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Bepridil and Amiodarone Simultaneously Target the Alzheimer's Disease β- and γ-Secretase via Distinct Mechanisms

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The two proteases β-secretase and γ-secretase generate the amyloid β peptide and are drug targets for Alzheimer's disease. Here we tested the possibility of targeting the cellular environment of β-secretase cleavage instead of the β-secretase enzyme itself. β-Secretase has an acidic pH optimum and cleaves the amyloid precursor protein in the acidic endosomes. We identified two drugs, bepridil and amiodarone, that are weak bases and are in clinical use as calcium antagonists. Independently of their calcium-blocking activity, both compounds mildly raised the membrane-proximal, endosomal pH and inhibited β-secretase cleavage at therapeutically achievable concentrations in cultured cells, in primary neurons, and *in vivo* in guinea pigs. This shows that an alkalinization of the cellular environment could be a novel therapeutic strategy to inhibit β-secretase. Surprisingly, bepridil and amiodarone also modulated γ-secretase cleavage independently of endosomal alkalinization. Thus, both compounds act as dual modulators that simultaneously target β- and γ-secretase through distinct molecular mechanisms. In addition to Alzheimer's disease, compounds with dual properties may also be useful for drug development targeting other membrane proteins.

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder. The amyloid hypothesis ascribes the pathogenesis of the disease to a cascade of events, starting with the generation and accumulation of the amyloid β peptide (Aβ), a proteolytic fragment of the amyloid precursor protein (APP) (Hardy and Selkoe, 2002). Formation of Aβ requires APP to be proteolytically processed by the two proteases, β- and γ-secretase. A third protease, α-secretase, cleaves APP within the Aβ domain and precludes Aβ generation. β-Secretase is the membrane-bound aspartyl protease BACE1 and cleaves APP at the N terminus of the Aβ domain (Rossner et al., 2006; Cole and Vassar, 2008). This cleavage generates the soluble APPsβ and a C-terminal fragment (C99), which undergoes a second cleavage at the C terminus of the Aβ domain by γ-secretase. γ-Secretase belongs to the GxGD-family of aspartyl proteases and cleaves APP within its hydrophobic transmembrane domain. γ-Secretase is a heterotetrameric protein complex consisting of presenilin, nicastrin, PEN-2, and APH-1 (Steiner et al., 2008). γ-Secretase mainly cleaves after amino acid 40 of the Aβ sequence, resulting in the generation of the Aβ40 peptide. To a lower extent, γ-secretase also generates Aβ38 and Aβ42. Although a minor product, Aβ42 is considered the key player in AD pathogenesis and is the main constituent of the amyloid plaques found in AD brains (Hardy and Selkoe, 2002).

β-Secretase is an obvious drug target for AD, but most β-secretase inhibitors do not reach sufficiently high concentrations in the brain (Ghosh et al., 2008; Meredith et al., 2008). It is therefore essential to identify alternative strategies for reducing β-secretase cleavage. One possibility is the addition of a membrane anchor to a β-secretase inhibitor, which has been shown to increase its local membrane concentration, resulting in a more efficient β-secretase inhibition than the free inhibitor (Rajendran et al., 2008). Here we tested the possibility of targeting the cellular environment of β-secretase cleavage instead of the β-secretase enzyme itself. β-Secretase has an acidic pH optimum and mostly cleaves APP in the mildly acidic endosomes (Rossner et al., 2006), where it can be blocked by the weak base ammonium chloride (Haass et al., 1993; Schrader-Fischer and Paganetti, 1996). Interestingly, several drugs in clinical use contain weakly basic amino groups and thus have the potential to raise the endosomal pH.

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We screened such compounds for an inhibition of β-secretase cleavage and identified the drugs bepridil and amiodarone as novel inhibitors of β-secretase cleavage. Both compounds are calcium channel blockers and calmodulin antagonists and are used for the treatment of heart disease. Here we found them to inhibit β-secretase cleavage and Aβ generation independently of their calcium channel- and calmodulin-blocking activities. The inhibition of β-secretase cleavage occurred at therapeutically achievable and nontoxic concentrations by raising the endosomal, membrane-proximal pH. Surprisingly, both compounds additionally modulated the cleavage specificity of γ-secretase in an inverse manner. Thus, bepridil and amiodarone define a new class of drugs—the dual modulators—which may be used for drug development targeting β- and γ-secretase, either individually or simultaneously.

Materials and Methods

Antibodies and reagents. The following antibodies were used: 192wt and 192swe (APPsβ wild-type and Swedish mutations, respectively, Elan Pharmaceuticals), W02 (APPsα) and 22C11 [full-length APP (fl.APP), provided by K. Beyreuther, University of Heidelberg, Heidelberg, Germany], 6687 (fl.APP, Eurogentec), 6E10 (Aβ1-16, Santa Cruz Biotechnology), 3552 (Aβ, Eurogentec), 2D8 (Aβ1-16, provided by E. Kremmer, Helmholtz Institute, Munich, Germany), α-Aβ38 MSD-Tag (Meso Scale Discovery), α-Aβ40 BAP24-TAG and α-Aβ42 BAP24-TAG (provided by M. Brockhaus, Roche, Basel, Switzerland), α-calmodulin (Millipore), and α-β-actin (Sigma). The reagents used were as follows: bepridil hydrochloride, amiodarone hydrochloride, chloroquine, nimodipine, and tamoxifen (Sigma); AMI-AcOH (provided by H.-R. Ha, University Hospital, Zurich, Switzerland); GL-189 (provided by K. Maskos and W. Bode, Max Plank Institute for Biochemistry, Martinsried, Germany); egg phosphatidylcholine (PC) (Avanti Polar Lipids); fluorescein phosphatidylethanolamine (FPE) (Invitrogen); and siRNA pools targeting Calmodulin genes *calm1* and *calm3* and nontargeting control pools (Dharmacon).

Cell culture, compound treatment, and immunoblots. Human embryonic kidney 293EBNA (HEK293) cells were cultured as described previously (Kuhn et al., 2007). Clonal H4 and U373 cells stably expressing APP751 from the vector pRC/CMV hAPPwt were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 200 μg/ml G418 (G418-medium) or supplemented with 15% FCS, respectively. Clonal HEK293 cells stably expressing APP695 from the vector pCEP4-APP695 (HEK293-APP) and polyclonal HEK293 cells stably expressing C99 from the vector pCEP4-SP-C99 were selected with 100 μg/ml hygromycin. HEK293-APPswe cells were cultured in G418-medium as described previously (Haass et al., 1995). Cells were plated into poly-D-lysine-coated 24-well plates (BD Biosciences) or 6 cm dishes (for Aβ analysis by immunoblot). At confluence, cells were pretreated with compound or vehicle for 45 min, then with fresh medium plus vehicle or compound for another 4–24 h. Conditioned media and lysates were collected and immunoblots were performed as described previously (Schobel et al., 2008).

