Structure−Activity Relationship of Hydroxamate-Based Inhibitors on the Secretases that Cleave the Amyloid Precursor Protein, Angiotensin Converting Enzyme, CD23, and Pro-Tumor Necrosis Factor-α†

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ABSTRACT: Multiple proteins are proteolytically shed from the membrane, including the amyloid precursor protein (APP) involved in Alzheimer's disease, the blood pressure regulating angiotensin converting enzyme (ACE), the low affinity IgE receptor CD23, and the inflammatory cytokine tumor necrosis factor-α (TNF-α). The inhibitory effect of a range of hydroxamic acid-based compounds on the secretases involved in cleaving and releasing these four proteins has been examined to build up a structure−activity relationship. Compounds have been identified that can discriminate between TNF-α convertase and the other three secretases (compound 15), between the shedding of CD23 and the shedding of APP and ACE (compound 21), and between the secretases and matrix metalloproteinase-1 (compound 22). The structure−activity relationship for the APP α-secretase and the ACE secretase were remarkably similar, and both secretases were activated in whole cell systems by the serine proteinase inhibitor 3,4-dichloroisocoumarin. The basal and carbachol-stimulated shedding of APP and ACE from human SH−SY5Y neuroblastoma cells could not be differentiated by any of the hydroxamate compounds, implying that the same or very similar activities are involved in the constitutive and regulated shedding of these two proteins. By utilizing a key discriminatory compound (compound 15) that potently inhibits TNF-α convertase but not α-secretase, we show that TNF-α convertase is not involved in the regulated shedding of APP from human neuronal cells. The compounds reported here will be useful in future studies aimed at identifying and validating candidate secretases.

Numerous integral membrane proteins are released from the cell surface through a posttranslational proteolytic cleavage event (reviewed in refs *1* and *2*). Proteins converted from a membrane-bound to a soluble form by such proteolytic cleavage include the Alzheimer's amyloid precursor protein (APP),[1] angiotensin converting enzyme (ACE), pro-tumor necrosis factor-α (proTNF-α), and the low affinity IgE receptor CD23. The proteolytic cleavage occurs on the extracellular face of the lipid bilayer in the membrane-proximal stalk region of the protein. The proteinases responsible for these cleavage events are referred to collectively as secretases, sheddases, or convertases.

In the nonamyloidogenic processing pathway, α-secretase cleaves APP within the amyloid *β*-peptide sequence, precluding deposition of intact amyloidogenic peptide (*3*, *4*). The large soluble N-terminal ectodomain cleaved from APP by α-secretase (sAPPα) is released from the cell surface and appears to have neuroprotective and memory enhancing properties (*5*−*7*). α-Secretase is a membrane-associated proteinase (*8*, *9*) that appears to act almost exclusively at the plasma membrane of neuronal cells (*10*). The cleavage of APP by α-secretase occurs constitutively in all cell lines examined, but can be upregulated by phorbol esters and activation of some G-protein coupled receptors, for example, stimulation of muscarinic (M_1 and M_3) receptors by muscarine or carbachol (*11*−*13*). The phorbol ester-induced release, but not the constitutive release, of sAPPα has been shown to be deficient in nonneuronal cells derived from TNF-α convertase knockout mice (*14*, *15*). TNF-α convertase is a member of the ADAM (*a d*isintegrin *a*nd *m*etalloproteinase) family of proteins (*16*, *17*) and was first identified as a functional enzyme responsible for processing pro-TNF-α to its mature form (*18*, *19*). Analysis of cells lacking TNF-α convertase has indicated that the enzyme might have an expanded role in the shedding of other cell-surface proteins including a TNF receptor, the L-selectin adhesion molecule and transforming growth factor-α (*20*).

Like APP, angiotensin converting enzyme (ACE; EC 3.4.15.1) is a type I integral membrane glycoprotein that is proteolytically cleaved and released from the cell surface by its cognate zinc metallosecretase (*21*). TNF-α convertase

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[1] Abbreviations: ACE, angiotensin converting enzyme; ADAM, a disintegrin and metalloproteinase; APP, amyloid precursor protein; CHO, Chinese Hamster ovary; DCI, 3,4-dichloroisocoumarin; MMP, matrix metalloproteinase; PVDF, poly(vinylidene) difluoride; sAPPα, soluble APP shed by α-secretase; TNF-α, tumour necrosis factor-α.

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does not efficiently cleave ACE (*22*), and the release of ACE is not reduced in cells derived from TNF-α convertase deficient mice (*23*), indicating that the ACE secretase is distinct from TNF-α convertase. CD23 or FcεRII, the low-affinity IgE receptor, is a type II integral membrane protein that has a role in the human immune response. Interleukin-4 stimulates proteolytic processing of CD23 to multiple soluble fragments (*24*) which are biologically active as immunostimulatory cytokines and regulate the synthesis of IgE. The secretase that cleaves and releases CD23 from the plasma membrane is also a zinc metalloproteinase (*25*).

Although these and numerous other cell surface proteins are known to be proteolytically shed, the identity of the secretase responsible is, in the majority of cases, not known. Many of these processes are inhibited by batimastat and structurally related hydroxamate compounds such as marimastat and BB2116 with IC_{50} values of 1−10 μM (*25*−*27*) (reviewed in ref *1*). However, such compounds were originally designed as active site directed inhibitors of the matrix metalloproteinases (MMPs) such as collagenase and gelatinase (*28*, *29*), and therefore are not selective for either a particular secretase or the secretase family as a whole. Even the TNF-α convertase inhibitors TAPI-2 and IC-3 are not selective, inhibiting other ADAMs and secretases (*26*, *30*). Thus, there is the urgent need to identify more selective and potent inhibitors of the various shedding events, to provide tools that can discriminate between individual secretases and that can be used to confirm the involvement of candidate secretases in particular shedding events.

This study is the first to compare the inhibitory effects of a range of hydroxamate-based compounds on multiple shedding events, including the α-secretase cleavage of APP, the secretases that cleave ACE and CD23, and TNF-α convertase in an attempt to identify compounds selective for particular shedding events. In addition, we have examined the effect of selected compounds against MMP-1 (interstitial collagenase; EC 3.4.24.7) to identify secretase-specific inhibitors. The remarkably similar inhibition profile with this large range of hydroxamate-based compounds, as well as the observation that both α-secretase and ACE secretase are stimulated by 3,4-dichloroisocoumarin (DCI), further underlines that these two secretases are very closely related activities. In addition, a subset of the compounds failed to differentiate between the constitutive and regulated release of either sAPPα or ACE, indicating that the same secretase is likely involved in both processes. Finally, a key discriminatory compound was used to show that TNF-α convertase is not involved in the regulated release of sAPPα from a human neuronal cell line.

