

RAC1 Inhibition Targets Amyloid Precursor Protein Processing by γ -Secretase and Decreases A β Production *in Vitro* and *in Vivo**

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β -Amyloid peptides (A β) that form the senile plaques of Alzheimer disease consist mainly of 40- and 42-amino acid (A β 40 and A β 42) peptides generated from the cleavage of the amyloid precursor protein (APP). Generation of A β involves β -secretase and γ -secretase activities and is regulated by membrane trafficking of the proteins involved in A β production. Here we describe a new small molecule, EHT 1864, which blocks the Rac1 signaling pathways. *In vitro*, EHT 1864 blocks A β 40 and A β 42 production but does not impact sAPP α levels and does not inhibit β -secretase. Rather, EHT 1864 modulates APP processing at the level of γ -secretase to prevent A β 40 and A β 42 generation. This effect does not result from a direct inhibition of the γ -secretase activity and is specific for APP cleavage, since EHT 1864 does not affect Notch cleavage. *In vivo*, EHT 1864 significantly reduces A β 40 and A β 42 levels in guinea pig brains at a threshold that is compatible with delaying plaque accumulation and/or clearing the existing plaque in brain. EHT 1864 is the first derivative of a new chemical series that consists of candidates for inhibiting A β formation in the brain of AD patients. Our findings represent the first pharmacological validation of Rac1 signaling as a target for developing novel therapies for Alzheimer disease.

Alzheimer disease (AD)² is the most common neurodegenerative disorder marked by progressive loss of memory and cognitive ability. The pathology of AD is characterized by the presence of amyloid plaques (1), intracellular neurofibrillary tangles, and pronounced cell death. The β -amyloid peptide (A β) (2) is the main constituent of senile plaques found in AD brains. Furthermore, extracellular A β 42 appears toxic to neurons *in vitro* and *in vivo* (reviewed in Ref. 3). A β is generated by proteolysis of an integral membrane protein, the amyloid precursor protein (APP), via at least two post-translational pathways. The amyloidogenic cleavage of APP is a sequential processing of APP initiated by

β -secretase (BACE), which cleaves APP within the luminal domain or at the cell surface, generating the N terminus of A β (4). This cleavage generates several membrane-bound proteolytic C-terminal fragments (CTFs), such as the 99-residue β -CTF (also called C99), as well as the secreted APP ectodomain sAPP β . The C terminus of A β is subsequently generated by intramembranous cleavage of CTFs by γ -secretase, producing either A β 40 or A β 42. The cleavages at residues 40–42 are referred to as γ -cleavage, and the cleavages at residues 49–52 are referred to as ϵ -cleavage (5). The nonamyloidogenic cleavage of APP, which precludes A β generation, is mediated by α -secretase, a disintegrin and metalloproteinase 10, and a disintegrin and metalloproteinase 17, in a reaction believed to occur primarily on the plasma membrane. This proteolytic cleavage by α -secretase occurs within the A β region and produces soluble APP (sAPP α), the dominant processing product, and the residual membrane-bound 10-kDa CTF (CTF α , also called C83). Like C99, C83 is a substrate for γ -secretase, which cleaves C83 to generate the nonamyloidogenic p3 fragment. APP is also a substrate of caspase activities that cleave its cytosolic domain (6).

Multiple lines of evidence suggest that APP processing and A β generation are determined by dynamic interactions of APP with membrane microdomains, known as lipid rafts, which facilitate the production of A β (7, 8). Lipid rafts are rich in cholesterol and sphingolipids and are also the principal compartment in which A β is found (9, 10). BACE and γ -secretase also localize to these lipid raft microdomains, in endosomes and post-Golgi compartments, enabling them to cleave APP (11, 12).

More generally, lipid rafts contribute to trafficking of proteins and lipids in the secretory and endocytic pathways by regulating vesicle formation and sorting. They also act as signaling platforms for various pathways including GTPase-dependent actin rearrangements (13) induced by small GTP-binding proteins from the Rho family such as Rac, Rho, and Cdc42. These small G proteins are activated by GTP/GDP exchange and regulate a wide variety of cellular functions such as gene expression, cytoskeletal reorganization, and vesicle/secretory trafficking. The activated Cdc42 or Rac then activates the PAK Ser/Thr kinase family. Recent studies showed the participation of Rho in the formation of stress fibers, whereas activated Cdc42 induces the formation of filopodia, thin finger-like extensions containing actin bundles. Rac regulates the formation of lamellipodia or ruffles, curtain-like extensions often formed along the edge of the cell (see Ref. 14 for a review). In the brain, these small G proteins participate in the morphological changes of neurons, localized in growth cones, axons, dendritic trunks, and spines (15).

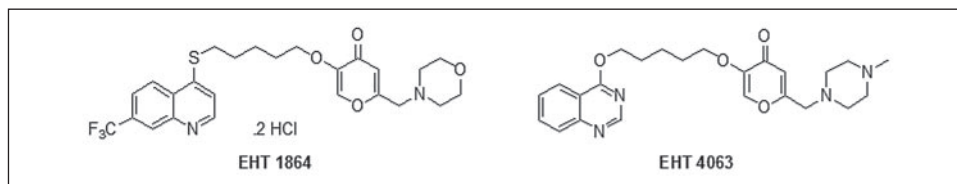
In the mature brain, it has been shown that Rac1, but not Rho nor Cdc42, is present in the raft domain of neuronal membranes (16). This was recently confirmed by an unbiased quantitative proteomics study revealing Rac1 as a raft-associated protein (17). Other studies showed that activation of Rac1 is associated with its rapid recruitment into the

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² The abbreviations used are: AD, Alzheimer disease; A β , β -amyloid; APP, amyloid precursor protein; sAPP α , soluble APP ectodomain; BACE, β -site APP-cleaving enzyme; CTF, carboxyl-terminal fragment; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-5-phenylglycine t-butyl ester; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEK, human embryonic kidney; NICD, Notch intracellular cytoplasmic domain; swAPP-HEK293, HEK cells expressing human APP695 containing the Swedish mutation; Pak1, p21-activated kinase 1; RBD, Rho-GTP binding domain; IL, interleukin; EHT 1864, 5-(5-(7-(trifluoromethyl)quinolin-4-ylthio)pentyl)oxy)-2-(morpholinomethyl)-4H-pyran-4-one dihydrochloride; EHT 4063, 5-(5-(quinazolin-4-yl)oxy)pentyl)-2-((4-methylpiperazin-1-yl)methyl)-4H-pyran-4-one; MES, 4-morpholine-ethanesulfonic acid; MCA, 7-methoxycoumarin-4-yl)acetyl; DNP, 2,4-dinitrophenyl; NMA, 2-(N-methylamino)benzoyl.

FIGURE 1. Structure of EHT 1864 and EHT 4063.



lipid rafts, whereas Cdc42 is not, and that Rac1, but not Rho or Cdc42, regulates the assembly and export to the cell membrane of Golgi-derived lipid rafts (18, 19).

A number of recent studies have implicated the Rho family of small G proteins, including Rac1 itself, in the modulation of APP processing. Interestingly, two different aspects of APP processing appear to be controlled by Rac1 and other small G proteins: ectodomain shedding (20), which is a prerequisite for β - γ -secretase cleavage, and the β - γ -secretase cleavage itself (21–23). In particular, overexpression of dominant negative (RacN17) or constitutively active (RacQL) mutants of Rac1 was demonstrated to inhibit or stimulate γ -secretase-mediated APP processing (21), respectively, which suggests that Rac1 is crucial for the homeostasis of endogenous A β production. It is now clear that APP processing is controlled by multiple pathways to provide a fine tuned processing of APP in physiological conditions. In the AD condition, consequent progress in the identification of dysregulated mechanisms controlling APP processing has been made. For example, Rac1, as well as other small G proteins, has been implicated in the pathways triggered by inflammatory mediators, such as interleukin (IL)-1 β , IL-6, or tumor necrosis factor- α , and by growth factors, such as transforming growth factor- β or platelet-derived growth factor, that have been found to be up-regulated in AD brains (24, 25). These signaling pathways have been shown to stimulate the generation of A β (26). The involvement of Rac1 in AD is further stressed by the observation that an up-regulation of neuronal Cdc42/Rac1 occurs in selected neuronal populations of the AD brain in comparison with age-matched controls (27). Therefore, an attractive hypothesis proposes a role for small G proteins, such as Rac1, in the control of APP processing and A β accumulation that occur in AD.