Aβ detection. For Aβ detection in the conditioned media, immunoprecipitation with antibody 3552 or 4G8 was performed before SDS-PAGE. Proteins were transferred to nitrocellulose membranes. Total Aβ was analyzed using a Tris-tricine gel (Schagger and von Jagow, 1987). Aβ species were separated on 11% urea gels (Wiltfang et al., 1997). Signals were quantified using a Fluorchem 8900 device with AlphaEaseFC software. Sandwich immunoassay of individual Aβ species using the Meso Scale Discovery Sector Imager 2400 was described previously (Page et al., 2008).

In vitro *BACE1 activity assay*. For *in vitro* analysis of BACE1 activity, an established fluorometric assay was used (Capell et al., 2002). The assay was performed in a volume of 100 μl containing 1 μl of purified BACE1 ectodomain, 1 μM substrate peptide (Cy3-SEV**NL**DAEFK(Cy5Q)-NH_2, GE Healthcare), and the indicated concentrations of bepridil, the BACE1 inhibitor GL-189, or just the vehicle DMSO in 40 mM sodium acetate buffer pH 4.5. Fluorescence was measured continuously over a period of 2 h at room temperature (Fluoroskan Ascent, excitation 530 nm, emission 590 nm, Labsystems).

Lactate dehydrogenase release assay. HEK293-APP cells were plated into poly-D-lysine-coated 96-well plates (BD Biosciences) at a density of 2×10^4 cells/well in medium containing heat-inactivated fetal calf serum. On the next day, the medium was changed and the cells were incubated with different concentrations of bepridil or vehicle only for 24 h (8 wells/condition). Tamoxifen was used as a positive control. Forty-five minutes before the end of the incubation, the cells from three wells of each condition were lysed with Triton X-100 (LDH_{max}). At the end of the incubation time, the amount of lactate dehydrogenase (LDH) in the conditioned media of the cells was analyzed using the CytoTox kit (Promega) according to the manufacturer's instructions. Toxicity was calculated as $ØLDH_{samples}/ØLDH_{max}$ for each condition (where Ø is mean value).

Primary mouse neuronal cultures. Neuronal cultures were derived from fetal Tg2576 mouse brain at gestation day 16 as described previously (Hartlage-Rübsamen et al., 2003). Briefly, brains from fetuses were prepared and suspended in DMEM/Ham's F12 by trituration with glass pipettes. Neurons were separated by sequential passage of the cell suspension through nylon membranes (150 and 20 μm), centrifuged for 5 min at 800 × *g*, and resuspended in seeding medium (DMEM containing 5% horse serum and 1× PSN antibiotic mixture, where PSN is penicillin, streptomycin, and neomycin). The cell number was determined and neurons were seeded at a density of 1.5 million cells per milliliter of culture medium in poly-L-lysine-coated 12-well plates. After 24 h of cultivation, the seeding medium was replaced by cultivation medium (25% astrocyte conditioned medium in DMEM/Ham's F12, N2 supplement, and 1× PSN). On day 3, *in vitro* neurons were used for pharmacological experiments. Cells were pretreated with compound or vehicle for 45 min then with fresh medium plus vehicle or compound for another 24 h.

Organotypic brain slice cultures. Organotypic brain slice cultures were established from adult APP transgenic Tg2576 mice as described previously (Reimers et al., 2007). Briefly, mice were decapitated and brains were quickly prepared and mounted in 2% agarose in distilled water. Brain sections (350 μm thick) were cut in the coronal plane using a vibratome (Leica). Brain sections were collected and maintained in culture plate inserts (Millicell CM, 30 mm diameter; Millipore) in DMEM/Ham's F12 medium containing 25% horse serum, 0.2% D-glucose, 2 mM L-glutamine, and 0.3 mM HEPES. The medium was changed every other day and pharmacological treatments were initiated after a cultivation period of 4 d. Slices were incubated for 16 h with serum-free medium, followed by pretreatment with vehicle or compound and then by incubation with fresh medium plus vehicle or compound for another 16 h. The conditioned medium and brain slices were collected and stored in aliquots at −20°C pending biochemical analyses.

Treatment of guinea pigs. Animal experiments were approved by Regierungspräsidium Leipzig (TVV 25/08). Female Duncin Hartley albino guinea pigs with a weight of 350–400 g were used (Charles River Laboratories). To account for variations in plasma Aβ levels among individual animals, baseline Aβ40 concentration was analyzed in each guinea pig. Blood samples (400 μl) were collected at 2:00 P.M. on the day before bepridil injection by puncture of the vena saphena lateralis. Heparin plasma was prepared by centrifugation at 2.500 × *g*, 10 min. The next day at 10:00 A.M., bepridil was injected intraperitoneally at 50 mg/kg body weight in a volume of 750 μl of 10% DMSO, 10% cyclodextran solution/100 g body weight. The positive control group received injections of DAPT (60 mg/kg body weight). The negative control group received vehicle injections. Four hours after injection, guinea pigs were killed by CO_2 inhalation, 1 ml of blood was collected, and plasma was prepared as described above and stored at −80°C. In the time course experiment, blood was taken 4 h after injection and again at 28 h after injection. In this case, guinea pigs were killed at 28 h after injection. Plasma Aβ40 concentrations before and after treatments were quantified by ELISA using the Biosource colorimetric assay kit according to the manufacturer's protocol. For each animal, the percentage amount of Aβ40 present after treat-

ment was calculated relative to baseline Aβ40. Brain levels of endogenous Aβ were below the detection limit.

Measurement of the pH in the endolysosomal system of living cells. HEK293-APP cells were incubated for 30 min with tetramethylrhodamine (TMR)-fluorescein-tagged dextran (5 mg/ml) and the respective concentration of bepridil, amiodarone, or DMSO as a negative control. Cells were then washed three times with serum-free medium and reincubated for 1 h in fresh medium containing the compounds. Cells were imaged with a Leica SP2 AOBS inverted confocal microscope using a 63× oil objective lens (numerical aperture = 1.3), exciting with a 488 nm and a 543 nm laser and emission bandwidths of 500–535 nm and 555–700 nm, respectively. The red:green ratio (TMR:fluorescein) of selected regions of interest (ROI) was calculated and pH values of each region were interpolated from a calibration curve. The calibration curve was generated by loading the cells as above with TMR-fluorescein-tagged dextran but without compounds and incubating them for 15 min in a triple-component calibration buffer containing HEPES, CHES, and citric acid and the ionophores nigericin (10 μM), valinomycin (10 μM), bafilomycin (0.1 μM), and monensin (1 μM). pH points between 4.5 and 7.0 with 0.5 increments were measured as described above (Di et al., 2006).

