for $C_{14}H_{13}ClNO_5$ (M+H$^+$) 310.0482 and 312.0453, found 310.0496 and 312.0516.

4.1.4. General procedure for the preparation of *N*-alkyl analogues of JLK6 (25–42)

The N-alkyl analogues of JLK-6 (**25**–**42**) were obtained by reductive amination of JLK-6 with the appropriate aldehydes. In a typical experiment, 100 mg (0.44 mmol) of JLK-6 and the aldehyde (1.2 equiv, 0.53 mmol) were solubilized in dry DCM (3 mL) and the reaction mixture was stirred at room temperature for 15 min. Then, $NaBH_3CN$ (2 equiv, 55 mg, 0.88 mmol) was added and stirring was continued for 16 h. After addition of a saturated aqueous $NaHCO_3$ solution, the mixture was extracted with DCM, and the organic layer was dried over MgSO4, filtered and concentrated in vacuo. When possible, the product was precipitated by addition of H_2O/CH_3CN (2:1). The yellow precipitate was filtered, washed with H_2O/CH_3CN (1:1) and dried. As an alternative, the product was purified by preparative reverse phase HPLC.

4.1.4.1. 4-Chloro-7-(ethylamino)-3-methoxy-isocoumarin [4-chloro-7-(ethylamino)-3-methoxy-1*H*-2-benzopyran-1-one] (25). Obtained from acetaldehyde (23 mg) and purified by reverse phase HPLC. Yellow solid. Yield, 9%. R_f (Hex/EtOAc, 4:1) 0.36. HPLC t_R = 2.15 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.51 (d, J = 8.7 Hz, 1H), 7.27 (dd, J = 8.7, 2.5 Hz, 1H), 7.20 (d, J = 2.4 Hz, 1H), 4.00 (s, 3H), 3.15 (q, J = 7.1 Hz, 2H), 1.23 (t, J = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 151.5, 148.6, 126.5, 123.9, 123.3, 119.4, 109.2, 92.0, 58.6, 38.4, 14.8. m/z (ES+) 254.1 and 256.1 (M+H$^+$). HRMS, calcd for $C_{12}H_{13}ClNO_3$ (M+H$^+$) 254.0584 and 256.0554, found 254.0597 and 256.0580.

4.1.4.2. 4-Chloro-7-(hexylamino)-3-methoxy-isocoumarin [4-chloro-7-(hexylamino)-3-methoxy-1*H*-2-benzopyran-1-one] (26). Obtained from hexanal (53 mg). Yellow solid. Yield, 9%. HPLC t_R = 3.43 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.46 (d, J = 8.5 Hz, 1H), 7.20 (dd, J = 8.5, 1.6 Hz, 1H), 7.13 (d, J = 1.6 Hz, 1H), 3.96 (s, 3H), 3.06 (t, J = 6.7 Hz, 2H), 1.50–1.62 (m, 2H), 1.19–1.44 (m, 6H), 0.87 (t, J = 5.3 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 151.4, 149.1, 126.0, 123.9, 123.0, 119.5, 108.7, 92.0, 58.6, 43.6, 32.0, 29.2, 27.2, 23.0, 14.8. m/z (ES+) 310.1 and 312.1 (M+H$^+$). HRMS, calcd for $C_{16}H_{21}ClNO_3$ (M+H$^+$) 310.1210 and 312.1180, found 310.1181 and 312.1205.

4.1.4.3. 4-Chloro-3-methoxy-7-(nonylamino)-isocoumarin [4-chloro-3-methoxy-7-(nonylamino)-1*H*-2-benzopyran-1-one] (27). Obtained from nonanal (76 mg) and purified by reverse phase HPLC. Yellow solid. Yield, 13%. HPLC t_R = 4.70 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.41 (d, J = 8.7 Hz, 1H), 7.18 (dd, J = 8.7, 2.4 Hz, 1H), 7.09 (d, J = 2.4 Hz, 1H), 3.92 (s, 3H), 3.01 (m, 2H), 1.51 (m, 2H), 1.21 (m, 12H), 0.81 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.7, 155.0, 148.5, 125.6, 123.4, 122.5, 119.0, 108.3, 91.7, 58.1, 43.2, 31.7, 29.4, 29.3, 29.1, 28.7, 27.0, 22.5, 14.3. m/z (ES+) 352.1 and 354.1 (M+H$^+$). HRMS, calcd for $C_{19}H_{27}ClNO_3$ (M+H$^+$) 352.1679 and 354.1650, found 352.1689 and 354.1671.