EXPERIMENTAL PROCEDURES

Compounds. Batimastat and compounds 11−30 (structures as shown in Figure 1) were synthesized as described previously (*31*−*33*). The compounds are numbered from 11 onward to avoid confusion with compounds 1−9 in our previous studies on this topic (*26*, *27*).

Cell Culture. The neuronal cell lines SH−SY5Y and IMR-32 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (50 units/mL), streptomycin (50 mg/mL), and 2 mM glutamate (all Gibco BRL, Paisley, UK). Cells were maintained at 37 °C in 5% CO_2 in air. When the cells were confluent, the medium was changed to Opti-MEM, and the cells were incubated with the indicated compounds for 7 h. The medium was then harvested, centrifuged at 1000*g* to remove cell debris, and concentrated approximately 50-fold using Vivaspin centrifugal concentrators (10 000 molecular weight cut off) (Vivascience Ltd., Lincoln, UK). The hydroxamate-based compounds were dissolved in dimethylsulfoxide and used at concentrations up to 100 μM, at which they did not affect cell viability.

The monocytic cell line THP-1 was maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum, nonessential amino acids, penicillin (50 units/mL), streptomycin (50 mg/mL) and 2 mM glutamate (all Gibco BRL, Paisley, UK) at 37 °C in 5% CO_2 in air. THP cells in a 24-well plate (10^6 cells/well) were incubated in fresh medium for 6 h. Conditioned medium from the cells was assayed for TNF-α by specific ELISA (Boehringer Mannheim, Lewes, East Sussex, UK) following the manufacturer's instructions.

Transfection of Cells. IMR-32 cells were grown to 70% confluence in 25 cm^2 flasks, washed once with 4 mL of Opti-MEM, and then transiently transfected using lipofectamine and 8 μg of DNA diluted in 2 mL of Opti-MEM. The expression vector pECE containing a C-terminal fragment of human ACE in which the N-terminal signal peptide was fused with the C-terminal domain (pECE hACE) (*34*) was used for transfection. After 5 h, 3 mL of growth medium was added to the flasks, and the cells were incubated overnight. The lipofectamine/medium mix was then replaced with 5 mL of fresh growth medium. After a further 24 h, the cells were washed with 4 mL of Opti-MEM and compounds were added, diluted as appropriate in 5 mL of Opti-MEM. After 7 h, the medium was removed from the cells, concentrated for analysis of sAPPα or used unconcentrated for determination of ACE activity.

SH−SY5Y cells were grown to 70% confluency, trypsinized, and stably transfected by electroporation with 30 μg of the DNA encoding the C-terminal fragment of human ACE in the vector pIRES*hyg* (*35*). Transfected cells were selected with hygromycin B (4 μg/mL). The cells were then incubated with compounds as described above.

α-*Secretase Assay.* Concentrated conditioned medium (25 μg of protein) was resolved on 10% or 7−17% polyacrylamide SDS gels and electroblotted onto Immobilon P poly(vinylidene) difluoride (PVDF) membranes (Millipore) (*27*). Membranes were probed for sAPPα using antibody 6E10 (Signet Laboratories Inc., Dedham, MA) or antibody Ab1−25, which recognize the N-terminal sequence of the amyloid β-peptide and thus only detect sAPPα, followed by a secondary horseradish peroxidase-conjugated antibody. Bound antibody was detected with the enhanced chemiluminescent detection system (Amersham, Little Chalfont, UK). IC_{50} values were determined by quantitative densitometric analysis of the immunoelectrophoretic blots. The dose−response curves were the best fit to the experimental points using the program Origin. Protein concentrations were determined using the bicinchoninic acid method (*36*).

ACE Secretase Assay. Porcine kidney cortex microvillar membranes were preincubated in 0.1 M borate buffer, pH 8.3, for 20 min at 4 °C in the absence or presence of inhibitors, and then incubated at 37 °C for 4 h. After the incubation, the secretase-cleaved form of ACE was separated