Measurement of the membrane-proximal pH using FPE. Twenty-five milligrams of egg PC were dissolved in low-salt buffer (20 mM HEPES-NaOH, pH 7.4) and hydrated for 1 h at room temperature (RT). Subsequently, small unilamellar vesicles (SUV) were prepared by sonication for 20 min at RT under a stream of argon gas as described previously (Kamp and Beyer, 2006). To incorporate the fluorescent probe FPE, a film of FPE was prepared by dissolving 36 μl of a stock solution of FPE (2.14 mg/ml in chloroform/methanol, 5:1 v/v) in 1 ml of chloroform and evaporating the solvents under a gentle stream of nitrogen gas in a round bottom flask followed by 1 h vacuum. The SUV suspension (1 ml, lipid concentration 25 mM) was injected on top of the FPE film and gently shaken overnight at RT. Remaining aggregates of unbound FPE were removed by 5 min tabletop centrifugation. The supernatant contained SUV loaded with FPE. Changes in the membrane-proximal pH were measured by diluting the SUV suspension in a stirred cuvette with 2.5 ml of buffer (final lipid concentration, 250 μM) and measuring the fluorescence of FPE (excitation at 490 nm, emission at 520 nm). Aliquots of the tested compounds were added with a Hamilton syringe through the pinhole of the fluorimeter (Jasco FP 6300).

Fluorescence-based measurement of intracellular calcium levels. Cytosolic Ca^{2+} was measured in HEK293-APP cells using the fura 2/AM dye (Invitrogen) as described previously (Hamid et al., 2007). Cells were viewed with an upright microscope (BX50WI, Olympus) using a 20× water-immersion objective. Excitation of the cells was performed at 340 nm for the Ca^{2+}-bound form and 380 nm for the unbound form of fura 2 and the ratio of the emissions was obtained using a digital imaging system (Till Photonics). Ca^{2+} concentrations were calculated from fluorescence-intensity ratios using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}] = K_d \times \beta[(R - R_{min})/(R_{max} - R)],$$

where K_d is the dissociation constant of fura 2 for Ca^{2+} ($K_d = 224$ nM), β is the fluorescence ratio of the 380 nm signal in the absence of Ca^{2+} to that in the presence of saturating Ca^{2+}, R is the fluorescence ratio obtained using a calcium calibration buffer kit (Invitrogen). All experiments were performed at room temperature and drugs were applied by bath perfusion.

Knockdown of calmodulin using siRNAs followed by compound treatment. HEK293-APP cells were plated at a density of 1×10^5 cells/well into poly-D-lysine-coated 24-well plates. After 4 h, cells were transfected with a mixture of siRNA pools against *calm1* and *calm3* or control pools using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, cells were treated with bepridil or vehicle as described above.

In vitro γ-*secretase assay.* Purification of γ-secretase and *in vitro* assays were performed as described previously (Winkler et al., 2009). The Q-Sepharose eluate fraction of purified γ-secretase was used as enzyme source.

Results

Alkalizing drugs inhibit β-secretase cleavage of APP at therapeutically relevant concentrations

First, we verified that the known alkalizing drugs ammonium chloride and chloroquine block β-secretase cleavage in HEK293-APP cells (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Next, we tested bepridil and amiodarone. Bepridil is in clinical use in France and Japan for the treatment of angina pectoris. Amiodarone is widely used for the treatment of arrhythmias. Both compounds are cationic amphiphilic drugs and have a hydrophobic core with two aromatic rings (Fig. 1*A*), which allows them to partition into the membrane. The hydrophobic core is linked through a short aliphatic spacer to a protonatable tertiary amino group (Fig. 1*A*, arrows), which acts as a weak base.

To investigate the effect of bepridil on β-secretase cleavage in detail, HEK293-APP cells were treated for 4 h with bepridil or with DMSO as a control. Secreted APP and Aβ were detected in the conditioned medium. Bepridil inhibited the generation of APPsβ and Aβ in a dose-dependent manner (Fig. 1*B–D*). In contrast to the inhibition of β-secretase cleavage, APPsα was largely unaffected by bepridil treatment. The amount of full-length APP in the cell lysate (immature and mature form) was not altered. Similar results for the inhibition of APPsβ generation were obtained for neuroglioma H4 and astroglioma U373 cells stably expressing APP and for the endogenous APPsβ derived from HEK293 cells (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). The IC_{50} for the inhibition of APPsβ and Aβ generation was ~6 μM (Fig. 1*C*,*D*), which is in the concentration range of bepridil found in the plasma of patients (3–10 μM) (Hollingshead et al., 1992). No cellular toxicity was observed below 50 μM, as determined in a lactate dehydrogenase release assay (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). This is in line with the fact that bepridil is used for chronic treatment. The inhibition of β-secretase cleavage occurred in an indirect manner, because bepridil was not able to block the activity of the β-secretase BACE1 in an established *in vitro* assay (Fig. 1*E*), which uses soluble BACE1 and measures the cleavage of a fluorophor-tagged APP-derived substrate peptide (Capell et al., 2002). In contrast, a known active site inhibitor of BACE1, GL-189 (Capell et al., 2002), fully blocked BACE1 activity in this assay (Fig. 1*E*). The indirect inhibition of β-secretase cleavage is in agreement with the proposed alkalinization of the endosomal pH being the mechanism of β-secretase inhibition. Amiodarone inhibited β-secretase cleavage in a similar manner as bepridil with an IC_{50} of ~ 30 μM (Fig. 1*F*,*G*). The therapeutically achievable plasma concentration of amiodarone is 1.5–6 μM (Freedman and Somberg, 1991). At this concentration, β-secretase cleavage was inhibited by 10–20% (Fig. 1*F*,*G*). From the data above, we conclude that, at therapeutically achievable concentrations, amiodarone and bepridil are novel inhibitors of β-secretase cleavage of APP and of Aβ generation.