4.1.4.4. 4-Chloro-7-(isobutylamino)-3-methoxy-isocoumarin [4-chloro-7-(isobutylamino)-3-methoxy-1*H*-2-benzopyran-1-one] (28). Obtained from isobutyraldehyde (19 mg) and purified by reverse phase HPLC. Yellow solid. Yield, 11%. HPLC t_R = 2.97 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.41 (d, J = 8.7 Hz, 1H), 7.20 (dd, J = 8.7, 2.4 Hz, 1H), 7.10 (d, J = 2.1 Hz, 1H), 3.92 (s, 3H), 2.86 (d, J = 6.6 Hz, 2H), 1.83 (sept., J = 6.3 Hz, 1H), 0.91 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.7, 150.9, 148.8, 125.5, 123.9, 122.4, 119.0, 108.3, 91.6, 58.2, 27.7, 20.8. m/z (ES+) 282.1 and 284.1 (M+H$^+$). HRMS, calcd for $C_{14}H_{17}ClNO_3$ (M+H$^+$) 282.0897 and 284.0867, found 282.0897 and 284.0878.

4.1.4.5. 4-Chloro-3-methoxy-7-[(3-methyl-butyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(3-methyl-butyl)amino]-1*H*-2-benzopyran-1-one] (29). Obtained from isovaleraldehyde (46 mg) and purified by reverse phase HPLC. Yellow solid. Yield, 20%. mp 52 °C. R_f (Hex/EtOAc, 4:1) 0.63. HPLC t_R = 3.21 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.51 (d, J = 8.7 Hz, 1H), 7.22–7.32 (m, 2H), 4.01 (s, 3H), 3.14 (t, J = 7.2 Hz, 2H), 1.68–1.84 (m, 1H), 1.52 (dd, J = 7.2, 7.2 Hz, 2H), 0.96 (d, J = 6.6 Hz, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 151.6, 148.2, 126.8, 123.9, 123.5, 119.4, 109.7, 91.9, 58.6, 42.3, 38.0, 26.2, 23.3 (2C). m/z (ES+) 296.1 and 298.1 (M+H$^+$). HRMS, calcd for $C_{15}H_{19}ClNO_3$ (M+H$^+$) 296.1053, found 296.1046.

4.1.4.6. 4-Chloro-7-[(2-ethyl-hexyl)amino]-3-methoxy-isocoumarin [4-chloro-7-[(2-ethyl-hexyl)amino]-3-methoxy-1*H*-2-benzopyran-1-one] (30). Obtained from 2-ethylhexanal (68 mg) and purified by flash column chromatography on silica gel (eluent hexane to hexane/EtOAc (90:10). Yellow solid. Yield, 7%. HPLC t_R = 4.81 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.41 (d, J = 8.7 Hz, 1H), 7.19 (dd, J = 8.7, 2.4 Hz, 1H), 7.11 (d, J = 2.4 Hz, 1H), 3.92 (s, 3H), 3.26 (s, 1H), 2.94 (m, 2H), 1.51 (m, 1H), 1.25–1.41 (m, 8H), 0.83 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.7, 150.8, 148.8, 125.4, 123.4, 122.5, 119.0, 108.1, 90.0, 58.2, 46.4, 38.4, 31.0, 28.8, 24.2, 23.0, 14.4, 11.1. m/z (ES+) 338.2 and 340.2 (M+H$^+$). HRMS, calcd for $C_{18}H_{25}ClNO_3$ (M+H$^+$) 338.1523 and 340.1493, found 338.1515 and 340.1508.