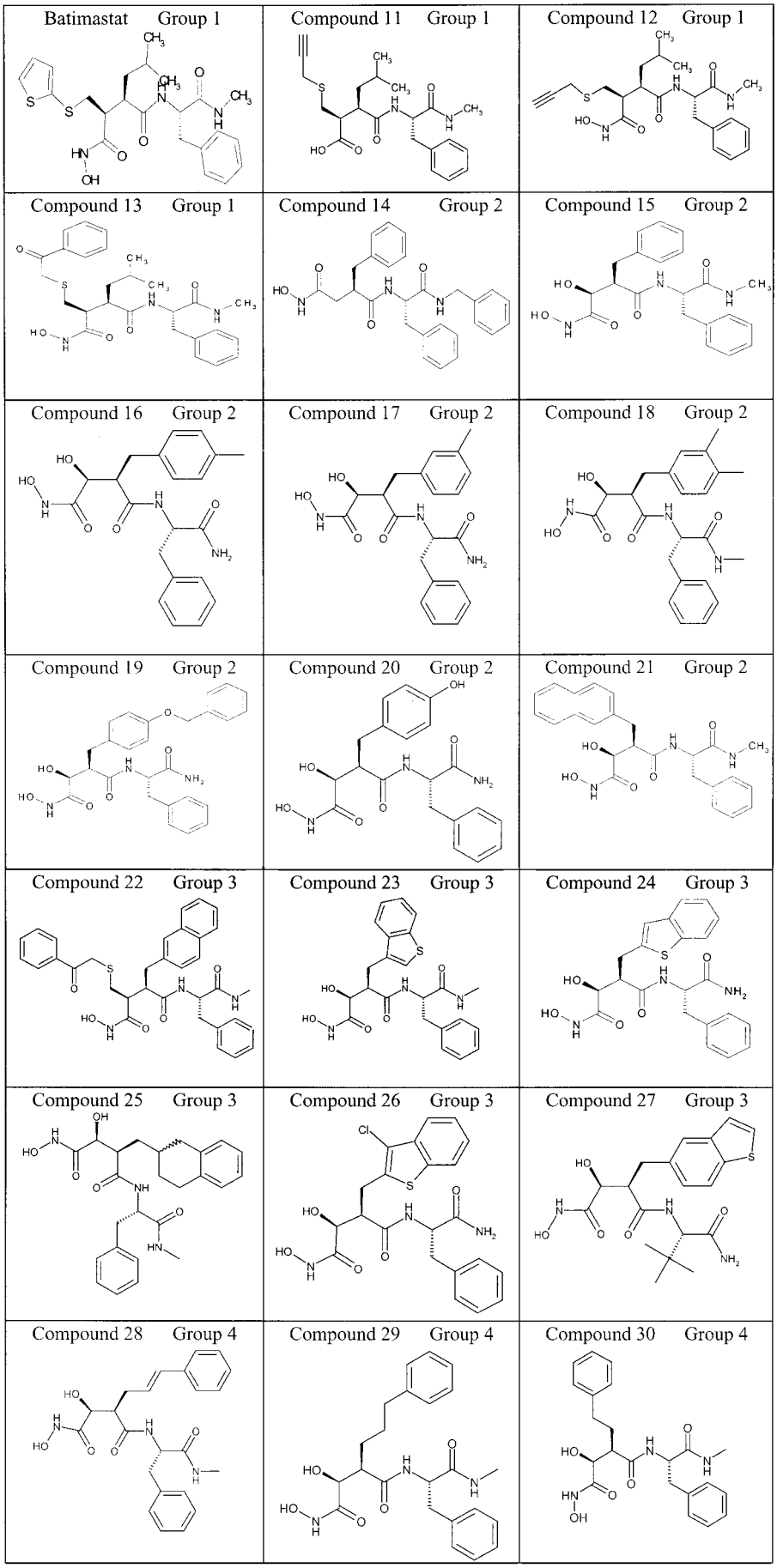

FIGURE 1: Structures of the hydroxamate-based compounds used in this study.

from the uncleaved membrane-bound form by temperature-induced phase separation in Triton X-114 (*26*, *37*). The resulting detergent-rich and aqueous phases were assayed for ACE enzymic activity with BzGly−His−Leu (5 mM) as substrate in 0.1 M Tris/HCl, 0.3 M NaCl, 10 μM $ZnCl_2$, pH 8.3. Reactions were terminated by heating at 100 °C for 4 min, and the substrate and reaction products were resolved and quantified by reverse-phase HPLC. (*38*). Secretase activity is equivalent to the amount of ACE in the final aqueous phase as a percentage of the total amount of ACE in both the aqueous and detergent-rich phases. ACE secretase activity in cell cultures was monitored by measuring the enzymic activity of the released ACE in the conditioned medium with BzGly−His−Leu as substrate.

CD23 Secretase Assay. Various concentrations of inhibitor were incubated for 1−3 h at 37 °C with plasma membranes prepared from RPMI 8866 human B-cells. The incubation was terminated by the addition of 10 μM batimastat and, following filtration, the amount of soluble CD23 was measured by ELISA (*25*).

TNF-α Convertase Assay and Western Blot Analysis. Recombinant TNF-α convertase was incubated with 833 μM TNF-α7 peptide (Ac-SPLAQAVRSSSR-NH_2; both provided by Dr. R. Black, Immunex Corporation, Seattle, USA) for 30 min in 10 mM Tris/HCl, pH 7.8. Following incubation at 37 °C, the reaction was stopped by heating at 100 °C, and the substrate and reaction products were resolved and quantitated by reverse phase HPLC (Waters Millennium system) with a C18 column using a linear gradient of 0−50% MeCN in 0.1% trifluoroacetic acid with detection at 214 nm. For Western blot analysis of TNF-α convertase a confluent 80 cm^2 flask of SH−SY5Y cells was harvested and resuspended in 2 mL of lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10 mM 1,10-phenanthroline, 1% (v/v) Triton X-100, 0.1% (v/v) Nonidet P-40). The cells were lysed by 30 passages through a 21-gauge needle, and insoluble material was removed by centrifugation at 11600*g* for 10 min. Protein (30 μg) was separated on an 8% polyacrylamide SDS gel and electroblotted onto PVDF membranes. The membrane was incubated in the absence or presence of a monoclonal anti-TNF-α convertase primary antibody (provided by Dr. R. Black, Immunex Corporation, Seattle, USA), followed by a secondary horseradish peroxidase-conjugated antibody. Bound antibody was detected with the enhanced chemiluminescent detection system (Amersham, Little Chalfont, UK).

MMP-1 Assay. MMP-1 (interstitial collagenase) was assayed using an internally quenched fluorogenic peptide substrate as described previously (*39*).

RNA Extraction, RT-PCR, and Northern Blot Analysis. Total RNA was extracted from SH−SY5Y cells and THP-1 cells using TRIzol reagent (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. The following primers were designed to human TNF-α convertase: TACEP1, 5′-CCG GAT GAC CCG GGC TTC GGC CCC CAC CAG-3′ (175 to 204 bp); TACEP2, 5′-GCC AAG CAA ACT TTA GAT GCT TCC TCA GC-3′ (reverse 1090 to 1118 bp); TACEP3, 5′-CAG AAT CAA CAC AGA TGG GGC CG-3′ (528 to 550 bp). Reverse-transcription PCR was performed using Titan One Tube RT-PCR system (Boehringer Mannheim, Lewes, East Sussex, UK). Briefly, 1 μg of total RNA was mixed with 0.2 mM dNTPs, 0.4 μM TNF-α convertase primers (TACEP1 and TACEP2), 5 mM dithiothreitol, 1.5 mM Mg^{2+}, and 1 μL of enzyme mix in 50 μL of reaction volume. PCR products were analyzed by electrophoresis on 1% agarose gels. For Northern blot analysis, total cellular RNA was size fractionated by formaldehyde gel electrophoresis, then blotted onto Hybond N+ membrane (Amersham, Slough, UK), and hybridized with ^{32}P-labeled TNF-α convertase cDNA fragment generated by RT-PCR as described above. The TNF-α convertase cDNA fragment was purified from the agarose with Geneclean II kit (BIO 101 Inc., Vista, CA).