Bepridil reduces Aβ levels in primary neurons, in organotypic brain slice cultures, and *in vivo* in the plasma of guinea pigs

Next, we tested whether the inhibition of β-secretase cleavage and Aβ generation by bepridil was also observed in primary neurons, in organotypic brain slice cultures, and in animals. Primary neurons were prepared from Tg2576 mice, which are an established mouse model of AD pathology and overexpress the Swedish mutant form of APP (Hsiao et al., 1996). Bepridil reduced APPsβ

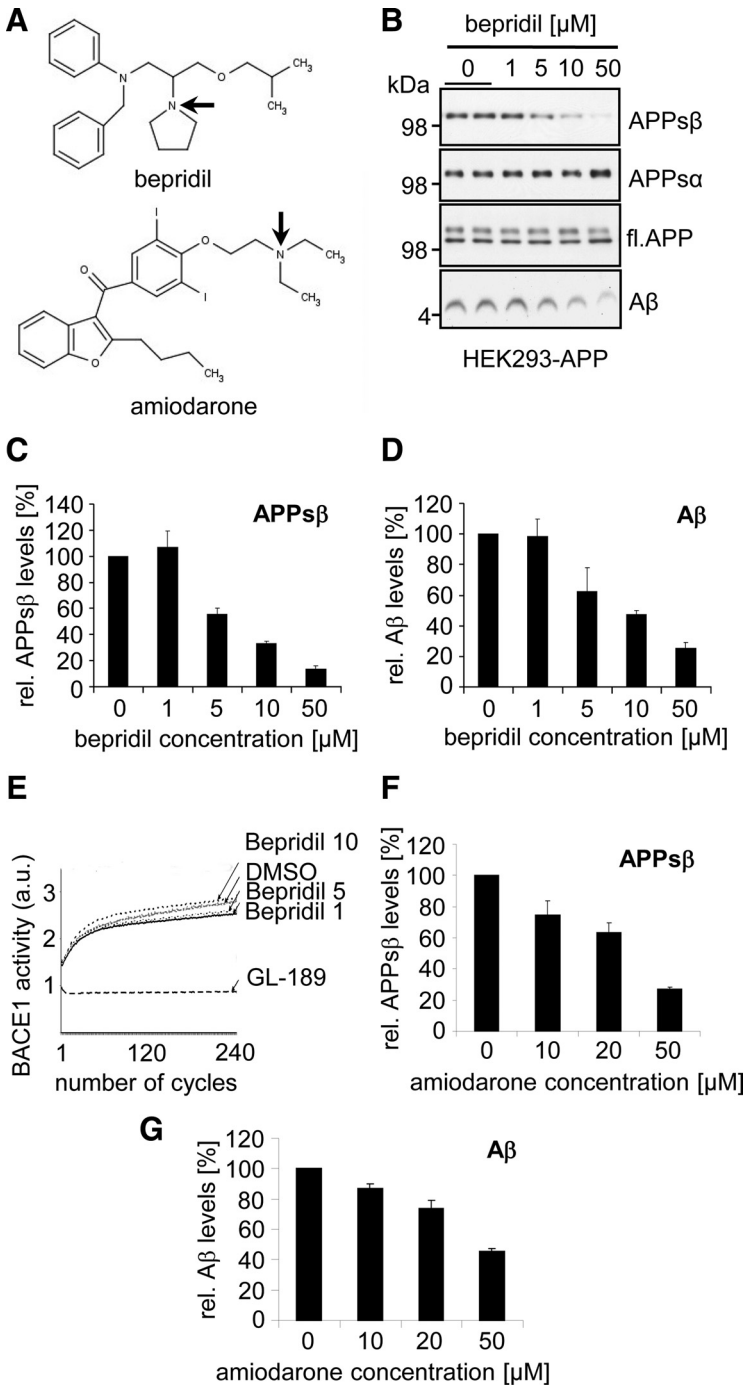

Figure 1. Bepridil and amiodarone are novel inhibitors of β-secretase cleavage. ***A***, Structure of bepridil and amiodarone. The protonatable amino group is marked (arrow). The second amino group in bepridil cannot be protonated due to a low pKa. ***B***, HEK293-APP cells were treated with bepridil for 4 h and the different APP species were analyzed by immunoblot. APPsβ and APPsα were detected in the conditioned medium using antibodies 192wt and W02, respectively. Aβ was detected using the antibody 6E10. fl.APP was detected in the cell lysate using the antibody 22C11. Bepridil caused a selective decrease in APPsβ and Aβ. ***C*** and ***D***, Results from ***B*** were quantified for APPsβ and Aβ. Shown are the mean and SD of four (***C***) and two (***D***) independent experiments. ***E***, The effect of bepridil was analyzed in an *in vitro* BACE1 activity assay using a fluorogenic APP-derived peptide substrate. Bepridil did not inhibit BACE1 activity, whereas the BACE1 inhibitor GL-189 completely blocked the activity of the enzyme. Concentrations of bepridil are given in μM. a.u., Arbitrary units; rel., relative. ***F*** and ***G***, Treatment of HEK293-APP cells with amiodarone as in ***B***. Shown are mean and SD of APPsβ and Aβ from three independent experiments.

and Aβ generation in the primary neurons in a dose-dependent manner and with similar IC_{50} values as in the cell lines (Fig. 2*A*,*B*). Then the effect of bepridil on Aβ generation was tested in organotypic brain slice cultures of Tg2576 mice. Also in this setting, bepridil reduced Aβ generation in a dose-dependent manner (Fig. 2*C*), demonstrating that the inhibitory effect is not only seen in cultured cells *ex vivo*, but also directly in the brain tissue. Bepridil lowered Aβ production to a similar extent as the well characterized γ-secretase inhibitor DAPT (Fig. 2*C*) (Dovey et al., 2001). Compared with cell lines and primary neurons, higher concentrations of bepridil and DAPT were required for inhibition in brain slices. Presumably, both compounds do not fully penetrate the 350-μm-thick slice at lower concentrations or are partly bound by lipids and proteins in the slice. At these concentrations, no toxicity was observed. Full-length APP and actin levels were not significantly changed in the lysates prepared from the slices (data not shown).

To test whether bepridil also reduces Aβ levels *in vivo*, guinea pigs were used. Compared with mice, guinea pigs have a larger body weight and blood volume, which allows one to draw blood before and after bepridil treatment and to determine Aβ changes within one animal. Guinea pigs were treated with bepridil (50 mg/kg, i.p.), which corresponds to a starting concentration of ~20 μM in plasma (Shiotani et al., 2005), or with the γ-secretase inhibitor DAPT (60 mg/kg) as a positive control (Lanz et al., 2003). Endogenous Aβ40 in the plasma was measured by ELISA. Plasma levels of endogenous Aβ38 and Aβ42 were below the detection limit. Initially, a time course pilot experiment was performed using two animals in the bepridil group and three animals in the control group. Plasma Aβ40 levels were reduced 4 h after bepridil injection and returned to control levels at 28 h after treatment (data not shown).