4.1.4.7. 4-Chloro-7-[(2,2-dimethyl-propyl)amino]-3-methoxy-isocoumarin [4-chloro-7-[(2,2-dimethyl-propyl)amino]-3-methoxy-1*H*-2-benzopyran-1-one] (31). Obtained from 2,2-dimethylpropanal (46 mg) and purified by preparative HPLC. Yellow solid. Yield, 34%. mp 141 °C. R_f (Hex/EtOAc, 4:1) 0.74. HPLC t_R = 3.44 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.48 (d, J = 8.7 Hz, 1H), 7.36 (d, J = 8.7 Hz, 1H), 7.27 (s, 1H), 4.00 (s, 3H), 2.94 (s, 2H), 1.01 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 151.3, 149.8, 125.8, 123.8, 122.9, 119.4, 108.8, 92.1, 58.6, 55.4, 33.1, 28.4 (3C). m/z (ES+) 296.1 and 298.1 (M+H$^+$). HRMS, calcd for $C_{15}H_{19}ClNO_3$ (M+H$^+$) 296.1053 and 298.1024, found 296.1048 and 298.1037.

4.1.4.8. 4-Chloro-7-[(2,2-dimethylbutyl)amino)]-3-methoxy-isocoumarin [4-chloro-7-[(2,2-dimethylbutyl)amino]-3-methoxy-1*H*-2-benzopyran-1-one] (32). Obtained from 2,2-dimethylbutanal (53 mg) and purified by reverse phase HPLC. Yellow solid. Yield, 16%. HPLC t_R = 3.84 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.42 (d, J = 8.7 Hz, 1H), 7.15 (dd, J = 8.7, 2.4 Hz, 1H), 7.10 (d, J = 2.4 Hz, 1H), 3.92 (s, 3H), 3.04 (m, 2H), 1.46 (m, 2H), 0.92 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.7, 150.9, 148.4, 125.8, 123.4, 122.7, 119.0, 108.4, 91.6, 58.1, 42.4, 39.7, 30.0, 29.7. m/z (ES+) 310.1 and 312.2 (M+H$^+$). HRMS, calcd for $C_{16}H_{21}ClNO_3$ (M+H$^+$) 310.1210 and 312.1180, found 310.1214 and 312.1186.

4.1.4.9. 4-Chloro-7-(cyclohexylmethylamino)-3-methoxy-isocoumarin [4-chloro-7-(cyclohexylmethylamino)-3-methoxy-1*H*-2-benzopyran-1-one] (33). Obtained from cyclohexylcarbaldehyde (60 mg) and purified by flash column chromatography on silica gel (Hex/EtOAc, 99:1). Yellow solid. Yield, 7%. R_f (Hex/EtOAc, 9:1) 0.73. HPLC t_R = 4.67 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.40 (d, J = 8.7 Hz, 1H), 7.18 (dd, J = 8.7, 2.4 Hz, 1H), 7.09 (d, J = 2.4 Hz, 1H), 3.92 (s, 3H), 2.89 (d, J = 7.5 Hz, 2H), 1.19–1.65 (m, 11H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.8, 153.3, 148.8, 123.5, 123.4, 122.5, 119.0, 108.2, 92.4, 58.2, 42.1, 37.1, 31.1, 28.0, 25.9. m/z (ES+) 322.1 and 324.1 (M+H$^+$). HRMS, calcd for $C_{17}H_{21}ClNO_3$ (M+H$^+$) 322.1210 and 324.1180, found 322.1196 and 324.1154.

4.1.4.10. 4-Chloro-3-methoxy-7-(phenylmethylamino)-isocoumarin [4-chloro-3-methoxy-7-(phenylmethylamino)-1*H*-2-benzopyran-1-one] (34). Obtained from benzaldehyde (56 mg) and purified by washing the solid with H_2O/MeCN (50:50). Yellow solid. Yield, 10%. mp 122 °C. R_f (Hex/EtOAc, 4:1) 0.52. HPLC t_R = 3.15 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.49 (d, J = 8.7 Hz, 1H), 7.24–7.44 (m, 6H), 7.21 (d, J = 2.4 Hz, 1H), 4.41 (s, 2H), 3.99 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.0, 151.6, 148.5, 140.0, 129.3 (2C), 128.1 (2C), 127.8, 126.7, 123.9, 123.5, 119.4, 109.7, 92.0, 58.6, 47.2. m/z (ES+) 316.1 and 318.1 (M+H$^+$). HRMS, calcd for $C_{17}H_{15}ClNO_3$ (M+H$^+$) 316.0740 and 318.0711, found 316.0740 and 318.0699.