We describe here a new molecule, EHT 1864, that inhibits Rac1 signaling and APP processing, lowering A β production *in vitro* and leading to a decrease in A β in the brain of guinea pigs. Since this molecule does not affect Notch processing and the neurotrophic α -secretase pathway, EHT 1864 represents a prototype of a new chemical series of interest for developing new treatments for AD.

EXPERIMENTAL PROCEDURES

Materials and Compounds—EHT 1864 and EHT 4063 (Fig. 1) were synthesized as described in Ref. 28.

All cell culture reagents were from Invitrogen (Cergy Pontoise, France) unless otherwise noted. NSC23766, *N*-[*N*-(3,5-difluorophenyl)-L-alanyl]-L-phenylglycine *t*-butyl ester (DAPT), BACE inhibitors, BACE, and γ -secretase fluorogenic substrates were obtained from Calbiochem (ClimSci, Montrouge, France).

Cell Culture and Treatments—Stably transfected HEK293 cells overexpressing human swAPP harboring the “Swedish” mutation (29) (swAPP-HEK293 cells) were maintained in modified Eagle’s medium plus Earle’s salt supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma), 1 \times nonessential amino acids, and antibiotics. GC cars NIH3T3 cells (LGC PromoChem) were grown in high glucose Dulbecco’s modified Eagle’s medium plus Glutamax supplemented with 10% newborn serum and antibiotics. Human astrocytomas U87MG (ATCC number HTB-14) were grown at 37 °C in Dulbecco’s modified

Eagle’s medium containing 1 mM glutamine, 10% FBS, and antibiotics. SH-SY5Y cells (ATCC number CRL-2266) were maintained in modified Eagle’s medium/F-12K (1:1, v/v) supplemented with 10% FBS, 2 mM L-glutamine, 1 \times nonessential amino acids, 1 \times sodium pyruvate, and antibiotics. HeLa cells (ATCC number CCL 2) were grown in modified Eagle’s medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. Cells were treated 48 h after plating in 10-cm plates with various concentrations of the indicated molecules or Me₂SO as the vehicle for 16 or 24 h. To do so, medium was replaced with 5 ml of new medium in which treatments were performed. Total Me₂SO dilution was 1:1000 in all cases. Cells were allowed to secrete in 5 ml of medium for 7 h in the presence of 1 μ M phosphoramidon.

Endogenous Rac GTPase Activation Assay—U87-MG cells were grown in a 150-mm diameter dish until they reached 80% confluence. The cells were then treated with the test compounds or the solvent only. Cells were then lysed in a buffer containing 0.5% Triton X-100, 10 mM Tris, pH 7.5, 25 mM KCl, 120 mM NaCl, and 1.8 mM CaCl₂. Lysates were clarified, the protein concentrations were normalized, and the GTP-bound Rac1 in the lysates were measured using the Rac activation assay Biochem kit (Cytoskeleton) as per the manufacturer’s recommendations.

Endogenous Rho GTPase Activation Assay—GST-Rhotekin Rho-GTP binding domain (RBD) fusion protein beads were prepared as follows, in laboratory of A. Hall (University College, London, UK). BL21 DE3 pLysS strain transformed with pGEX2T Rhotekin RBD grown overnight in LB containing 50 μ g/ml ampicillin and 25 μ g/ml chloramphenicol was pelleted and then resuspended in GTLB I buffer (50 mM Tris, pH 8, 40 mM EDTA, 25% (w/v) sucrose, and 1 mM phenylmethylsulfonyl fluoride). Suspension was rotated on a wheel at 4 °C for 10–20 min. GTLB II buffer (50 mM Tris pH 8, 100 mM MgCl₂, 0.2% (w/v) Triton X-100) was added, and suspension was rotated again for a further 10 min. Bacteria were sonicated on ice at 15 μ s in 10-s bursts and centrifuged. Supernatant was carefully removed and transferred to 50-ml Falcon tubes. 1 ml of 50% glutathione-agarose beads was added, and the suspension was rotated on a wheel at 4 °C for 1 h. Beads were spun down for 20 s at no more than 2500 rpm. Supernatant was discarded, and beads were washed with cold wash buffer (50 mM Tris, pH 7.6, 50 mM NaCl, 5 mM MgCl₂). Beads were transferred to Eppendorf tubes and spun down again, and the last traces of buffer were removed. Beads were then resuspended in wash buffer containing 50% glycerol (final glycerol concentration 25%), aliquoted, and stored at –80 °C. Pull-down experiments were performed as described for the Rac-GTPase activation assay except that the detection antibody was an anti-RhoA antibody (Tebu, Le Perray en Yvelines, France) used at a 1:750 dilution.

Transient Expression Reporter Assays—Transcriptional activation of luciferase gene expression constructs was performed as described previously (30). Briefly, 250,000 NIH3T3 cells/well were seeded in 6-well plates and were co-transfected 24 h later with plasmids prK5-RacV12 and reporter constructs using Lipofectamine Plus (Invitrogen). The compound of interest was added after the incubation with Lipofectamine. 24 h after transfection, cells were starved for an additional 24 h with Dulbecco’s modified Eagle’s medium supplemented with 0.5% FBS together with the appropriate doses of test compounds or the sol-

vent only. Analyses of the cell lysates of the transiently transfected NIH3T3 cells were performed using the luciferase assay system (Promega) and Fluoroscan Ascent FL plate reader (Thermo LabSystems). All assays were performed in duplicate, and results shown represent the mean \pm S.E. of four independent experiments for each reporter gene. We did not use an internal standard in the transfections, since all three promoters tested responded to active Rac overexpression to varying extents. However, consistent and reproducible data were obtained in different assays performed using several plasmid preparations, and we monitored protein concentration for yield in the cell extracts as well as expression of the tagged, exogenous protein by Western blotting.

The reporter constructs 5 \times Gal4-Luc plus Gal4-c-Jun, HIV-Luc bearing NF- κ B binding sites (30), and cyclin D1-Luc (31) were described previously and are a kind gift of Dr. Channing J. Der (University of North Carolina, Chapel Hill, NC). The expression plasmid pRK5-RacV12 was described previously (32) and is a kind gift of Dr. Alan Hall (University College, London, UK).

Western Blot Analyses—swAPP-HEK293 cells were scraped and lysed in CelLytic-M (Sigma). Protein concentrations were determined by the Bradford procedure. Equal protein quantities were separated on a 10% SDS-polyacrylamide gel and transferred to Hybond-C (Amersham Biosciences) membranes. After transfer, membranes were blocked with 5% nonfat milk and incubated overnight with the primary antibody anti-APP antibody at 1:1000 (AHP538; Serotec), allowing the detection of both APP (resolved as doublets in some experiments) and C99 CTF. For sAPP α detection, cells were allowed to secrete for 7 h. Media were collected and cleared by centrifugation, and then equal amounts were loaded on 10% SDS-PAGE and subjected to Western blot with 6E10 monoclonal antibody (1:1000). Immunological complexes were revealed with an anti-mouse peroxidase (1:5000; Jackson Laboratories) antibody followed by ECL (Amersham Biosciences).

Notch Δ E Transfection and Notch-1 Cleavage Assays in HeLa Cells—HeLa cells in 10-cm plates were transiently transfected with the expression vector pSC2+ Δ E3MV-6MT, which overexpresses truncated Notch-1, lacking most of the Notch extracellular domain, and has a C-terminal Myc tag, (Notch Δ E). This truncated form of Notch is the substrate of γ -secretase (33). 1 day post-transfection, cultures were preincubated with EHT 1864 or the γ -secretase inhibitor DAPT for 18 h at the indicated concentrations, and then CelLytic-M lysates were processed for detection of the Notch intracellular domain (NICD) by Western blotting using anti-Myc antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000.