Next, we examined a larger number of guinea pigs ($n = 8$) at the 4 h time point. Twenty hours before treatment as well as 4 h after compound treatment, blood was drawn from all animals and analyzed for Aβ40. Bepridil reduced plasma levels of Aβ40 by ~50% compared with control-treated animals (Fig. 2*D*). A similar reduction of Aβ40 was observed for the positive control DAPT (Fig. 2*D*).

Together, the above experiments demonstrate that bepridil is able to block β-secretase cleavage and lower Aβ generation in primary neurons, in organotypic brain slice cultures, and *in vivo* in guinea pigs.

Bepridil and amiodarone raise the pH in endocytic compartments

Next, we tested whether bepridil and amiodarone are able to raise the pH in the acidic compartments of the endocytic pathway in

living cells, where β-secretase cleavage takes place (Rossner et al., 2006). To this aim, HEK293-APP cells were loaded with dextran carrying two different fluorescent tags. The pH-sensitive fluorescein acts as a pH sensor, whereas tetramethylrhodamine serves as a loading control and allows for ratiometric measure of pH. The TMR-dextran-fluorescein molecule is taken up into cells by endocytosis and labels endosomes and lysosomes. Treatment with bepridil led to a concentration-dependent increase of the pH from 5.1 to 5.5 in the endolysosomal pathway (Table 1). This pH increase is in agreement with the observed inhibition of BACE1, because BACE1 has a narrow pH optimum at pH 4.5–4.8, and its activity can be inhibited by small changes in the pH (Shimizu et al., 2008). Amiodarone had an effect on the pH comparable to bepridil (supplemental Table 1, available at www.jneurosci.org as supplemental material). Together, these results demonstrate that bepridil and amiodarone mildly alkalinize endosomal and lysosomal compartments of living cells.

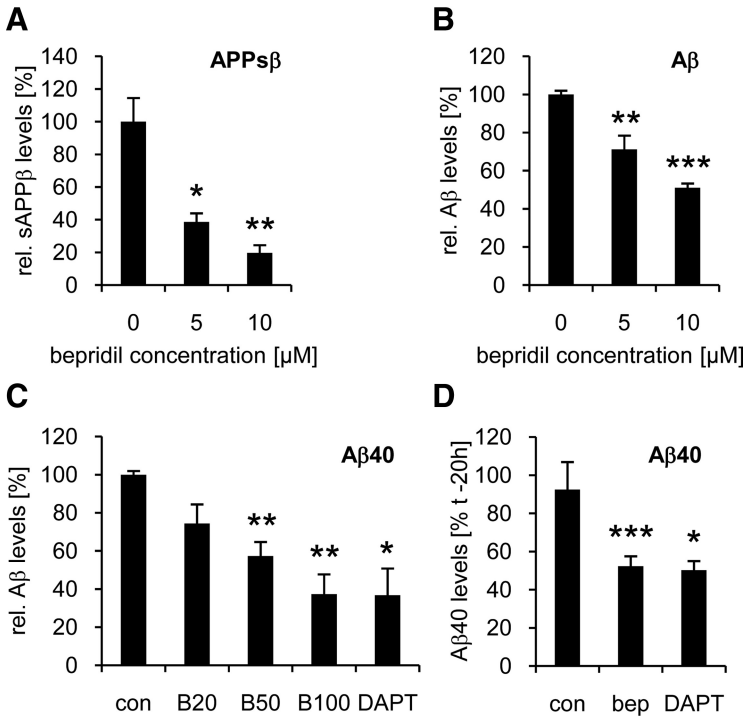

Figure 2. Bepridil inhibits Aβ generation in primary neurons, organotypic brain slice cultures, and in guinea pigs. ***A*** and ***B***, Primary neurons from Tg2576 mice were treated for 24 h with bepridil. Full-length APP and APP cleavage products were detected by immunoblot and quantified. Aβ was measured by sandwich immunoassay. Bepridil inhibited Aβ and APPsβ generation in the primary neurons. Shown are the mean and SD of APPsβ (***A***) and Aβ (***B***) from six experiments. ***C***, Organotypic brain slice cultures from Tg2576 mice were treated with vehicle control (con); with 20, 50, or 100 μM bepridil (B20, B50, B100); or with 25 μM γ-secretase inhibitor DAPT. Aβ40 was measured as in ***A*** and normalized to full-length APP levels in the lysates prepared from the slices. Bepridil reduced Aβ generation in a dose-dependent manner. Shown are the mean and SD from five experiments. ***D***, Guinea pigs were treated intraperitoneally with 50 mg/kg bepridil (bep) ($n = 8$) or vehicle control ($n = 8$). DAPT (60 mg/kg) was used as a positive control ($n = 3$). Levels of endogenous Aβ40 in plasma were determined after 4 h and are presented as percentage of Aβ40 plasma levels determined 20 h before treatment in the same animal. Bepridil and the positive control DAPT caused a highly significant decrease of Aβ40 compared with animals injected with control medium only. rel., Relative. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with control, determined with unpaired two-tailed *t* test.

Bepridil and amiodarone raise the membrane-proximal pH

Bepridil and amiodarone are cationic amphiphilic drugs, which penetrate into biological membranes. As a consequence, the positively charged, basic amino group localizes closely to the membrane surface where it can repel protons, leading to a local pH increase. This suggests that bepridil and amiodarone may increase the pH primarily in the membrane-proximal zone, where the β-secretase cleavage site in the APP sequence is located, without affecting the bulk pH. Direct experimental proof in living cells is difficult, because the fluorescently labeled dextran used above does not allow one to distinguish between pH changes occurring in the bulk of the endolysosomal lumen versus those that are restricted to the membrane-proximal area. To test for possible pH changes in the membrane-proximal area, an *in vitro* assay was used. A lipid-linked pH sensor was incorporated into lipid vesicles prepared from egg phosphatidylcholine (for a schematic drawing, see supplemental Fig. 4, available at www.jneurosci.org as supplemental material). The sensor consists of the pH-sensitive fluorophor fluorescein coupled to phosphatidylethanolamine. The unprotonated form of fluorescein, which is present under basic conditions, shows a higher fluorescence than the protonated form, which is predominant under acidic conditions. This system has been successfully used in the past for the measurement of changes in surface potential and pH in the membrane-proximal zone (Teissie et al., 1985; Simard et al., 2008). Bepridil and amiodarone were used at concentrations of 10 and 20 μM, which corresponds to the concentrations at which β-secretase inhibition was observed in the cellular assay. Bepridil and amiodarone increased the fluorescence of fluorescein in a dose-dependent manner compared with control (Fig. 3*A*). The increase in fluorescence was equivalent to an increase of the membrane-proximal pH by 0.4 or 0.6 U for 20 μM bepridil or amiodarone, respectively, as determined by a pH titration curve (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). This increase is similar to the change observed in the cellular system above (Table 1). Importantly, the compounds did not affect the pH of the bulk solution, as measured with a pH electrode. As a control for the assay, the lipid 1-tetradecylamine was used, which caused an increase in the membrane-proximal pH, as expected for this amino group containing basic lipid (Fig. 3*B*). As an additional control, a derivative of amiodarone (AMI-AcOH) was used, which contains a carboxylic acid group instead of the amino group of amiodarone (Fig. 3*C*). AMI-AcOH lowered the membrane-proximal pH (Fig. 3*B*). As expected from these data, AMI-AcOH did not block β-secretase cleavage (Fig. 3*D*), which further supports that the

Table 1. Bepridil raises pH in endocytic pathway

Concentration (μM)	pH
0	5.1 ± 0.02
5	5.2 ± 0.05
10	5.3 ± 0.03
15	5.3 ± 0.04
20	5.4 ± 0.05
30	5.5 ± 0.05

Mean ± SEM, $n > 50$.