4.1.4.11. 4-Chloro-3-methoxy-7-[(4-methoxyphenylmethyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(4-methoxyphenylmethyl)amino]-1*H*-2-benzopyran-1-one] (35). Obtained from 4-methoxybenzaldehyde (72 mg) and purified by washing the solid with H_2O/MeCN (50:50) and cold diethylether. Yellow solid. Yield, 52%. mp 141–142 °C. R_f (Hex/EtOAc, 4:1) 0.35. HPLC t_R = 3.12 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 7.47 (d, J = 8.9 Hz, 1H), 7.33 (d, J = 8.4 Hz, 2H), 7.28 (dd, J = 8.9, 2.1 Hz, 1H), 7.20 (d, J = 2.1 Hz, 1H), 6.93 (d, J = 8.4 Hz, 2H), 4.32 (d, J = 5.3 Hz, 2H), 3.99 (s, 3H), 3.76 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 159.2, 151.5, 148.7, 131.8, 129.3 (2C), 126.5, 123.8, 123.3, 119.3, 114.7 (2C), 109.5, 91.9, 58.6, 55.9, 46.6. m/z (ES+) 346.1 and 348.1 (M+H$^+$). HRMS, calcd for $C_{18}H_{17}ClNO_4$ (M+H$^+$) 346.0846 and 348.0817, found 346.0850 and 348.0851.

4.1.4.12. 4-Chloro-3-methoxy-7-[(4-nitrophenylmethyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(4-nitrophenylmethyl)amino]-1*H*-2-benzopyran-1-one] (36). Obtained from 4-nitrobenzaldehyde (80 mg) and purified by washing the solid with H_2O/MeCN (50:50) and cold diethylether. Yellow solid. Yield, 30%. R_f (Hex/EtOAc, 4:1) 0.12. HPLC t_R = 3.05 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.16 (d, J = 8.1 Hz, 2H), 7.58 (d, J = 8.1 Hz, 2H), 7.41 (d, J = 8.7 Hz, 1H), 7.21 (d, J = 8.7 Hz, 1H), 7.08 (s, 1H), 4.50 (d, J = 5.4 Hz, 2H), 3.90 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.5, 159.0, 148.3, 147.7, 147.0, 128.5, 126.5, 124.0, 123.5, 122.9, 118.9, 109.2, 91.3, 58.1, 46.1. m/z (ES+) 361.0 and 363.0 (M+H$^+$). HRMS, calcd for $C_{17}H_{14}ClN_2O_5$ (M+H$^+$) 361.0591 and 363.0562, found 361.0604 and 363.0592.

4.1.4.13. 4-Chloro-3-methoxy-7-[(2-nitrophenylmethyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(2-nitrophenylmethyl)amino]-1*H*-2-benzopyran-1-one] (37). Obtained from 2-nitrobenzaldehyde (80 mg) and purified by washing the solid with H_2O/MeCN (50:50) and cold diethylether. Yellow solid. Yield, 29%. R_f (Hex/EtOAc, 4:1) 0.2. HPLC t_R = 3.05 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.04 (d, J = 8.1 Hz, 1H), 7.65 (m, 1H), 7.54 (m, 1H), 7.42 (d, J = 8.7 Hz, 1H), 7.20 (dd, J = 8.7, 2.1, 1H), 7.10 (d, J = 1.8 Hz, 1H), 6.94 (m, 1H), 4.67 (d, J = 6.0 Hz, 2H), 3.91 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.5, 148.7, 147.8, 134.9, 134.2, 132.3, 129.6, 128.8, 126.6, 125.4, 123.5, 122.6, 118.9, 109.1, 93.1, 58.1, 44.1. m/z (ES+) 361.0 and 363.0 (M+H$^+$). HRMS, calcd for $C_{17}H_{14}ClN_2O_5$ (M+H$^+$) 361.0591 and 363.0562, found 361.0603 and 363.0597.