RESULTS

Structure−Activity Relationship for Hydroxamate-Based Compounds on α-Secretase, ACE Secretase, CD23 Secretase, and TNF-α Convertase. The inhibitory effect of a range of hydroxamate-based compounds on the activity of the constitutive α-secretase was assessed by monitoring the basal release of sAPPα from the IMR32 neuronal cell line. Dose−response curves were generated for each compound, and the IC_{50} values were determined (Table 1). Three compounds, 13, 22, and 24, were the most potent inhibitors of α-secretase, with IC_{50} values lower than that of batimastat (IC_{50} 1.2 μM) (Table 1). The inhibitory effect of the compounds on the constitutive ACE secretase was determined by monitoring the release of ACE from porcine kidney microvillar membranes (*26*). As with α-secretase, two of the most potent inhibitors of ACE secretase were compounds 13 and 22, although compound 24 was slightly less potent on ACE secretase as compared to α-secretase (Table 1). On the whole, the inhibitory effect of the compounds on α-secretase was remarkably similar to their effect on ACE secretase. Significant differences in inhibitory potency were only observed with those compounds that were relatively weak inhibitors of both activities, i.e., with IC_{50} values above 10 μM against one of the secretases, e.g., compounds 14, 15, 18, 20, and 21.

The inhibitory effect of the compounds on the constitutive CD23 secretase was determined by monitoring the release of CD23 from membranes derived from RPMI 8866 cells (*25*). Although compounds 13, 22, and 24 were again the most potent compounds at inhibiting CD23 secretase, they were all at least an order of magnitude more potent on CD23 secretase than on either α-secretase or ACE secretase (Table 1). Compound 21 inhibited the CD23 secretase with an IC_{50} of 0.4 μM, but was at least 20-fold less potent an inhibitor of α-secretase and ACE secretase. Of the subset of compounds tested against TNF-α convertase, the most discriminatory inhibitor was compound 15, which inhibited TNF-α convertase with an IC_{50} of 0.20 μM, but which was a relatively poor inhibitor of the other three secretases, particularly α-secretase (IC_{50} 38 μM) and the ACE secretase (IC_{50} 16 μM) (Table 1).

As batimastat is a broad spectrum inhibitor of MMPs, we examined whether some of the other hydroxamate-based compounds inhibited MMP-1. Clear differences were observed with several compounds between their effect on MMP-1 and the secretases cleaving APP, ACE, and CD23. For example, compound 15 was a relatively weak inhibitor of α-secretase, ACE secretase, and CD23 secretase (IC_{50} 5−38 μM) but was a potent inhibitor of MMP-1 (IC_{50} 0.09

Table 1: Effect of Hydroxamate-Based Compounds on α-Secretase, ACE Secretase, CD23 Secretase, TNF-α Convertase, and MMP-1

		IC_{50} (μM)				
compd no.	group	α-secretase[a]	ACE secretase[b]	CD23 secretase[c]	TNF-α convertase[d]	MMP-1[e]
Batimastat	1	1.2	1.6[f]	0.1	0.019[g]	0.005
11	1	>20	>100	>20	n.d.	n.d.
12	1	2.05 ± 0.46	2.21 ± 1.00	0.055	n.d.	<0.03
13	1	0.45 ± 0.12	0.43 ± 0.13	0.016 ± 0.005	1.34 ± 0.64	<0.03
14	2	64.00 ± 8.62	9.03 ± 0.86	5.0	n.d.	0.5
15	2	37.67 ± 5.78	15.63 ± 6.96	5.0	0.20 ± 0.01	0.09
16	2	74.67 ± 19.20	66.00 ± 6.03	5.3 ± 1.0	n.d.	n.d.
17	2	>100	>100	20	n.d.	n.d.
18	2	26.67 ± 17.53	>100	>10	n.d.	n.d.
19	2	>20	>100	>10	n.d.	n.d.
20	2	25.33 ± 8.45	>100	4.2 ± 0.7	n.d.	n.d.
21	2	7.63 ± 4.19	19.99 ± 5.67	0.4	n.d.	>1.0
22	3	0.44 ± 0.03	0.23 ± 0.03	0.02 ± 0.007	0.61 ± 0.03	>10
23	3	>100	>100	>1	n.d.	n.d.
24	3	0.32 ± 0.05	1.06 ± 0.31	0.02	0.08 ± 0.04	0.14
25	3	3.67 ± 0.99	5.97 ± 2.20	0.14 ± 0.07	n.d.	0.36
26	3	>100	>100	>10	n.d.	n.d.
27	3	3.83 ± 1.93	3.17 ± 1.02	0.1 ± 0.01	n.d.	10
28	4	2.83 ± 0.46	4.87 ± 0.67	0.15 ± 0.04	n.d.	0.25
29	4	1.63 ± 1.28	0.84 ± 0.16	0.02 ± 0.005	0.16 ± 0.05	<0.1
30	4	2.43 ± 1.34	13.30 ± 3.21	1	n.d.	0.04

[a] IMR32 cells were incubated in the presence of the indicated compounds as described in the experimental section. Medium was harvested, and constitutively released sAPPα was quantified by densitometric analysis of the Western blots. [b] The effect of the compounds on the activity of the constitutive ACE secretase was determined using the colocalized porcine kidney microvillar membrane assay as described in the experimental section. [c] The effect of compounds on the activity of CD23 secretase was determined using membranes derived from RPMI 8866 cells as described in the experimental section. [d] The effect of compounds on TNF-α convertase was determined using recombinant enzyme and the TNF-α7 peptide substrate as described in the experimental section. [e] The effect of compounds on MMP-1 was determined using a fluorogenic peptide substrate as described in the experimental section. Results are the mean (± SEM) of three independent dose−response curves. n.d., not determined. [f] Data from ref *27*. [g] Data from ref *22*.

μM). In contrast, compounds 22 and 27 were relatively potent inhibitors of the three secretases but less potent inhibitors of MMP-1. In particular, compound 22 was one of the most potent compounds against α-secretase, ACE secretase, and CD23 secretase (IC_{50} 0.02−0.4 μM) but failed to inhibit MMP-1 up to a concentration of 10 μM.

α-Secretase and ACE Secretase Are Stimulated by 3,4-Dichloroisocoumarin. The release of ACE from CHO cells has been reported to be stimulated by the serine protease inhibitor 3,4-dichloroisocoumarin (DCI) (*40*). Due to the previously noted similarities between ACE secretase and α-secretase (reviewed in ref *41*), which are reinforced here with a larger range of inhibitory compounds, we examined whether DCI would promote the release of sAPPα from the IMR32 cells. For direct comparison, ACE was transfected into the cells, and the effect of DCI on the release of both ACE and sAPPα was examined. Incubation of the IMR-32 cells with DCI (1 or 10 μM) resulted in a significant increase in the release of both sAPPα and ACE (Figure 2A). The DCI-stimulated release of both sAPPα and ACE was completely blocked by batimastat (Figure 2B). In contrast, incubation of porcine kidney microvillar membranes with DCI up to concentrations of 10 μM failed to cause an increase in the release of ACE above the basal level (data not shown).