In Vivo Delivery of Inhibitors—EHT 1864 or vehicle (physiological saline) were injected in male Hartley albino guinea pigs, weighing 250–270 g at delivery and obtained from Charles River Laboratories (L'Arbresle, France), once a day for 15 consecutive days by the intraperitoneal route. 1 h after the final administration, the guinea pigs were killed; brains were immediately extracted and immersed in an oxygenated (95% O₂, 5% CO₂) physiological saline bath placed on ice (1–2 °C); and superficial vessels were removed. The whole brains were dissected to provide left and right cortices, which were weighed, snap-frozen in liquid nitrogen, and stored at –80 °C, separately. The maximum time between sacrifice and snap freezing was less than 15 min.

Measurements of A β 40 and A β 42—Stably transfected swAPP-HEK293 cells or confluent SH-SY5Y cells were incubated for 7 h in the presence of phosphoramidon (1 μ M) (Sigma). Media and cell lysates were collected as above, centrifuged, normalized to total protein, and assayed for A β 40 and A β 42 by sandwich ELISA according to the manufacturer's instructions (BIOSOURCE International). For A β 42 detection, samples were concentrated on YM3 Microcon columns (Mil-

lipore Corp.). For *in vivo* samples, the protocol ensured a final concentration of guanidine of <0.1 M, as recommended by the manufacturer, and ELISA standards included guanidine. Right cortices were homogenized for 3 h at room temperature in 5 M guanidine HCl, 50 mM Tris-HCl, and pH 8 with a protease inhibitor mixture (Roche Applied Science). Tissue homogenates were diluted 1:1 (v/v) in BSAT-DPBS buffer (Dulbecco's phosphate-buffered saline with 5% bovine serum albumin and 0.03% Tween 20), pH 7.4, and were centrifuged at 20,000 \times g for 20 min at 4 °C. Supernatants were diluted 1:1 (v/v) in ELISA kit sample buffer, normalized to total protein, and assayed for A β 40 and A β 42 by sandwich ELISA according to the manufacturer's instructions. For A β 42 detection, samples were concentrated on YM3 Microcon columns (Millipore).

BACE Assay—Human BACE1 cDNA was generated by reverse transcription-PCR from human brain mRNA samples (Biocat) and cloned into the pcDNA3 expression vector. Subsequently, a HEK293 cell line stably expressing BACE1 was generated and used as a source of BACE1. An *in vitro* assay was developed based on previous studies (34, 35) using a quenched fluorogenic substrate containing the Swedish mutation MCA-SEVNLDAEFK(DNP)-NH₂ (Substrate V; Calbiochem). Proteins were extracted in 20 mM MES, 1% Triton X-100 plus protease inhibitor mixture by incubation on ice for 30 min. The assay was carried out in black 96-well plates (ATGC) in a volume of 200 μ l of reaction buffer (25 mM MES, 25 mM sodium acetate, 25 mM Tris, pH 4.4), containing 25 μ l of the preparation plus 15 μ M peptide Substrate V. Excitation was performed at 320 nm, and the reaction kinetics were monitored by measuring the fluorescence emission at 420 nm on a Fluoroscan Ascent FL plate reader (Thermo LabSystems). Controls included purified recombinant human BACE501 protein (R&D Systems) diluted at 1 μ g/well in 200 μ l of 0.1 M sodium acetate buffer (pH 4.4), the BACE substrate analog inhibitor III (Glu-Val-Asn-statine-Val-Ala-Glu-Phe-NH₂; Calbiochem), or substrate alone, and background fluorescence was subtracted from recorded BACE activity. Final Me₂SO concentration was 1% (v/v) and did not affect the fluorescence or BACE activity.

γ -Secretase Assay—Here, we implemented a γ -secretase assay allowing *de novo* A β generation *in vitro*, using cell membranes as the source of γ -secretase and endogenous C99 generated from swAPP as the substrate. Preparation of solubilized γ -secretase fractions was performed essentially as described previously (36, 37) with the above modifications. All incubations were performed in the presence of Complete protease inhibitor mixture. Confluent plates of swAPP-HEK293 cells were lysed in 1 ml of ice-cold CelLytic-M (Sigma) and incubated for 15 min at 4 °C on a shaker. Cell debris and nuclei were removed by centrifugation at 1000 \times g for 15 min at 4 °C. For membrane isolation, the supernatant solutions were centrifuged at 20,000 \times g for 1 h at 4 °C. After centrifugation, the ensuing pellets were resuspended in 100 μ l of activity buffer (150 mM sodium citrate, pH 6.4) per cell plate and were defined as solubilized γ -secretase, as previously shown in Ref. 33. Solubilized γ -secretase activity was induced at 37 °C for 2 h with or without the indicated treatments, and A β 40 generated *de novo* was quantified by ELISA. Control experiments used the internally quenched fluorogenic γ -secretase substrate NMA-GGVVIAIVK(DNP)-DRDRDR-NH₂ (λ_{ex} = 355 nm; λ_{em} = 440 nm) from Calbiochem, which contains the C-terminal β -APP amino acid sequence that is cleaved by γ -secretase and the γ -secretase inhibitor DAPT (Calbiochem).

Cytotoxicity Assays—Cell viability and cytotoxicity of the tested compounds were routinely assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or a released lactate dehydrogenase assay using the CytoTox 96 assay according to the manufacturer's instructions (Promega).

Statistical Analysis—Mann-Whitney *U* test and Wilcoxon test were used to determine the significance between the data means. Significance values are as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus corresponding control.

RESULTS

EHT 1864 Inhibits Rac1/Pak1 Interaction—To test whether EHT 1864 might affect Rac1 activity, U87-MG cells were treated with different concentrations of EHT 1864. We used a GST fusion protein containing the p21-binding domain of human p21-activated kinase 1 (Pak1) to affinity-precipitate endogenous active Rac1 (GTP-Rac1) from cell lysates in order to monitor the activation of the small GTPase Rac1. The GST-Pak-p21-binding domain fusion protein was incubated with cell lysate, and the effector pulled down active or GTP-Rac1 was detected by Western blot analysis using a specific Rac1 antibody. The Rhotekin protein specifically recognizes and binds to the active, GTP-bound, form of RhoA, RhoB, or RhoC protein via its RBD. We monitored the activation of RhoA (GTP-RhoA) using Rhotekin RBD beads to affinity-precipitate endogenous active RhoA from lysates of cells treated with EHT 1864. As shown in Fig. 2A, EHT 1864 strongly inhibited, in a dose-dependent manner, the ability of Rac1 to interact with its effector Pak1. In contrast, even at the highest dose tested (25 μ M), EHT 1864 did not affect the activation status of RhoA.

EHT 1864 Inhibits Rac1 Signaling—It has been reported that Rho family members can drive transcription from reporter constructs Gal4-c-Jun plus 5 \times Gal4-Luc, HIV-Luc bearing NF- κ B binding sites, and cyclin D1-Luc (30–32). In order to confirm that EHT 1864 can inhibit Rac1-dependent functions, we cotransfected the constitutively active mutant Rac1-Val12 (RACV12) with the different reporter constructs in the presence of different concentrations of the compound. As shown in Fig. 2, RACV12 elicited transcriptional responses from the NF- κ B, c-Jun, and cyclin D1 reporter constructs (Fig. 2, B–D, respectively). -Fold inductions for the c-Jun, NF- κ B, and cyclin D1 reporter constructs were 5, 15, and 4, respectively. Treatment of cells with EHT 1864 dose-dependently reduced luciferase activity from all three reporter constructs, and for each, the 50% inhibition dose came at \sim 5 μ M. These results indicated that EHT 1864 is able to interfere with Rac1-mediated transduction pathway, probably by inhibiting its ability to recruit its effectors.

As a control, EHT 4063, an EHT 1864-related molecule that is unable to disrupt the Rac1/Pak1 interaction in the pull-down experiments (data not shown), was inactive in the gene reporter assays using the three different reporter constructs. EHT 1864 and EHT 4063 had no effect on cell viability of NIH3T3 cells, as demonstrated by lactate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (data not shown).

EHT 1864 Prevents A β 40 and A β 42 Production in Vitro—We have examined the effect of EHT 1864 and EHT 4063 on the production of A β 40 using SH-SY5Y cells endogenously expressing wild type APP. Confluent cells were allowed to secrete in 5 ml of medium for 18 h. In our conditions, the total amount of secreted A β 40 in untreated samples was 21.4 ± 3.9 pg/ml, and A β 42 levels were below the detection limit of the ELISA assay. Incubation of cells for 18 h with 2, 10, or 20 μ M EHT 1864 resulted in 79.8, 30.1, and 12.9% reduction in secreted A β 40 levels, respectively. In contrast, EHT 4063 was strictly inactive in reducing secreted A β levels. We calculated EHT 1864 IC₅₀ as being 5.44 μ M using the Prism software (Fig. 3A).