4.1.4.14. 7-[(4-Benzyloxyphenylmethyl)amino]-4-chloro-3-methoxy-isocoumarin 7-[(4-benzyloxyphenylmethyl)amino]-[4-chloro-3-methoxy-1*H*-2-benzopyran-1-one] (38). Obtained from 4-benzyloxybenzaldehyde (113 mg) and purified by washing the solid with H_2O/MeCN (50:50) and cold diethylether. Yellow solid. Yield, 13%. HPLC t_R = 3.48 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 6.79–7.37 (m, 12H), 5.02 (s, 2H), 4.23 (m, 1H), 3.90 (s, 3H), 3.25 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.6, 157.7, 151.0, 148.2, 137.6, 131.6, 128.9, 128.8, 128.2, 128.0, 126.0, 123.3, 122.8, 118.9, 115.1, 109.0, 92.3, 69.6, 58.1, 46.1. m/z (ES+) 422.2 and 424.2 (M+H$^+$). HRMS, calcd for $C_{24}H_{21}ClNO_4$ (M+H$^+$) 422.1159 and 424.1130, found 422.1170 and 424.1152.

4.1.4.15. 4-Chloro-3-methoxy-7-[(1-naphthalenylmethyl) amino]-isocoumarin [4-chloro-3-methoxy-7-[(1-naphthalenylmethyl)amino]-1*H*-2-benzopyran-1-one] (39). Obtained from 1-naphthaldehyde (83 mg) and purified by washing the solid with cold MeCN. Yellow solid. Yield, 34%. mp 162 °C. R_f (Hex/EtOAc, 4:1) 0.38. HPLC t_R = 3.55 min. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.19 (d, J = 7.7 Hz, 1H), 8.01 (dd, J = 8.7, 2.0 Hz, 1H), 7.89 (d, J = 7.7 Hz, 1H), 7.44–7.65 (m, 4H), 7.35 (dd, J = 8.7, 2.0, 1H), 7.27 (d, J = 2.0 Hz, 1H), 6.95–7.02 (m, 1H), 4.84 (d, J = 5.2 Hz, 2H), 3.99 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 151.5, 148.9, 134.9, 134.4, 132.0, 129.4, 128.5, 127.1, 126.7, 126.6, 126.3, 125.9, 124.5, 123.9, 123.2, 119.4, 109.3, 92.0, 58.6, 45.4. m/z (ES+) 366.0

and 368.0 ($M+H^+$). HRMS, calcd for $C_{21}H_{17}ClNO_3$ ($M+H^+$) 366.0897 and 366.0867, found 366.0898 and 368.0899.

4.1.4.16. 4-Chloro-3-methoxy-7-[(2-naphthalenylmethyl) amino]-isocoumarin [4-chloro-3-methoxy-7-[(2-naphthalenylmethyl) amino]-1*H*-2-benzopyran-1-one] (40). Obtained from 2-naphthaldehyde 83 mg) and purified by flash column chromatography on silica gel (Hex/EtOAc, 9:1). Yellow solid. Yield, 19%. R_f (Hex/EtOAc, 9:1) 0.81. HPLC t_R = 4.47 min. ^1H NMR (300 MHz, DMSO-d_6): δ 7.79–7.87 (m, 4H), 7.44–7.51 (m, 3H), 7.41 (d, J = 8.4 Hz, 1H), 7.26 (dd, J = 8.7, 2.4 Hz, 1H), 7.16 (d, J = 2.4 Hz, 1H), 4.50 (s, 2H), 3.89 (s, 3H), 3.29 (sl, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.6, 151.0, 148.2, 137.2, 133.4, 132.6, 128.5, 128.0, 127.9, 126.6, 126.1 (2C), 126.0, 125.6, 123.4, 122.9, 118.9, 109.1, 91.4, 58.1, 46.9. m/z (ES+) 366.1 and 368.1 ($M+H^+$). HRMS, calcd for $C_{21}H_{17}ClNO_3$ ($M+H^+$) 366.0897 and 366.0867, found 366.0890 and 368.0887.