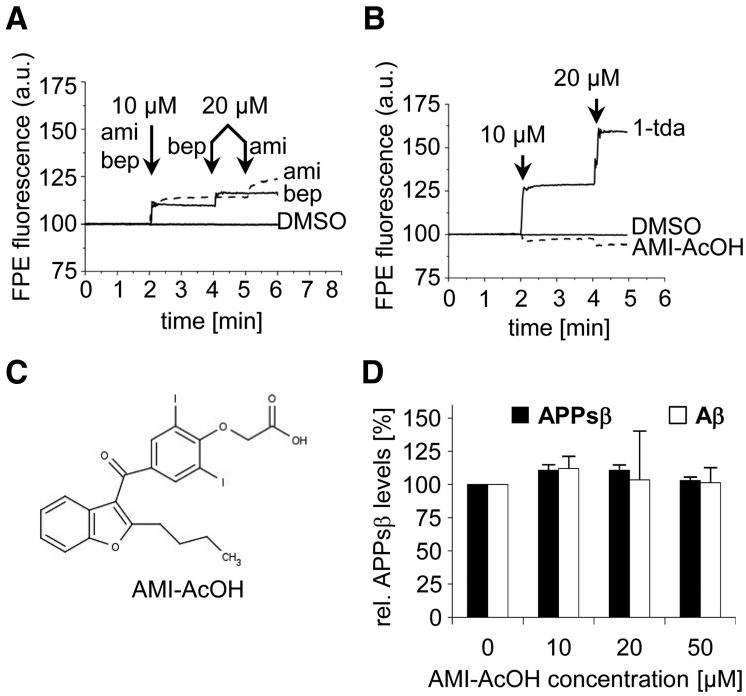

Figure 3. Bepridil and amiodarone raise the membrane-proximal pH. ***A*** and ***B***, Measurements of the fluorescence of FPE incorporated into membrane-like lipid vesicles. Bepridil (bep) or amiodarone (ami) (***A***) or the controls 1-tetradecylamine (1-tda) or AMI-AcOH (***B***) were added at the indicated time points. Concentrations of 10 and 20 μM were used. Bepridil, amiodarone, and the control 1-tda caused an increase in the fluorescence intensity of FPE, showing that they alkalinize the pH in the membrane-proximal zone. In contrast, AMI-AcOH caused a decrease in the membrane-proximal pH. rel., Relative; a.u., arbitrary unit. ***C***, Structure of AMI-AcOH. ***D***, HEK293-APP cells were treated for 4 h with AMI-AcOH. AMI-AcOH did not lead to a decrease of APPsβ and Aβ, demonstrating that the basic amino group is essential for the inhibition of β-secretase cleavage. Shown are the mean and SD of two independent experiments.

amino group and the alkalizing activity are required for blocking β-secretase cleavage.

Inhibition of β-secretase cleavage is independent of calcium changes and calmodulin

Bepridil and amiodarone are known calcium channel blockers and calmodulin (CaM) antagonists, but their mechanism of action is not fully understood. If the inhibitory effect on β-secretase cleavage is indeed due to the compounds' alkalizing activity, it should be independent of calcium channel inhibition and CaM. Indeed, the specific calcium channel blocker nimodipine, which is unrelated to bepridil and amiodarone, did not affect APP processing at concentrations where calcium channels in HEK293 cells are potently blocked (supplemental Fig. 6*A*, available at www.jneurosci.org as supplemental material) (Berjukow et al., 1996), which is in agreement with a previous publication (Facchinetti et al., 2006). Furthermore, bepridil did not alter intracellular calcium levels as determined in HEK293-APP cells loaded with the calcium-indicator dye fura 2 (supplemental Fig. 6*B*, available at www.jneurosci.org as supplemental material). Moreover, bepridil still potently inhibited APPsβ generation in CaM knockdown cells (supplemental Fig. 6*C*, available at www.jneurosci.org as supplemental material). Together, these experiments exclude that changes in cellular calcium or CaM are responsible for the inhibition of β-secretase cleavage by bepridil and amiodarone.

Bepridil and amiodarone modulate cleavage specificity of γ-secretase

Generation of Aβ requires the action of both β- and γ-secretase. Because γ-secretase has multiple cleavage sites, Aβ species of different length are generated—mainly Aβ40 and, to a lower extent, Aβ38 and Aβ42. Some small molecule drugs, called γ-secretase modulators (GSM), shift the γ-secretase cleavage site. Straight GSMs (sGSMs) lower Aβ42 and raise Aβ38 (Weggen et al., 2001), whereas inverse GSMs (iGSM) do the opposite (Kukar et al., 2005). To investigate whether bepridil—in addition to its inhibitory effect on β-secretase cleavage—modulates γ-secretase cleavage, C99-expressing HEK293 cells were used. C99 is the C-terminal APP fragment arising through β-secretase cleavage and allows monitoring γ-cleavage independently of β-cleavage. Total levels of Aβ as well as levels of Aβ38, Aβ40, and Aβ42 were measured. Surprisingly, bepridil increased Aβ42 by approximately twofold and reduced Aβ38 to ~50%, whereas levels of Aβ40 and total Aβ remained unchanged (Fig. 4*A*,*B*). A similar result was obtained in an *in vitro* γ-secretase assay, where purified γ-secretase, reconstituted into lipid vesicles together with C99 as a substrate, was incubated with bepridil (Fig. 4*C*). Generation of the APP intracellular domain (AICD), which is the second γ-secretase cleavage product, was not affected in the *in vitro* assay (supplemental Fig. 7, available at www.jneurosci.org as supplemental material), revealing that bepridil does not inhibit γ-secretase activity, but instead modulates the cleavage site of γ-secretase and the Aβ species being generated. Together, these experiments demonstrate that bepridil acts as an iGSM.