The Regulated and Constitutive Release of sAPPα and ACE Are Inhibited Similarly by Hydroxamate Compounds. We next compared the effect of a number of the hydroxamate compounds at inhibiting the constitutive and regulated secretion of sAPPα and ACE. The release of sAPPα from the human neuroblastoma SH−SY5Y cells is known to be stimulated by activation of muscarinic receptors with either muscarine or the muscarinic agonist carbachol, and thus this cell line has been widely used for investigating the regulated release of sAPPα (*27*, *42*). For a direct comparison of the effect of the compounds on the release of sAPPα and ACE, the SH−SY5Y cells were stably transfected with the single domain form of ACE (*43*). The transfected cells were then stimulated either with (regulated release) or without (constitutive release) carbachol. A subset of the hydroxamate compounds were incubated with the cells at a single concentration of 20 μM (Figure 3). The extent of inhibition exerted by each of the compounds on the constitutive and regulated cleavage of APP was remarkably similar (Figure 3A). Similarly, the extent of inhibition exerted by each compound on the constitutive and regulated cleavage of ACE was again very similar (Figure 3B). Both the constitutive and carbachol-stimulated release of sAPPα were inhibited by batimastat in a dose-dependent manner. Quantitative densitometric analysis of the immunoblots revealed that batimastat inhibited the constitutive release of sAPPα from the SH−SY5Y cells with an IC_{50} of 1.2 μM. In carbachol-stimulated cells, the IC_{50} for inhibition of sAPPα release by batimastat was 0.3 μM. Thus, none of the compounds examined clearly differentiated between the constitutive and regulated release of either sAPPα or ACE.

TNF-α Convertase Is Expressed in the Human Neuroblastoma SH−SY5Y Cells But Is not Involved in the Regulated Release of sAPPα. Initially, we determined whether TNF-α convertase is expressed in the SH−SY5Y cells by reverse-transcription PCR of total cellular RNA using TNF-α convertase-specific primers. As a positive control, RNA extracted from THP-1 cells, which are known to express TNF-α convertase (*18*), was used. Electrophoresis

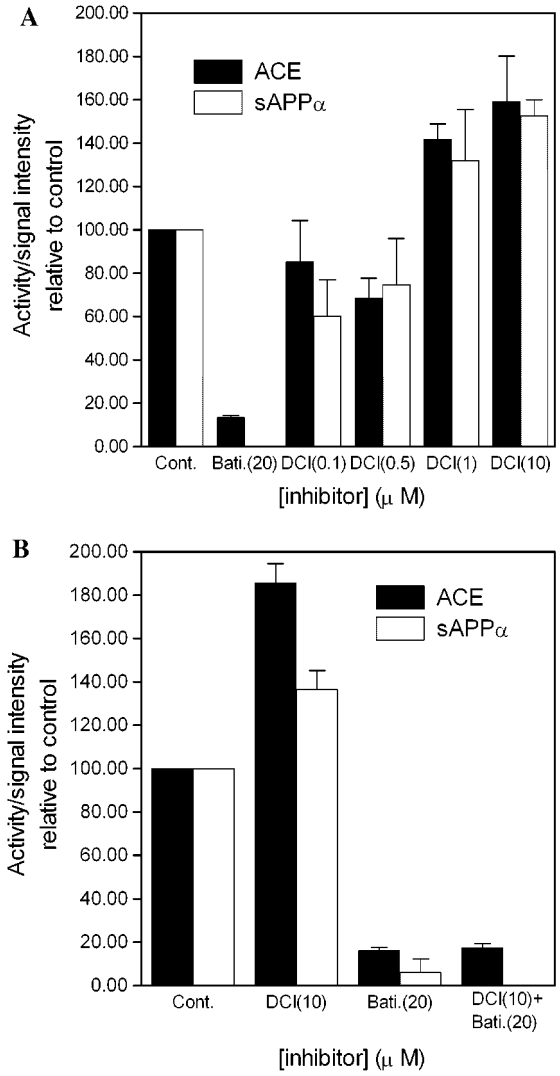

FIGURE 2: DCI stimulates the release of sAPPα and ACE. IMR-32 cells, transfected with the cDNA encoding ACE, were incubated for 7 h in Opti-MEM containing the indicated concentrations of DCI and/or batimastat (panels A and B). ACE activity measured in the medium is represented in nmoles of BzGly produced per 40 μL of medium per 5 h. sAPPα was quantitated in the medium by densitometric analysis of the immunoblot using antibody 1−25. Results (± SEM) are the mean of triplicate determinations.