4.1.4.17. 4-Chloro-3-methoxy-7-[(3-phenylpropyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(3-phenylpropyl)amino]-1*H*-2-benzopyran-1-one] (41). Obtained from 3-phenylpropanal (71 mg) and purified by HPLC. Yellow solid. Yield, 20%. mp 98 °C. R_f (Hex/EtOAc, 4:1) 0.60. HPLC t_R = 3.37 min. ^1H NMR (300 MHz, DMSO-d_6): δ 7.46 (d, J = 8.7 Hz, 1H), 7.20–7.32 (m, 6H), 7.14 (d, J = 2.4 Hz, 1H), 3.96 (s, 3H), 3.09 (t, J = 6.9 Hz, 2H), 2.70 (t, J = 7.5 Hz, 2H), 1.88 (dt, J = 7.5, 6.9 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.2, 151.5, 148.8, 142.6, 129.2 (4C), 126.7, 126.3, 123.9, 123.1, 119.5, 109.0, 92.0, 58.6, 43.2, 33.5, 31.0. m/z (ES+) 344.1 and 346.1 ($M+H^+$). HRMS, calcd for $C_{19}H_{19}ClNO_3$ ($M+H^+$) 344.1053 and 346.1024, found 344.1071 and 346.1020.

4.1.4.18. 4-Chloro-3-methoxy-7-[(3-phenyl-2-propen-1-yl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(3-phenyl-2-propen-1-yl)amino]-1*H*-2-benzopyran-1-one] (42). Obtained from 3-phenyl-2-propenal (70 mg) and purified by washing the solid with H_2O/MeCN (40:60). Yellow solid. Yield, 41%. mp 142 °C. R_f (Hex/EtOAc, 4:1) 0.32. HPLC t_R = 3.34 min. ^1H NMR (300 MHz, DMSO-d_6): δ 7.51 (d, J = 8.7 Hz, 1H), 7.41–7.46 (m, 2H), 7.22–7.39 (m, 5H), 6.65 (d, J = 16.0 Hz, 1H), 6.40 (dt, J = 16.0, 5.3 Hz, 1H), 3.85–4.08 (m, 5H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 160.1, 151.5, 148.7, 137.5, 131.3, 129.5 (2H), 128.3, 127.9, 127.0 (2C), 126.5, 123.9, 123.2, 119.4, 109.5, 92.0, 58.6, 45.5. m/z (ES+) 342.1 and 344.1 ($M+H^+$). HRMS, calcd for $C_{19}H_{17}ClNO_3$ ($M+H^+$) 342.0897 and 344.0867, found 342.0899 and 344.0873.

4.1.5. General procedure for the preparation of *N*-aryl analogues of JLK6 (43 and 44)

To a solution of JLK-6 (100 mg, 0.44 mmol) in dry DCM containing 4 Å molecular sieves, were added the arylboronic acid (2 equiv), $Cu(OAc)_2$ (1.5 equiv) and Et_3N (2 equiv). After 3 days stirring at room temperature, the suspension was filtered and the filtrate was evaporated to dryness. The residue was taken up in H_2O/MeCN (50:50) to precipitate the desired product.[52]

4.1.5.1. 4-Chloro-3-methoxy-7-(phenylamino)-isocoumarin [4-chloro-3-methoxy-7-(phenylamino)-1*H*-2-benzopyran-1-one] (43). Obtained from phenylboronic acid (108 mg) and purified by washing the solid with H_2O/MeCN (50:50). Brown solid. Yield, 64%. R_f (Hex/EtOAc, 4:1) 0.46. HPLC t_R = 3.23 min. ^1H NMR (300 MHz, DMSO-d_6): δ 7.74 (m, 1H), 7.62 (m, 2H), 7.36 (m, 2H), 7.18 (d, J = 7.5 Hz, 2H), 6.99 (m, 1H), 4.02 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 159.8, 152.4, 143.8, 142.8, 130.3 (2C), 129.4, 125.9, 124.2, 122.2, 119.2, 119.0 (2C), 113.6, 91.2, 58.4. m/z (ES+) 302.0 and 304.0 ($M+H^+$). HRMS, calcd for $C_{16}H_{13}ClNO_3$ ($M+H^+$) 302.0584 and 304.0554, found 302.0611 and 304.0611.