Human APP harboring the Swedish mutation (swAPP) is more prone to processing than wild type APP. To test whether similar inhibition of A β levels was also observed following swAPP processing, we used

swAPP-HEK293 cells that secrete high quantities of both A β 40 and the more amyloidogenic peptide A β 42. A β 40 and A β 42 released in the conditioned medium were quantified after an 18-h treatment with 2, 10, or 20 μ M EHT 1864. The total amount of secreted A β 40 was \sim 10-fold higher than the total amount of secreted A β 42. Fig. 3B shows that EHT 1864 dose-dependently inhibits both A β species with a similar activity at all three concentrations tested and suggests that inhibition of A β peptide occurs independently of the wild type or Swedish mutation conditions. At 50 μ M or above, EHT 1864 led to A β 40 and A β 42 levels below the detection limit of the ELISA tests. EHT 4063 was strictly inactive in reducing A β 40 and A β 42 levels in swAPP-HEK293 cells.

It is now established and accepted in the field that there are two cellular pools of A β , both intracellular and extracellular (secreted), that behave independently of one another. Therefore, we next determined cell-associated A β levels as an indication of the effect of EHT 1864 on intracellular A β in swAPP-HEK293 cells. We harvested cells in Cel-Lytic-M buffer and assayed cell lysates for levels of intracellular A β 40 and 42 species by specific ELISAs. As observed for secreted A β 40 and A β 42, EHT 1864 treatment caused a dose-dependent decrease in both A β 40 and A β 42 intracellular levels after normalization of A β levels to cellular protein content (Fig. 3C). EHT 4063 was strictly inactive in reducing both intracellular and extracellular A β 40, as for A β 42 levels (data not shown). Thus, our results thus far indicated that EHT 1864 induced a decrease in APP processing and subsequent A β generation independently of APP mutation. Furthermore, in both SH-SY5Y and HEK293 cells, cell viability was unaffected by EHT 1864 or EHT 4063 as measured by both lactate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (data not shown).

EHT 1864 Does Not Affect BACE and α -Secretase Pathways—Previous studies have shown that α - and β / γ -secretase pathways may compete for APP substrate under certain conditions. Therefore, increased sAPP α levels may explain decreased A β levels. Thus, we tested the effect of EHT 1864 on the α -secretase pathway by monitoring levels of sAPP α secreted in the culture medium of swAPP-HEK293 cells. The antibody used here for sAPP α recognizes the last 16 residues of sAPP α that are not present in sAPP β . As shown in Fig. 4A, exposure to 2, 10, or 20 μ M EHT 1864 did not affect sAPP α levels, establishing the absence of direct or indirect modulation of α -secretase activity by EHT 1864. The level of full-length cytosolic and membranous APP was also found to be unchanged upon treatment with EHT 1864, excluding the possibility of altered APP holoprotein expression, maturation, or trafficking to the membrane.

We then tested the effect of EHT 1864 on BACE1 activity, the rate-limiting enzyme in A β production. To rule out a direct inhibitory effect on BACE activity, a BACE-specific fluorogenic assay was implemented using recombinant human BACE protein diluted at 1 μ g/well in 0.1 M sodium acetate buffer, pH 4.4, which cleaved the quenched fluorogenic Substrate V containing the Swedish mutation, resulting in increased fluorescence. The BACE substrate analog inhibitor III abolished the cleavage, whereas incubation of EHT 1864 at 2, 10, or 20 μ M (Fig. 4B) as well as higher concentrations (data not shown) did not affect fluorescence, establishing that EHT 1864 is unable to affect BACE activity.

Next, we tested whether EHT 1864 could indirectly affect cellular BACE activity using HEK293 cells stably expressing BACE1. BACE activity was present in homogenate proteins from transfected cells. In contrast, very low substrate cleavage activity was detected in control cells (data not shown). The activity was inhibited by the well characterized BACE inhibitor III, and incubation of cells for 24 h with EHT 1864 at 2, 10, or 20 μ M did not affect fluorescence (Fig. 4C), suggesting that there is no indirect effect of EHT 1864 on BACE activity. In fact, immu-

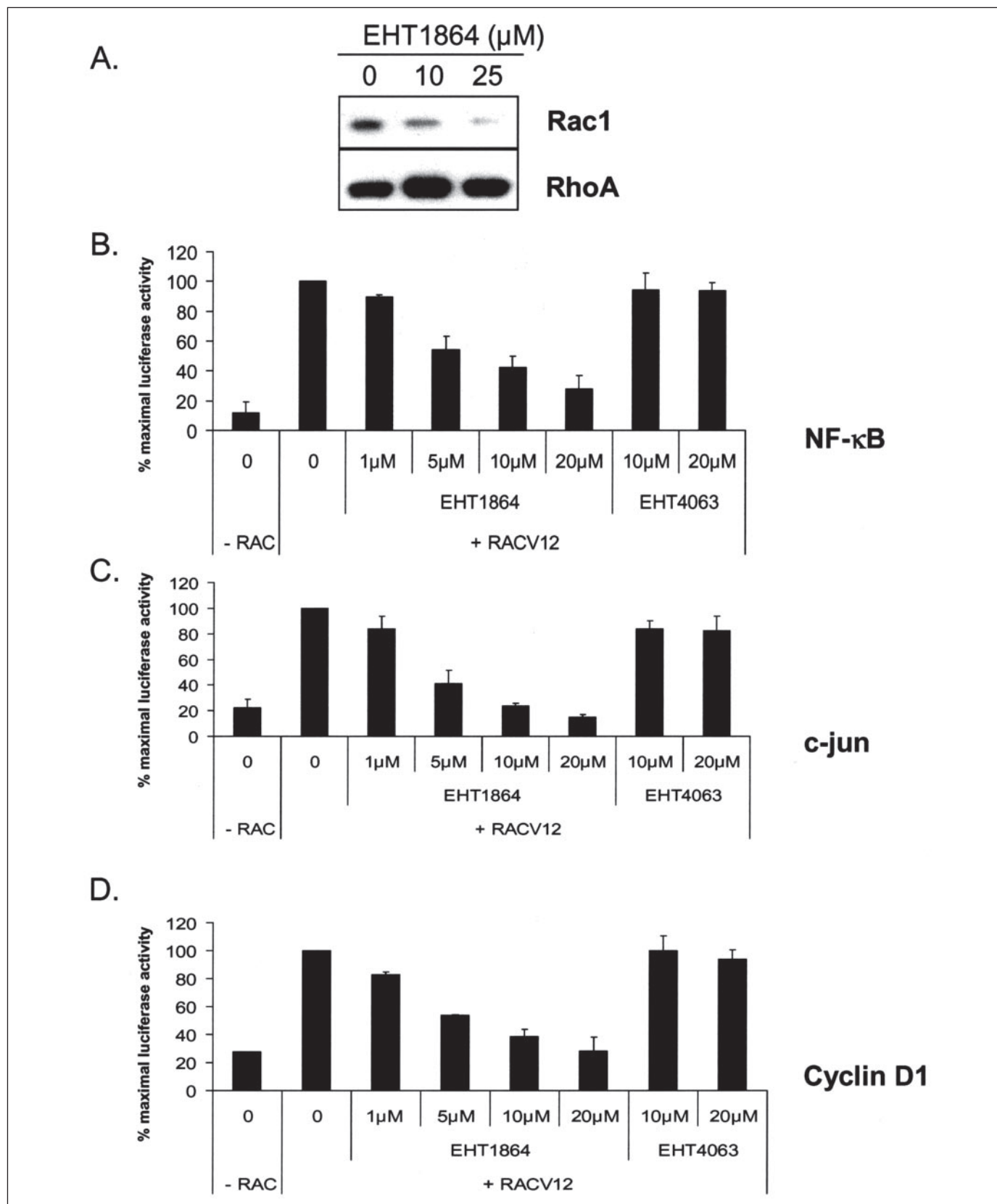


FIGURE 2. *A*, U87-MG cells were incubated for 5 min in the absence or presence of EHT 1864, and then levels of activated (GTP-bound) Rac1 or RhoA in the lysates were measured using GST-Pak1 pull-down or Rhotekin RBD pull-down, respectively. *B–D*, EHT 1864 reduces Rac1V12-dependent transient expression reporter assay. NIH3T3 cells were transiently transfected with constitutively active mutant Rac1V12 plus the following reporter system: HIV-Luc, bearing NF- κ B binding sites (*B*), Gal4-c-Jun plus 5 \times Gal4-Luc (*C*), and cyclin D1-Luc (*D*). The compound of interest was added at the indicated dose just after transfection and again after 24 h, when the medium was changed. Cell lysates were analyzed for Luciferase activity in duplicate, and results shown represent the mean \pm S.E. of four independent experiments for each reporter gene.