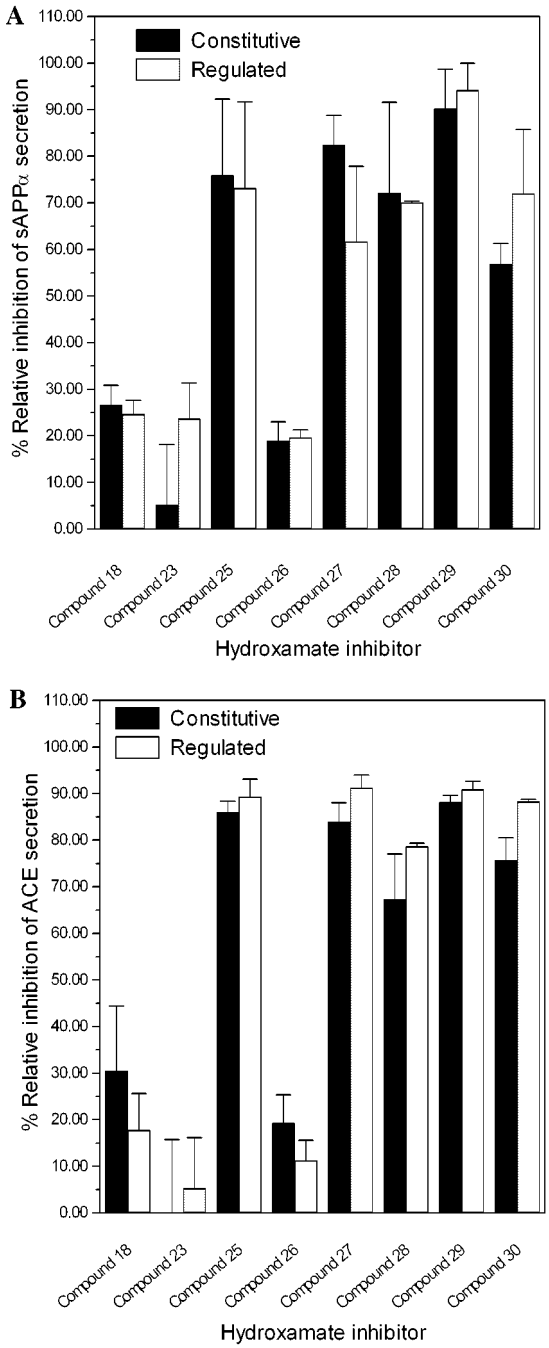

FIGURE 3: Effect of hydroxamate compounds on the constitutive and regulated release of sAPPα and ACE. SH−SY5Y cells stably expressing ACE were incubated in the presence of 20 μM inhibitor (constitutive secretion) or 20 μM carbachol + 20 μM inhibitor (regulated secretion) for 7 h in Opti-MEM. The conditioned medium was harvested and concentrated, and equal amounts of protein were either electrophoresed on 7−17% polyacrylamide SDS gels and then Western blotted with antibody 6E10 followed by densitometric analysis to quantify sAPPα (panel A) or assayed with BzGly−His−Leu to quantify ACE (panel B). The results are the mean (± SEM) of three experiments.

of the PCR products on agarose gels showed one band of approximately 1 kb in both THP-1 and SH−SY5Y cells, consistent with cDNA generated from the TNF-α convertase-specific primer pair. To eliminate the possibility that the cDNA product was a result of DNA contamination, the reverse transcriptase enzyme was inactivated by preincubation at 100 °C for 10 min. Subsequent RT-PCR with SH−SY5Y total RNA did not yield any cDNA. The specificity of the PCR was enhanced by using the 1 kb cDNA PCR product as template cDNA for a second PCR using the original reverse TNF-α convertase-specific primer (TACEP2) and a third TNF-α convertase-specific primer (TACEP3) approximately 350 base pairs downstream of primer TACEP1. Purified template cDNA from both THP-1 cells and SH−SY5Y cells yielded a 600 bp cDNA product, consistent with PCR using primers TACEP2 and TACEP3. The relative amounts of TNF-α convertase RNA species in THP-1 cells and SH−SY5Y cells were found to be similar by Northern blot analysis using the 1 kb cDNA product as probe (Figure 4A). The expression of TNF-α convertase protein in the SH−SY5Y cells was confirmed by Western blot analysis of the cell lysate with a monoclonal antibody raised against murine TNF-α convertase (*18*). The antibody recognized a polypeptide of 120 kDa that corresponds to the full-length form of the enzyme containing the prodomain and a polypeptide of 100 kDa that corresponds to the mature form lacking the prodomain. The band at 65 kDa recognized by the antibody