4.1.5.2. 4-Chloro-3-methoxy-7-[(3-nitrophenyl)amino]-isocoumarin [4-chloro-3-methoxy-7-[(3-nitrophenyl)amino]-1*H*-2-benzopyran-1-one] (44). Obtained from 3-nitrophenylboronic acid (148 mg) and purified by washing the solid with MeCN and cold diethylether. Brown solid. Yield, 16%. R_f (Hex/EtOAc, 4:1) 0.32. HPLC t_R = 3.19 min. ^1H NMR (300 MHz, C_3D_6O): δ 8.34 (s, 1H), 7.99 (m, 2H), 7.80 (m, 3H), 7.62 (d, J = 6.0 Hz, 2H), 4.13 (s, 3H). ^{13}C NMR (75 MHz, C_3D_6O): δ 161.9, 159.5, 144.8, 131.6, 131.2, 127.3, 124.3, 123.3, 119.1, 116.3, 115.4, 110.9, 109.9, 102.9, 92.4, 58.3. m/z (ES+) 347.0 and 349.0 ($M+H^+$). HRMS, calcd for $C_{16}H_{12}ClN_2O_5$ ($M+H^+$) 347.0435 and 349.0405, found 347.0420 and 349.0413.

4.1.6. Peptide boronates (45–52)

The peptide boronates were prepared by the general method of Kettner and Shenvi[57] and Matteson and Sadhu.[56] Their physicochemical properties are reported in the Supplementary data.

4.2. Biology

4.2.1. Effect of compounds on Aβ production by stably transfected HEK293 cells overexpressing βAPPswe (Swedish mutant K670N/M671L)

HEK cells have been established as described.[53] Cellular in vitro Aβ production was measured by following the expression of Aβ-40 and Aβ-42 by means of FCA3340 and FCA3542 antibodies as previously described.[63] These antibodies, developed by us are particularly useful for immunoprecipitation of Aβ and Western blot analysis. Briefly, the cells were incubated for 7 h in secretion medium (OptiMem supplemented with 1% FBS) in the presence of various inhibitor concentrations. Media were collected, completed with one tenth of 10× RIPA buffer and incubated with a 200-fold dilution of antibodies overnight at 4 °C under agitation. Protein A-Agarose was then added to the media and stirred three more hours at 4 °C. After a quick centrifugation step to remove supernatants, agarose beads were boiled 5 min in Tris–Tricine loading buffer. Recovered proteins were resolved on 16.5% Tris–Tricine poly-acrylamide gels, transferred onto nitrocellulose membranes, which were heated in boiling 1× PBS for 5 min and blocked in 5% skimmed milk in 1× PBS containing 0.05% Tween 20. Membranes were then incubated overnight with 6E10 monoclonal antibody and blots were visualized by enhanced chemiluminescence (ECL, Amersham). Images were acquired using Fuji's Luminescence Image Analyser LAS-3000 (Raytest, Courbevoie, France) and quantified using Aida analyser software.

4.2.2. In vitro γ-secretase assay on membrane preparations

The production of the recombinant γ-secretase substrate (C100-Flag), the preparation of the 'solubilized' membranes fraction and the in vitro γ-secretase assay were described in details in a previous study.[61]

In brief, 'solubilized membranes' (5 μL) obtained from HEK293 cells were first diluted in sodium citrate buffer and then incubated with 10 μL of reaction buffer containing recombinant C100-Flag (50 μg/mL). The 20 μL resulting reaction mixtures were incubated over constant agitation for 16 h at 37 °C (or 4 °C for negative controls). Samples were then supplemented with 2× Tris–Tricine loading buffer, boiled for 5 min and subjected to western blot as described above.

4.2.3. Cytotoxicity

Cell toxicity of the different compounds was analyzed using an in vitro lactate deshydrogenase (LDH) release assay (CytoTox-One, Promega).

Briefly, cells were grown on 24-wells plates and treated with the compounds at the maximal concentrations as described above. After treatment, 20 μL of media were collected and analyzed for

LDH content according to the manufacturer's instructions. These values were compared to both total LDH activity contained in one well (cell lysate) and the corresponding LDH release in the medium of a control well.

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Supplementary data

Supplementary data (S1, the hypothesized mechanism of inhibition of serine proteases by isocoumarins (Fig. 1S); S2, characterization data for compounds **1**–**6**; S3, synthetic pathway (Scheme 1S) and characterization data and biological evaluation of peptide-boronates **45**–**52** (Figs. 2S and 3S); S4, structure of biotinylated probes of JLK-6 (Scheme 2S)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.045.

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