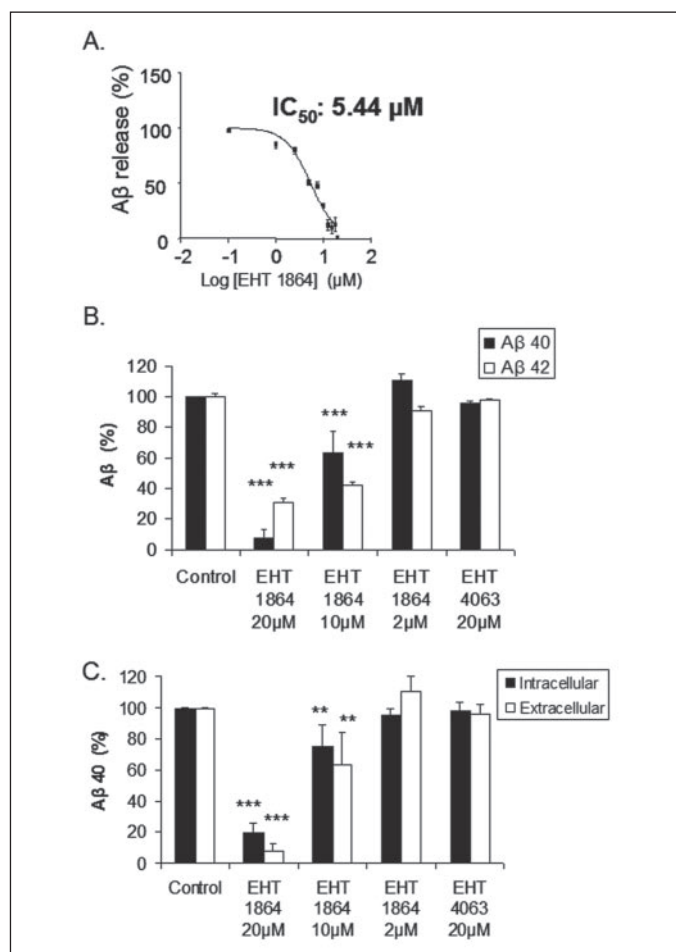


FIGURE 3. EHT 1864 reduces production of A β 40 and 42 amyloid peptides. *A*, dose-dependent reduction of A β 40 levels in supernatants of SH-SY5Y cells expressing wild type APP treated with various doses of EHT 1864 (subtoxic concentrations). Supernatants were processed using A β 40-specific ELISA, and percentage of A β release and IC₅₀ were determined using Prism software. *B*, dose-dependent reduction of A β 40 and A β 42 levels in cell supernatant of HEK293 cells expressing swAPP treated with various doses of EHT 1864 or EHT 4063. Supernatants were processed using A β 40- or A β 42-specific ELISAs, and A β levels were determined. *C*, dose-dependent inhibition of intracellular (cell-associated) A β 40 levels, which parallels that of secreted A β 40 in swAPP HEK293 cells. Results shown represent the mean \pm S.E. of three independent experiments.

no fluorescence studies aimed at determining the subcellular and membranous localization of BACE using antibodies raised against the N-terminal (amino acids 46–65) or C-terminal (amino acids 485–501) parts of BACE in the presence or absence of Triton failed to detect any changes in BACE immunoreactivity following EHT 1864 treatment (data not shown), suggesting no effect of EHT 1864 on BACE cellular localization as well.

EHT 1864 Targets γ -Secretase Activity—C99, the product of APP cleavage by BACE, is generated at high levels in SH-SY5Y cells overexpressing BACE. EHT 1864 treatment caused a dose-dependent increase in C99 (Fig. 5A), which is indicative of an inhibitory effect on γ -secretase, resulting in protection of C99 from proteolysis. As for swAPP-HEK293 cells, EHT 1864 had no effect on holo-APP levels (data not shown).

To further address the question of whether EHT 1864 is inhibiting γ -secretase activity through a direct or an indirect mechanism, we used an established γ -secretase assay allowing *de novo* A β generation *in vitro*, using cell membranes as the source of γ -secretase (36–39). Solubilized γ -secretase fractions are activated in a γ -secretase buffer (see “Experimental Procedures”), and activity is monitored following either the

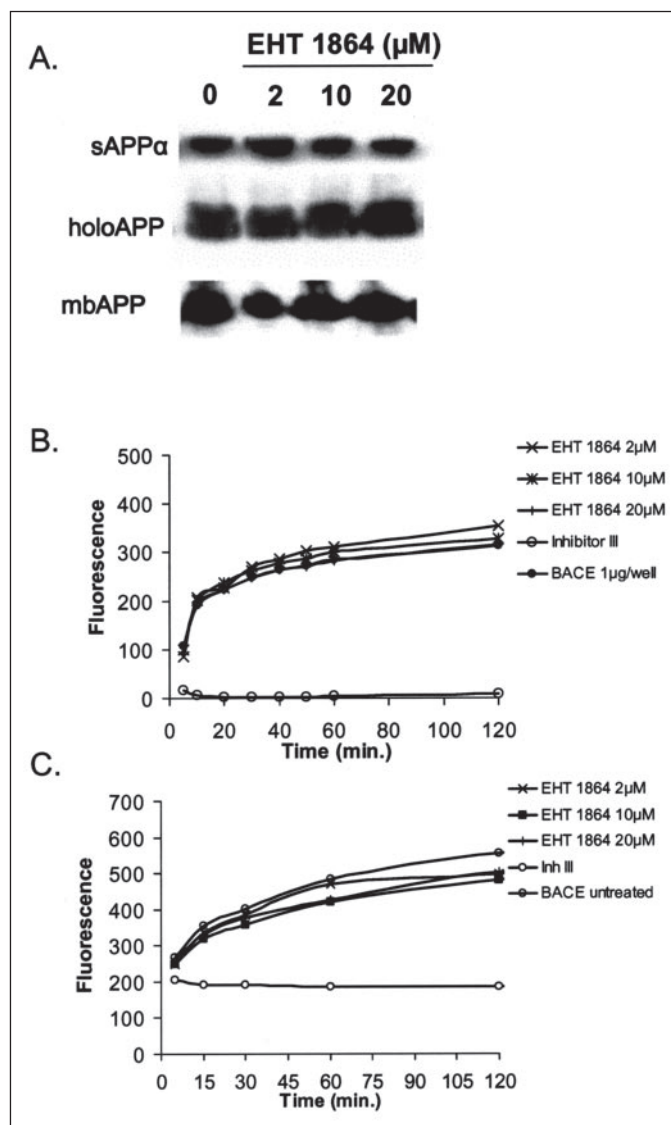
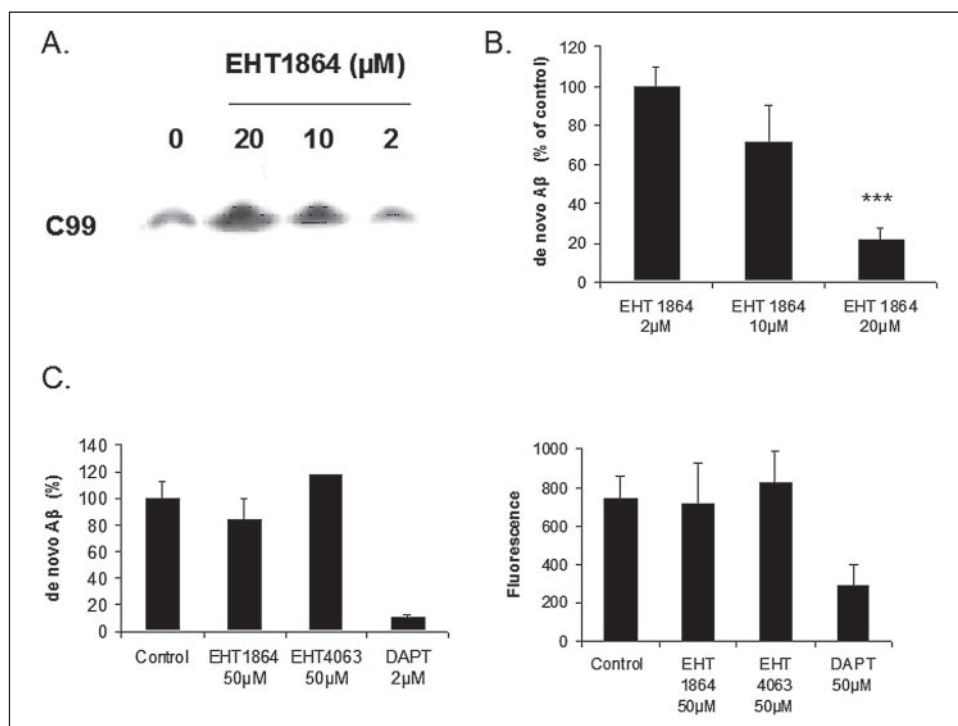