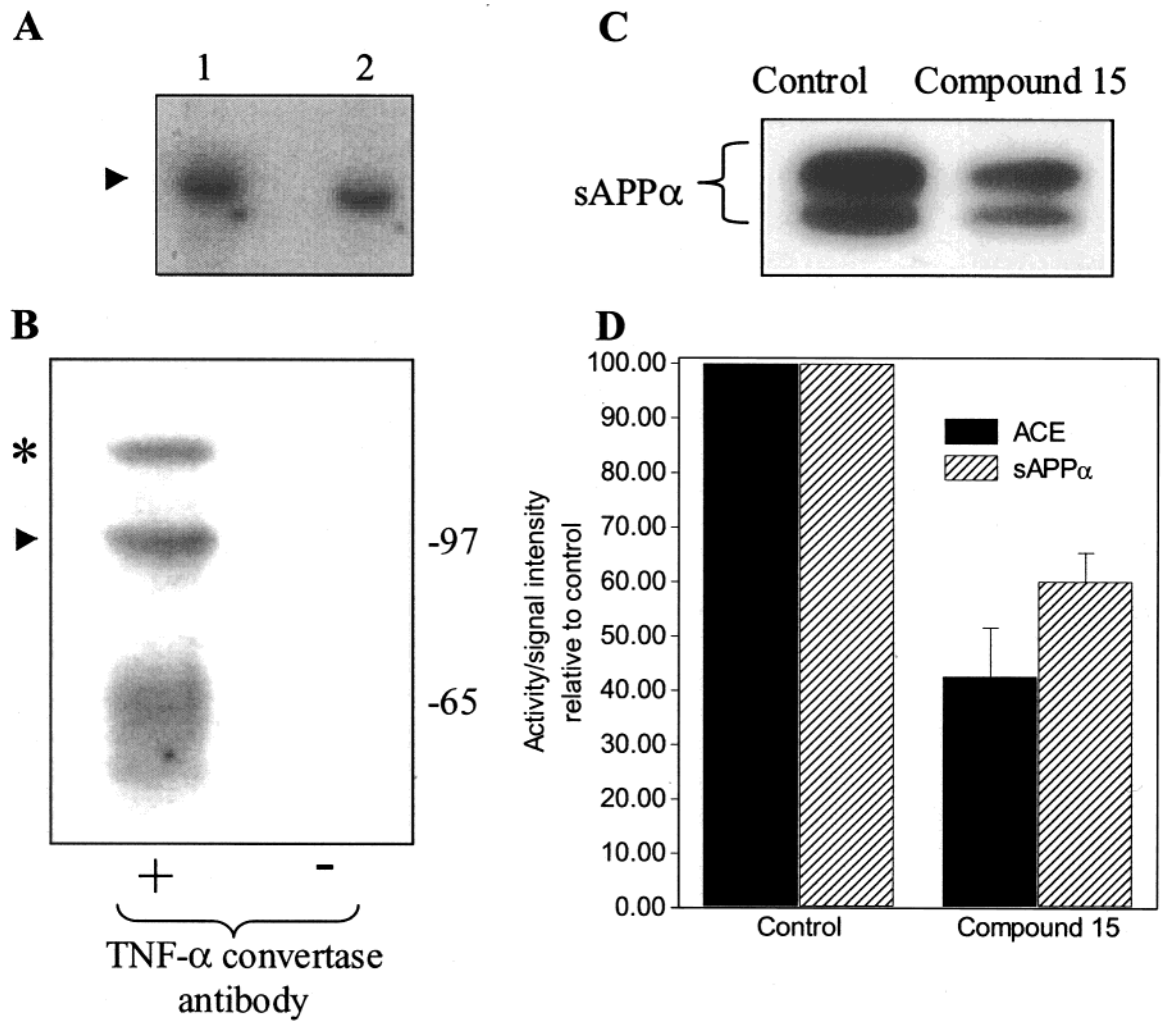

FIGURE 4: TNF-α convertase is expressed in SH−SY5Y cells but is not involved in the regulated cleavage of APP. (A) Northern blot analysis for TNF-α convertase in SH−SY5Y cells. Total cellular RNA (7 μg/lane) was resolved on a formaldehyde gel, blotted, and hybridized with ^{32}P-labeled TNF-α convertase-specific cDNA generated by RT-PCR. Lane 1, THP-1 cells; lane 2, SH−SY5Y cells. Arrowhead indicates the position of the TNF-α convertase mRNA. (B) Western blot analysis for TNF-α convertase in SH−SY5Y cells. Cell lysates were prepared as described in the experimental section, and protein (30 μg) was separated on an 8% polyacrylamide gel, transferred on to a PVDF membrane, and subjected to Western blot analysis in the presence (+) or absence (−) of the monoclonal anti-TNF-α convertase antibody. The full-length form of TACE containing the prodomain is denoted by an asterisk and the mature form lacking the prodomain is denoted by an arrowhead. (C) SH−SY5Y cells were stimulated with 20 μM carbachol in either the absence or the presence of 20 μM compound 15. Concentrated conditioned medium was subject to immunoelectrophoretic blot analysis with antibody 6E10 to detect sAPPα. (D) Quantitation of the effect of 20 μM compound 15 on the carbachol-stimulated release of ACE and sAPPα from SH−SY5Y cells stably transfected with ACE. sAPPα was detected with antibody 6E10 and quantified by densitometric analysis. ACE was quantified by assaying the conditioned medium with BzGly−His−Leu.

is likely a C-terminally truncated form of the protein as reported previously (*44*).

Next we exploited our observation that compound 15 is a potent inhibitor of TNF-α convertase but a much weaker inhibitor of the α-secretase and ACE secretase (Table 1). To examine the effect of compound 15 on the regulated release of sAPPα and ACE, SH−SY5Y cells stably transfected with ACE were stimulated with carbachol in the presence of 20 μM compound 15 (a concentration 100-fold greater than the observed IC_{50} with TNF-α convertase, but a concentration near to the IC_{50} observed for the constitutive release of sAPPα and ACE; Table 1). The carbachol-stimulated release of both sAPPα and ACE was inhibited by only 40−60% by 20 μM compound 15 (Figure 4C,D). Thus, although TNF-α convertase is expressed in the SH−SY5Y cells, it does not appear to be involved in the carbachol-stimulated release of either sAPPα or ACE.

DISCUSSION

Previously, we have reported that the α-secretase responsible for the nonamyloidogenic cleavage of APP displays a similar inhibition profile with batimastat and a limited number of structurally related hydroxamate-based compounds to the secretase that cleaves ACE (*27*). In the present study, we have examined the ability of a much more extensive range of compounds to inhibit α-secretase, and compared their effect on the secretases that cleave ACE, CD23, and pro-TNF-α to build up a structure−activity relationship, and to identify more potent and selective compounds.

The compounds in group 1 (see Table 1) in which P_1' is isobutyl, clearly show the importance of the hydroxamic acid moiety that coordinates to the zinc ion at the active site, for inhibitory activity toward the α-secretase, ACE secretase, and CD23 secretase (compare compound 11 vs compound 12). In addition, this group of compounds shows that the

phenacylthiomethyl group (the "α" substituent, adjacent to the hydroxamic acid) in compound 13 seems to enhance potency relative to the analogous substituents in either compound 12 or batimastat. In group 2, the compounds have the P_1' substituent derived from benzyl, and most have an hydroxy group as the α substituent. These compounds were not as potent toward the secretases cleaving APP, ACE, and CD23 as those in group 1. However, compounds having a small para-substituent on the aromatic ring (compounds 16 and 20) were better tolerated than either large para-groups (compound 19) or meta-substitutents (compound 17). Compound 21, with a flat P_1' group, showed enhanced potency over other closely related moieties, notably, the bulkier compound 18. Compound 21 was 19- and 50-fold more potent an inhibitor of CD23 secretase than either the α-secretase or ACE secretase, respectively.

The group 3 compounds are analogues of compound 21, in that they also have bicyclic P_1' groups. They explore the size and orientation of the S_1' pocket relative to the inhibitor backbone. Thus, comparison of the two parent benzothiophene analogues, compounds 23 and 24, shows that the orientation of the bicyclic moiety derived from the 2 position of the thiophene ring (compound 24) is preferred for all the secretases, over that of the 3-substituted compound (compound 23). Furthermore, substitution on the thiophene ring (compound 26) was deleterious to activity across all of the secretases. Of the other bicyclic P_1' groups the "reversed" benzothiophene compound 27 and tetrahydronaphthyl compound 25 show similar activities, demonstrating that α-secretase, ACE secretase, and CD23 secretase enzymes have some overlapping tolerances in their respective S_1' pockets. Again the α phenacylthiomethyl compound (compound 22) was the most potent for inhibition of the secretase activities and clearly differentiates the secretase activities from MMP-1. In contrast to this, the group 4 compounds have long, relatively flexible P_1' groups. This gives rise to potent, but relatively nonselective compounds that inhibit all the secretases and MMP-1.

Overall, there is a good deal of overlap in the inhibition of the various secretases. However, there are certain compounds that can clearly differentiate between MMP-1 and the various shedding enzymes (compound 22), between TNF-α convertase and the other secretases (compound 15), and between the CD23 secretase and the shedding of APP and ACE (compound 21). Although the rank order of potency for the compounds on CD23 secretase is essentially the same as that for α-secretase and ACE secretase, all of the compounds were significantly more potent against CD23 secretase. This does not appear to be due to differences in the cell lines used, as the potency of a subset of the compounds toward ACE secretase was identical using the kidney membrane cell-free assay (as in Table 1) or the SH−SY5Y whole cell assay (as in Figure 3) (data not shown). With the exception of compound 30, none of the compounds examined were both potent inhibitors and able to differentiate between the α-secretase and ACE secretase. Although compounds 14, 18, and 20 appeared to have substantial potency differences between the two activities, none of them were particularly potent against either one activity.