Next we measured whether the iGSM effect was also observed in cells expressing full-length APP instead of C99. To measure the Aβ species in a sandwich immunoassay, HEK293-APPswe cells were used, which produce more Aβ than HEK293-APP wild-type cells. In contrast to C99-expressing cells, bepridil reduced total Aβ levels (Fig. 4*D*) due to an inhibition of β-secretase cleavage, which is in agreement with the findings in Figure 1*D*. Generation of the main Aβ species Aβ40 was inhibited to a similar extent as total Aβ. However, Aβ38 was even more strongly inhibited, whereas Aβ42 was only mildly affected by bepridil (Fig. 4*D*). Thus, Aβ42 levels were highest, followed by Aβ40 and Aβ38, which is comparable to the effects observed in the C99-expressing cells (Fig. 4*A*,*B*). Similar results were observed for bepridil in primary neurons obtained from Tg2576 mice (Fig. 4*E*). An even stronger iGSM effect than for bepridil was observed for amiodarone. This compound strongly increased Aβ42, mildly reduced Aβ40, and more strongly lowered Aβ38 in HEK293-APPswe cells (Fig. 4*F*). Compared with bepridil, the milder effect of amiodarone on the main Aβ species Aβ40 is in agreement with the higher IC_{50} value for β-secretase inhibition. Together, the results demonstrate that bepridil and amiodarone act as dual modulators. They inhibit β-secretase cleavage of APP and simultaneously modulate γ-secretase cleavage.

To determine whether the GSM effect—similar to the inhibition of β-secretase cleavage—requires the amino group, we tested the effect of the amiodarone derivative AMI-AcOH on the generation of the individual Aβ species. AMI-AcOH lacks an amino

group and is not able to raise the membrane-proximal pH (Fig. 3*B*,*C*). AMI-AcOH still modulated the γ-secretase cleavage (Fig. 4*G*). This reveals that the amino group is not required for GSM activity, which is in agreement with the observation that the GSM activity of bepridil was also observed in the γ-secretase *in vitro* assay (Fig. 4*C*). Surprisingly, AMI-AcOH lowered Aβ42 and raised Aβ38 (Fig. 4*G*) and thus had the opposite GSM effect (sGSM) compared with amiodarone (iGSM). When comparing the structure of other known sGSMs and iGSMs (Weggen et al., 2001; Kukar et al., 2005), we observed that both classes have a hydrophobic, aromatic core, but that sGSMs additionally have a free carboxylic acid group as it is found in AMI-AcOH (Fig. 3*C*). This suggests that the carboxylic acid group determines whether a GSM acts as an iGSM or a sGSM and thus defines a structural feature required for sGSM activity.

Discussion

The proteases β- and γ-secretase directly generate the Aβ peptide and are considered main drug targets for Alzheimer's disease. However, clinically suitable inhibitors for both proteases have been difficult to find. Our study identifies and mechanistically characterizes the two clinically used compounds bepridil and amiodarone, which we found to act as dual modulators on APP processing. They inhibit β-secretase cleavage and simultaneously modulate γ-secretase cleavage. The mechanistic analysis shows that both effects occur through distinct molecular mechanisms and require different chemical groups of the compounds. This provides a blueprint for the development of new drugs indirectly inhibiting β-secretase, modulating γ-secretase, or targeting both enzymes simultaneously.

β-Secretase inhibitors need to reach the endosome where β-secretase cleavage of APP mostly takes place. For example, a recent study showed that membrane-anchoring of a BACE1 inhibitor allowed its endocytosis, increased its local membrane concentration in the endosome, and inhibited BACE1 activity more efficiently than the free inhibitor (Rajendran et al., 2008). Here, we report that inhibition of β-secretase cleavage can also be achieved using the alkalizing properties of bepridil and amiodarone. For the first time we show that both compounds mildly raise the pH in acidic compartments of living cells and increase the membrane-proximal pH in an *in vitro* membrane assay. Interestingly, bepridil and amiodarone recently have been shown to also block another pH-sensitive membrane-proximal endosomal process—the insertion of anthrax toxin into endosomal membranes—by raising the endosomal pH (Sanchez et al., 2007). We found that the inhibition of β-secretase cleavage both in cells and in guinea pigs occurred at concentrations of bepridil and amiodarone that are therapeutically achievable in the plasma of patients.