FIGURE 4. EHT 1864 does not affect α -secretase and BACE pathways. *A*, determination of sAPP α secretion, APP expression and maturation, and membranous APP levels by Western blotting. swAPP HEK293 cells were treated with various doses of EHT 1864 or not, and supernatants were analyzed for sAPP α secretion levels using 6E10 antibody, whereas cell lysates and membrane preparations were concomitantly processed for total holo-APP or membranous APP (mbAPP) detection, respectively, using AHP538 antibody. *B*, EHT 1864 does not directly act as an inhibitor of BACE in a fluorogenic substrate cleavage assay. EHT 1864 (2, 10, or 20 μ M) was added to human BACE protein (diluted at 1 μ g/well in 0.1 M sodium acetate buffer, pH 4.4) in the presence of 15 μ M quenched fluorogenic substrate. Results shown represent the mean \pm S.E. of three independent experiments. Substrate cleavage, resulting in increased fluorescence, is not changed by EHT 1864, whereas the BACE substrate analog inhibitor III abolishes the cleavage. *C*, EHT 1864 is not acting as an indirect inhibitor of BACE activity. BACE activity was determined in homogenate proteins from HEK293 cells stably expressing BACE1 that were treated or not with various doses of EHT 1864 for 24 h. Results shown represent the mean \pm S.E. of three independent experiments. EHT 1864 at 2, 10, or 20 μ M did not affect fluorescence, whereas very low substrate cleavage activity was detected in cells treated with BACE inhibitor III.

cleavage of an internally quenched fluorogenic γ -secretase substrate or endogenous C99 itself. The well characterized cell-permeable γ -secretase inhibitor DAPT (40) was included as control. C99 cleavage by γ -secretase was measured by *de novo* A β 40 generation. *De novo* A β 40 production increased with time, was optimal at 37 $^{\circ}$ C, and was completely inhibited by the γ -secretase inhibitor DAPT (data not shown). Incubation of cells with EHT 1864 for 16 h resulted in a dose-dependent reduction in *de novo* A β production from C99 (Fig. 5B), consistent with

FIGURE 5. EHT 1864 targets γ -secretase activity but is not acting as a direct inhibitor of γ -secretase. A, C99 accumulation in BACE-SH-SY5Y cells due to EHT 1864 treatment is dose-dependent. BACE-SH-SY5Y was treated for 24 h with EHT 1864 at 2, 10, or 20 μ M, and C99 accumulation was monitored by Western blotting using AHP538 antibody. B, γ -secretase assay, as monitored using A β 40-specific ELISA. Incubation of cells with EHT 1864 for 16 h results in a dose-dependent reduction in γ -secretase activity. C, EHT 1864 added to solubilized γ -secretase preparations obtained from untreated cell shows no direct inhibitory effect on γ -secretase activity on two different substrates, endogenous C99 (as monitored by *de novo* A β 40 generation) (left graph) and fluorogenic γ -secretase peptide substrate (as monitored by fluorescence recording) (right graph). Results shown represent the mean \pm S.E. of three independent experiments.



an action on γ -secretase activity. In contrast, EHT 4063 did not affect γ -secretase activity at any of the concentrations tested.

To test whether EHT 1864 could act as a direct γ -secretase inhibitor, EHT 1864 was added to solubilized γ -secretase preparations obtained from untreated swAPP-HEK293 cells. Alternatively, we used HeLa cells, which endogenously present high γ -secretase activity as compared with other cell types (38), as the source of solubilized γ -secretase in the fluorogenic γ -secretase substrate cleavage assay. At 50 μ M, EHT 1864 did not change *de novo* A β 40 generation from membrane preparations from swAPP-HEK293 cells (Fig. 5C, left graph). A similar lack of effect was seen with concentrations as high as 4 mM (not shown), and this result was confirmed using the fluorogenic γ -secretase substrate that contains the C-terminal β -APP amino acid sequence cleaved by γ -secretase solubilized from HeLa cells (Fig. 5C, right graph), establishing that EHT 1864 is not a direct competitive inhibitor of γ -secretase. Rather, since EHT 1864 inhibited A β 40 production in cells and in the γ -secretase assay, our results suggest that EHT 1864 modulates γ -secretase activity, so that the A β production pathway is blocked, through an indirect mechanism.

EHT 1864 Does Not Inhibit Notch-1 Cleavage—Many γ -secretase inhibitors, including DAPT, also inhibit the cleavage of the γ -secretase substrate Notch-1, the signaling of which is required in the adult organism for ongoing differentiation processes of the immune system and the gastrointestinal tract. In contrast, agents that modulate γ -secretase activity and reduce A β 42 levels, such as nonsteroidal anti-inflammatory drugs or Gleevec, do not inhibit Notch-1 cleavage. To determine whether EHT 1864 inhibits Notch cleavage, we used HeLa cells because of their high γ -secretase activity as compared with other cell types (38). HeLa cells were transiently transfected to overexpress N-terminally truncated Notch-1 (Notch Δ E) and exposed for up to 16 h to various concentrations of EHT 1864 or of the γ -secretase inhibitor DAPT (100 nM). Detection of Notch Δ E and the γ -secretase cleavage product NICD by Western blot showed that EHT 1864 did not affect Notch cleavage at any concentration tested (Fig. 6), confirming that EHT 1864 is not a