The similarity between the secretases that constitutively cleave APP and ACE is further supported by the observation that the basal release of both sAPPα and ACE is stimulated by DCI. This compound appears to be activating the same, or a closely related secretase, as the increased release could be completely blocked by batimastat. Previously it has been reported that another serine proteinase inhibitor, 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride, caused an increase in sAPPα secretion from a variety of cell lines at concentrations from 0.3 to 1 mM (*45*), and that DCI enhances the release of ACE from CHO cells by an unknown mechanism involving activation of a TAPI-inhibitable secretase (*40*). In the latter case, DCI appeared to increase the release of a preexisting pool of ACE from the cell surface. However, the effect of DCI would appear not to be directly on the secretase or substrate protein in the membrane, as no effect was observed when kidney microvillar membranes, which contain the endogenous active secretase (*21*), were incubated with DCI. Thus, some other cellular/cytosolic factor must be required for this effect, which is removed upon isolation of the membranes. This increase in sAPPα production and ACE shedding could be due to inhibition of a serine proteinase, thus providing more full-length APP and ACE for cleavage by the zinc metallosecretase. However, we consider that this is unlikely to be the explanation for the observed increase in ACE release by DCI, as there is no evidence for wild-type ACE being cleaved by a serine proteinase, even upon inhibition of the metallosecretase by batimastat. This is in marked contrast to the effect of a point mutation in the stalk region of ACE which invoked the action of an intracellular serine proteinase (*43*). In addition, the stimulatory effect of DCI does not appear to be due to inhibition of the degradation of the released forms of sAPPα and ACE, as both these proteins are stable to degradation by conditioned medium (data not shown). The precise molecular mechanism by which DCI stimulates the release of sAPPα and ACE awaits to be determined.

Using the human neuronal SH−SY5Y cell line, in which the release of sAPPα is upregulated on activation of muscarinic receptors (*27*, *42*), we investigated whether a subset of the hydroxamate compounds could differentiate between the constitutive and regulated release of sAPPα. Compounds that were potent inhibitors of the constitutive α-secretase were also effective inhibitors of the regulated activity, while compounds that were ineffective at inhibiting the constitutive activity also failed to inhibit the regulated α-secretase. Similar results were obtained on the constitutive and regulated cleavage of ACE tranfected into the SH−SY5Y cells. These data support the hypothesis that it is either the same zinc metalloproteinase, or two very similar activities that cannot be differentiated between using the wide range of hydroxamates described in this study, that is involved in both the constitutive and regulated cleavages of APP and ACE.

The regulated, phorbol ester-induced release of sAPPα was shown to be deficient in primary embryonic fibroblasts derived from mice in which the gene encoding TNF-α convertase had been disrupted by homologous recombination (*14*, *15*). In both studies, the uninduced, constitutive release of sAPPα by these cells was not affected, leading to the conclusion that TNF-α convertase is responsible for the regulated α-secretase cleavage of APP, at least in nonneuronal murine cells. However, it has not been demonstrated that this enzyme is involved in the release of sAPPα from neuronal cells. Thus, we examined whether TNF-α conver-

tase is involved in the carbachol-induced release of sAPPα from the human neuronal SH−SY5Y cell line. Reverse-transcription PCR, Northern blot analysis, and Western blot analysis revealed that TNF-α convertase is expressed in the SH−SY5Y cells. However, although TNF-α convertase is potently inhibited by compound 15 (IC_{50} 0.2 μM), incubation of the SH−SY5Y cells with 20 μM compound 15 did not completely block the carbachol-stimulated release of sAPPα. Further evidence for the lack of involvement of TNF-α convertase in the regulated release of sAPPα from the SH−SY5Y cells comes from the observed inhibitory effect of batimastat. This hydroxamate compound inhibits TNF-α convertase with an IC_{50} of 19 nM (*22*) and a K_i of 11 nM (*46*), with complete inhibition being observed at 100 nM in a cell-based assay (*47*). However, both the constitutive and carbachol-stimulated release of sAPPα from the SH−SY5Y cells were significantly less responsive to batimastat inhibition, with IC_{50} values of 0.3 and 1.2 μM, respectively. Thus, the lack of effect of the potent and selective TNF-α convertase inhibitor compound 15, and the difference in inhibitory potency of batimastat, on the carbachol-stimulated release of sAPPα clearly show that TNF-α convertase is not involved in the regulated α-secretase cleavage of APP in the human SH−SY5Y cells. This is consistent with the recent observation that in human embryonic kidney (HEK-293) cells TNF-α convertase is probably not responsible for the muscarine-receptor-activated release of sAPPα (*48*). Similarly, TNF-α convertase was not involved in the regulated release of ACE from the SH−SY5Y cells, consistent with the observation that the release of ACE is not abrogated in cells derived from TNF-α convertase knock out mice (*23*).

A recent in situ hybridization study in mice revealed only partial overlap of the expression of APP and TNF-α convertase (*49*), supporting the conclusion that TNF-α convertase is not likely to be a major brain α-secretase. Two other ADAM proteinases, ADAM 9 and ADAM 10, have been reported to be involved in the secretion of sAPPα from COS cells and human embryonic kidney cells, respectively (*50*−*52*). ADAM 10 but not TNF-α convertase was also shown to be responsible for the constitutive release of sAPPα in furin-deficient LoVo cells (*53*). The key discriminatory compounds identified in the present study will be invaluable in ascertaining the roles of these, or other zinc metalloproteinases, in both the α-secretase cleavage of APP in neuronal cells and the shedding of other membrane proteins such as ACE and CD23.

In conclusion, we report the structure−activity relationships for a range of hydroxamate-based compounds on the secretases that cleave APP, ACE, and CD23. This is the most comprehensive structure−activity relationship study for inhibitors of multiple secretases to date. In it we have not only identified compounds for discriminating between certain individual secretases, but have also identified a compound that discriminates between secretases and other zinc metalloproteinases, particularly the matrix metalloproteinases. Using this range of compounds, we were unable to differentiate either between the constitutive and regulated α-secretase cleavage of APP, or between the secretases that cleave APP and ACE. This reinforces and extends our earlier conclusion that there is probably a single zinc metalloproteinase involved in the constitutive and regulated proteolytic processing of these two membrane proteins. In addition, using a key discriminatory compound we show that TNF-α convertase is not involved in the regulated release of sAPPα from human neuronal cells. The compounds reported here will be useful in future studies aimed at identifying and validating candidate secretases.

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