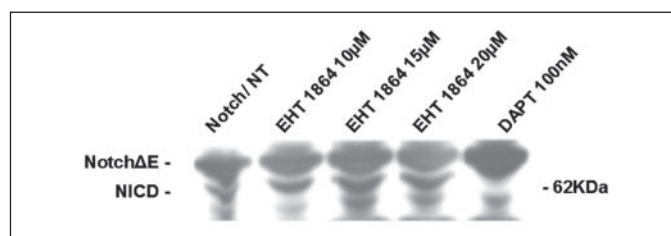
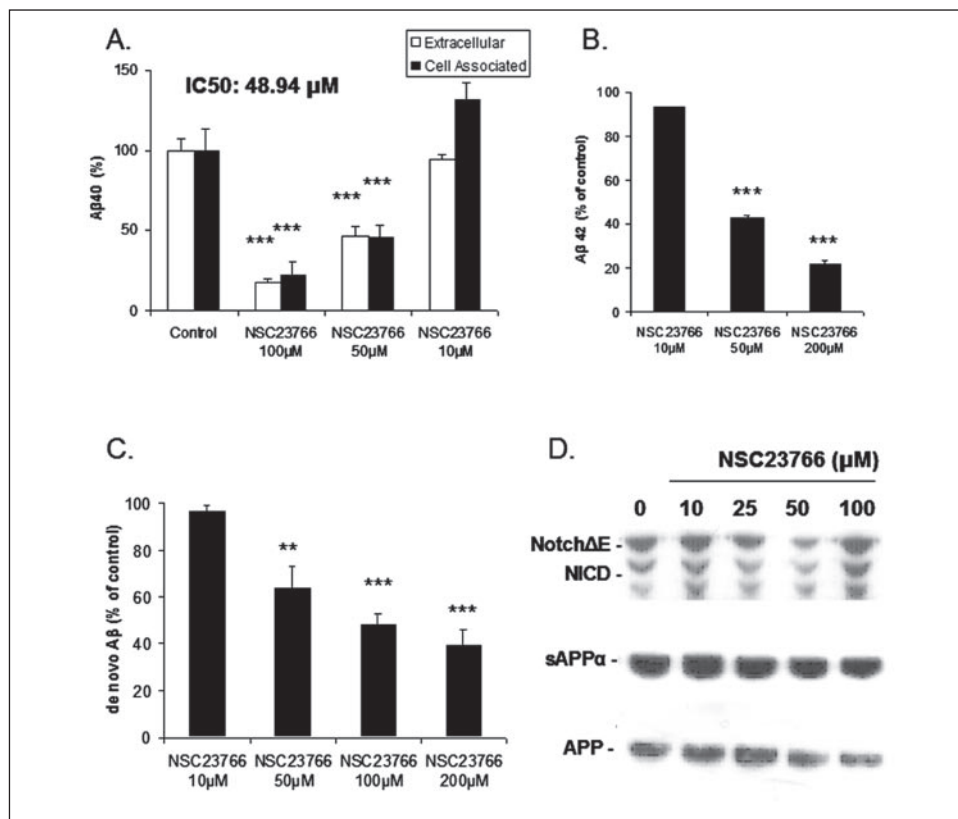


FIGURE 6. EHT 1864 does not affect Notch proteolysis. HeLa cells were transiently transfected to overexpress N-terminally truncated Notch-1 (Notch Δ E) and exposed for 16 h to the indicated concentrations of EHT 1864 or of the γ -secretase inhibitor DAPT. Cell extracts were probed by Western blot using anti-c-Myc antibody to detect Notch Δ E and the γ -secretase cleavage product NICD.

direct γ -secretase inhibitor. As a control, DAPT potently inhibited Notch cleavage, leading to virtually undetectable NICD levels.

A Commercially Available Rac1 Inhibitor, NSC23766, Also Prevents A β 40 and A β 42 Production in Vitro without Affecting Notch and sAPP α —Very few Rac1-specific inhibitors are commercially available. Recently, Gao *et al.* (41) described NSC23766, a cell-permeable Rac1-specific inhibitor with an IC₅₀ of \sim 50 μ M, which was shown not to affect the activity of Cdc42 or RhoA. On swAPP-HEK293 cells, treatment with various concentrations of NSC23766 dose-dependently reduced levels of secreted and intracellular A β 40 (Fig. 7A). Interestingly, the IC₅₀ was 48.94 μ M, in line with its reported effects on Rac1 inhibition (41). Based on IC₅₀ determination, EHT 1864 is 10-fold more potent than NSC23766. Accordingly, extracellular A β 42 levels were also dose-dependently decreased, with 57.97% inhibition of released A β 42 at a concentration of 50 μ M (Fig. 7B). Treatment of cells with various concentrations of NSC23766 for 24 h resulted in a dose-dependent reduction in *de novo* A β 40 generation from solubilized γ -secretase (Fig. 7C), suggesting that NSC23766 prevented γ -secretase activity in cells. When directly added to the γ -secretase reaction mixture, NSC23766 did not reduce A β 40 levels, suggesting that NSC23766 did not act as a direct γ -secretase inhibitor (data not shown). In addition, up to 400 μ M, we

FIGURE 7. The commercially available Rac1 inhibitor NSC23766 prevents A β 40 and A β 42 production *in vitro* without affecting Notch and sAPP α . A, swAPP-HEK293 cells were treated with subtoxic concentrations of Rac1-specific inhibitor NSC23766 described by Gao *et al.* (41) for 16 h, and supernatants and cell lysates were processed for A β 40 dosage by ELISA for the determination of released and intracellular (cell-associated) A β 40 levels, respectively. A β release and IC₅₀ were determined using the Prism software. B, reduction of A β 42 levels in cell supernatant of swAPP-HEK293 cells treated with NSC23766. C, γ -secretase assay, as monitored using an A β 40-specific ELISA to quantitate *de novo* A β 40 production by γ -secretase. Incubation of cells with NSC23766 for 24 h results in a dose-dependent reduction in γ -secretase activity. Results shown represent the mean \pm S.E. of three independent experiments. D, NSC23766 has no effect on Notch Δ E/NICD, APP, and sAPP α . swAPP-HEK293 and pSC2+ Δ E3MV-6MT-transfected HeLa cells were treated for 16 h with the indicated concentrations of NSC23766, and cell lysates or supernatants were probed by Western blot using antibody anti-c-Myc (Notch Δ E/NICD), 6E10 (sAPP α), or AHP538 (APP).



found no evidence of cytotoxicity measured by both lactate dehydrogenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (data not shown), suggesting that decreased A β levels were not due to cell viability impairment and that Rac1 inhibition itself is not deleterious for cell survival.

Finally, we tested the effect of NSC23766 on Notch Δ E/NICD, sAPP α , and APP in pSC2+ Δ E3MV-6MT and swAPP-HEK293-transfected HeLa cells, respectively, treated for 16 h with the indicated concentrations of NSC23766 and found no alterations in APP levels, the α -secretase pathway, or Notch processing, as determined by Western blotting (Fig. 7D).

Thus, NSC23766 recapitulates the effect of EHT 1864 on APP processing and A β production. This strengthens our hypothesis that the ability of EHT 1864 to prevent A β 40 and A β 42 production *in vitro* by specifically inhibiting γ -secretase-dependent APP cleavage without affecting Notch and the neurotrophic α -secretase pathway is likely to rely on its ability to interfere with Rac1 signals.

EHT 1864 Prevents A β 40 and A β 42 Production *in Vivo*—The effects of EHT 1864 were tested in the guinea pig to determine whether the observed reductions in A β 40 and A β 42 observed in cell lines overexpressing wild type and human mutant APP can be reproduced *in vivo*. We used normal wild type albino guinea pigs as a model, because guinea pigs are an established model for physiological APP processing and A β production (42). In addition, their A β 40 and A β 42 peptides are identical to human A β and can be readily detected by the BIOSOURCE sandwich ELISA.

Preliminary experiments performed in rats showed that EHT 1864 after oral administration displays good tolerability, brain penetrance, and no genotoxicity (Ames test).³ We opted for a straightforward delivery mode in guinea pigs and delivered EHT 1864 over 15 days by means

of daily intraperitoneal injections at two concentrations (10 and 40 mg/kg). We used a guanidine-based extraction protocol to ensure recovery of both Triton-soluble and Triton-insoluble A β fractions. In control animals, recovered A β 40 concentration was 1220 pg/mg proteins. EHT 1864 (40 mg/kg/day) lowered brain A β 40 by 37% with $p < 0.05$ (by the Wilcoxon test) (Fig. 8A). For A β 42, despite a high variability in measurement, probably due to the smaller amounts of peptide, the same dose of the compound EHT 1864 (40 mg/kg/day) caused a 23.6% decrease in A β 42 levels. At 10 mg/kg, EHT 1864 also led to a small reduction in the amount of A β 40 and A β 42 in the brain (12.8 and 6%, respectively).

No significant changes in full-length APP and sAPP α levels were detected in treated animals (Fig. 8B), consistent with an inhibitory action at the level of APP cleavage. No obvious signs of behavioral or anatomic abnormalities were observed for any of the treated animals at the indicated doses, and normal weight gain was observed for all animals.

DISCUSSION

γ -Secretase is a key event in the amyloidogenic cleavage of APP. γ -Secretase also plays an essential role in the processing of a number of targets, including Notch and its ligands. Accordingly, treatment with γ -secretase inhibitors results in clear Notch-based toxic effects on the immune system and the gastrointestinal tract (43) and may possibly generate learning and memory deficits (44). Thus, it now seems clear that the ideal γ -secretase inhibitor for AD therapy should lower A β levels without affecting Notch.

In the present study, we report that EHT 1864, a new chemical entity, can reduce both extracellular and intracellular levels of A β peptides. Our results indicate that this compound does not induce a general down-regulation of APP expression but rather decreases A β production by inhibiting the γ -secretase-dependent cleavage of APP, without

³ V. Picard and D. Drouin, unpublished observations.

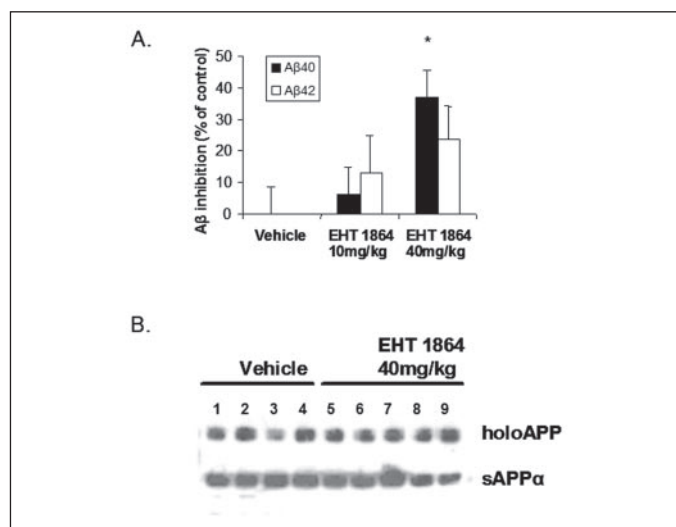


FIGURE 8. Reduction in A β 40 levels in adult albino guinea pig brain by EHT 1864. A, A β 40 levels in cortex after administration of EHT 1864 at 10 and 40 mg/kg. B, APP expression and maturation and sAPP α levels in cortex after administration of EHT 1864 at 40 mg/kg. Lanes 1–4, vehicle (saline)-treated animals; Lane 5–9, EHT 1864-treated animals (40 mg/kg daily for 15 consecutive days and by the intraperitoneal route).

affecting Notch cleavage. We also present data indicating that EHT 1864 specifically impairs the ability of Rac1 to interact with effectors and interfere with Rac1-dependent signals. In fact, EHT 1864 also blocks Rac1-dependent cytoskeleton rearrangements such as ruffles induced by osmotic shock.⁴

Since several previous reports have stressed that Rac1 is a central player of signaling pathways that control APP processing (20–22), it is likely that the ability of EHT 1864 to inhibit the γ -secretase-dependent production of A β peptides relies on its inhibitory effects on Rac1 signaling. Rac1, as well as other small G proteins, is involved in several transduction pathways that have been found to be up-regulated in AD brains, like those triggered by inflammatory mediators, such as IL-1 β , IL-6, tumor necrosis factor- α , and by transforming growth factor- β or platelet-derived growth factor. Since these factors promote the synthesis and the processing of APP, which leads to an increase in A β peptide levels (21–26, 44–47), small G proteins can be envisioned as attractive targets to control the pathological increase of A β 40 and A β 42 levels. Interestingly, several molecules that have been suggested for AD treatment, such as statins and nonsteroidal anti-inflammatory drugs, interfere with small G protein functions. Indeed, in addition to the fact that they can directly inhibit γ -secretase activity, nonsteroidal anti-inflammatory drugs may also interfere, like statins, with small G proteins activities by altering the isoprenoid pathway. The isoprenoid pathway, which includes lipid modification of various members of the Rho family of small G proteins, such as RhoA, -B, and -C and Rac, controls the proper translocation of these small G proteins to the proper organelle membrane, essential for activating downstream effectors. For example, statins inhibit RhoA and Rac1 isoprenylation (see Ref. 48 for a review), inducing changes in the actin cytoskeleton, assembly of focal adhesion complexes, and decreasing efficiency of vesicular transport. Accordingly, statins induce a general, nonspecific down-regulation of the activity of small G proteins, which leads not only to a decrease of A β secretion but also to an intracellular accumulation of APP and of A β peptides, resulting in plaque formation and cellular damage (49). Our

results show that inhibition of Rac1 by EHT1864 reduces both extracellular and intracellular amounts of A β peptides. Therefore, interfering with Rac1 signaling does not lead to the same intracellular accumulation as the general inhibition of small G proteins by statins.

The importance of Rac1-specific functions for APP processing can be explained by specific cellular roles of this protein. In the mature brain, Rac1, but not Rho nor Cdc42, is present in the raft domain of neuronal membranes (16, 17). Rac1 recruitment into the lipid rafts occurs upon its activation through various signals regulating the assembly and export to the cell membrane of Golgi-derived lipid rafts (18, 19). Lipid rafts participate in a number of important biological functions including the trafficking of proteins and lipids in the secretory and endocytic pathways. Raft-associated proteins cycle between the cell surface and Golgi by raft-mediated endocytosis characterized by clathrin independence and dynamin dependence. Biochemically, rafts are characterized by their insolubility in nonionic detergents, such as Triton X-100, and by this method several proteins relevant to A β production have been shown to be present in raft domains. These proteins include a small proportion of APP, BACE, and the γ -secretase complex (7–12), but not ADAM-10 and ADAM-17, prompting the hypothesis that amyloidogenic processing of APP takes place in lipid rafts, whereas APP outside rafts probably undergoes cleavage by α -secretase. Therefore, it is likely that Rac1 regulates the display and functionality of lipid rafts where APP processing takes place and A β is generated, relaying the different signaling pathways to the assembly of the functional lipid rafts. The new chemical entity, which specifically inhibits Rac1 signals, may act to disorganize complexes containing the γ -secretase and its APP substrate in both the secretory and endocytic compartments. This hypothesis is supported by our observation that EHT 1864 interferes with the γ -secretase activity, leading to a decrease in both intracellular and extracellular levels of A β .

On the other hand, γ -secretase cleavage of Notch and of its ligands occurs at the cell surface in nonraft membrane domains (45). Since our data show that EHT 1864 does not impact Notch processing, we can suggest that the interaction of the γ -secretase with its substrates at the plasma membrane is not controlled by Rac1 activity.

In vivo, administration of EHT 1864 to guinea pigs leads to a 30% reduction of A β peptide production, indicating that this compound can cross the blood-brain barrier. It is interesting to note that in patients with early onset AD, due to mutations in APP or in presenilin, A β 42 peptide levels are increased by as little as 30%. In addition, the same small increase in A β 42 levels in transgenic mice is sufficient to markedly accelerate A β deposition (50, 51). Therefore, given the progressive nature of the Alzheimer disease, a small reduction in A β production could have a profound impact on the evolution of the pathology, by lowering A β peptide levels below concentrations required for plaque formation and for neurodegeneration. These arguments and our data prompt us to consider EHT 1864 as the prototype of a series of molecules suitable for AD treatment.

Rac1 is involved in many biological processes, and it has been reported that deletion of Rac1 can interfere with normal biological phenomenon such as neutrophil functions (52). Decreasing Rac1 activity by a chemical entity to progressively inhibit A β peptide accumulation is likely to be compatible with mild interference with normal Rac1-dependent biological functions. In fact, statins that inhibit small G proteins, including Rac1, have proven to be safe and useful drugs to inhibit HMG-CoA reductase in humans. In addition, other benefits of inhibiting Rac1 signaling can be envisioned in the context of this disease. In fact, several mechanisms that are involved in AD have been shown to be regulated by Rac1 activity. This is the case for A β -induced neuronal

⁴ L. Désiré, J. Bourdin, N. Loiseau, H. Peillon, V. Picard, C. De Oliveira, F. Bachelot, B. Leboulton, T. Tavernier, E. Beausoleil, S. Lacombe, D. Drouin, and F. Schweighoffer, unpublished observations.

death, astrocytic inflammation, Cdk5-dependent abnormal tau phosphorylation, and impairment of *N*-methyl-D-aspartate-dependent LTP (53–55). Thus, EHT 1864 may therefore not only reduce A β production and plaque formation but also rescue neurons against A β toxicity, inhibit the formation of neurofibrillary tangles, and enhance cognitive functions. These aspects of AD pathology and of EHT 1864 pharmacology will be addressed using the triple transgenic mouse models that recapitulate many of the AD symptoms.

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