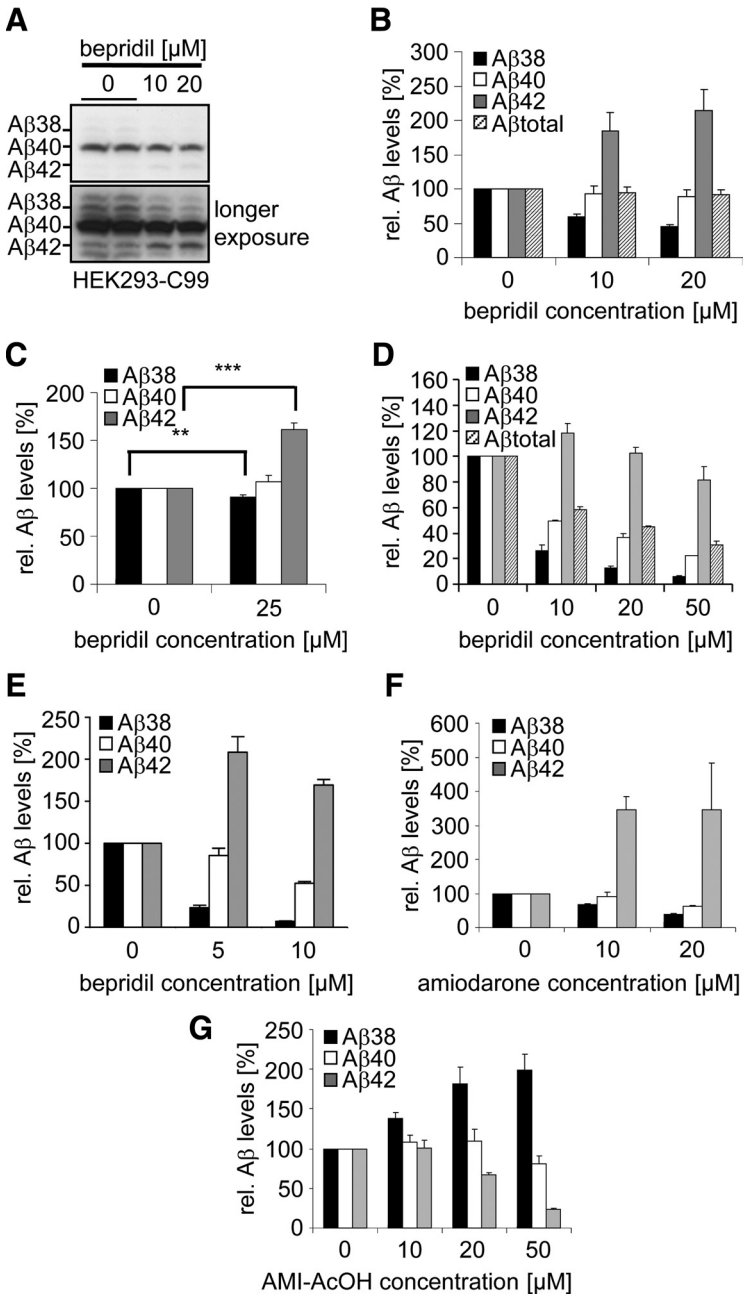

Figure 4. Bepridil and amiodarone modulate γ-secretase cleavage. ***A*** and ***B***, HEK293 cells expressing C99 were treated for 16 h with the indicated concentrations of bepridil. ***A***, The different Aβ species were analyzed on an 11% urea gel. ***B***, Quantification of the results from ***A***. Indicated are the mean and SD of two independent experiments. Bepridil acts as iGSM, decreasing Aβ38 and increasing Aβ42. Aβ40 and total Aβ were unaffected. ***C***, iGSM effect of bepridil in an *in vitro* γ-secretase assay using purified, reconstituted γ-secretase and recombinant C99 as a substrate. Aβ species were determined by sandwich immunoassay ($n = 4$). ***D***, HEK293-APPswe cells were treated as above with bepridil and Aβ species were determined by sandwich immunoassay. The dual modulatory effect led to a strong decrease in Aβ38 and Aβ40 and only minor changes of Aβ42. ***E***, Primary neurons from Tg2576 mice were incubated as in ***D*** with bepridil, which resulted in a strong reduction of Aβ 38, a milder reduction of Aβ40, and an increase of Aβ42. ***F***, HEK293 APPswe cells were treated with amiodarone as in ***D***. For amiodarone, the decrease in Aβ38 and Aβ40 is less pronounced compared with bepridil in ***D*** and Aβ42 is further increased. ***G***, HEK293-APPswe cells were treated as above with the amiodarone derivative AMI-AcOH. In contrast to amiodarone, AMI-AcOH acts as a straight GSM increasing Aβ38 and decreasing Aβ42. rel., Relative. $^{**}p < 0.01$; $^{***}p < 0.001$, determined with unpaired, two-tailed *t* test.

Whether both compounds also block β-secretase cleavage in the brain remains to be tested. Bepridil and amiodarone are chronically tolerated in patients. This suggests that a mild rise in the endosomal pH may be a clinically acceptable strategy. In fact, a similar approach is used for the treatment of the bacterial infection chronic Q fever, where the antimalarial drug chloroquine and its derivatives are used to raise the lysosomal pH (Rolain et al., 2007).

Weak bases, such as bepridil and amiodarone, become concentrated in acidic organelles, where they are protonated and retained. This principle may also help in the development of active site-directed BACE1 inhibitors. One such compound was found to have a higher potency in cells when it contained an additional amino group (Yang et al., 2006). Although the reason for the amino group requirement is not fully understood, it is possible that the amino group targets the inhibitor to the endosome, resulting in higher local inhibitor concentrations at the site of β-secretase cleavage and a more efficient inhibition of β-secretase. Potentially, such inhibitors also raise the endosomal pH and thereby additionally inhibit β-secretase cleavage.

Another outcome of our study is that amiodarone and bepridil raised the membrane-proximal pH in the membrane assay, but did not affect the bulk pH. This situation may also be found in endosomes in living cells. Direct experimental proof is difficult, because the available technology to determine the pH in the endolysosomal system in living cells, including the one used in this study, does not allow to distinguish between pH changes occurring in the bulk of the endosomal lumen versus those that are restricted to the membrane-proximal area. However, the compounds' chemical properties fit well with an effect primarily on the membrane-proximal pH. By inserting into the membrane, amiodarone and bepridil repel protons from the membrane surface and thereby raise the membrane-proximal pH. This puts BACE1 in an environment where the pH is no longer favorable for its activity. This localized area of action of bepridil and amiodarone may be helpful when the use of such compounds is considered for therapeutic purposes, as other processes in the endosomal and lysosomal lumen may occur normally. In fact, the processing of the soluble proenzyme procathepsin L, which takes place in the lumen of late endosomes, is not affected by amiodarone (Stadler et al., 2008).

Surprisingly, we found that amiodarone and bepridil—in addition to their inhibitory effect on β-secretase cleavage—additionally modulate γ-secretase in an inverse manner (iGSM). Together with their inhibitory effect on β-secretase cleavage, this resulted in a strong reduction of Aβ38, a prominent reduction of Aβ40, and only a minor reduction of Aβ42. The compounds' modulatory effect on γ-secretase seems to be independent of their alkalizing ability, because the effect also occurred in the cell-free γ-secretase assay. Moreover, the basic amino group was not required for the modulation of γ-secretase, since AMI-AcOH, which lacks the amino group, still modulated the cleavage specificity of γ-secretase. Additionally, other alkalizing compounds, such as ammonium chloride, which inhibit β-secretase cleavage, do not affect the cleavage specificity of γ-secretase (Vingtdeux et al., 2007). Furthermore, other compounds with GSM activity, such as fenofibrate and flurbiprofen, do not contain amino groups (Kukar et al., 2008). Thus, the dual modulators amiodarone and bepridil are likely to use two different parts of the molecule for the inhibition of β-secretase (amino group) and the modulation of γ-secretase (probably the hydrophobic core).

The first GSMs were identified in 2001 (Weggen et al., 2001). Proposed mechanisms of action include a direct binding to C99 (Kukar et al., 2008) and to the γ-secretase complex (Eriksen et al., 2003) and a change in the dimerization strength of C99 (Munter et al., 2007). GSMs are a chemically heterogeneous group of compounds. When comparing the structure of published GSMs, we found that sGSMs contain a free carboxylic acid group, which is absent in iGSMs. In line with this observation, we found that the addition of a carboxyl group to amiodarone (AMI-AcOH) was able to invert the specificity to γ-secretase. A similar result was observed in a recent study, which showed that a hydrophobic compound with iGSM activity could be converted to a sGSM by the addition of a free carboxylic acid group (Narlawar et al., 2007). Thus, the addition of a free acidic group may be a more generally applicable strategy to convert an iGSM to an sGSM.

Mechanistically, it is not yet clear why the carboxyl group is required for the sGSM effect. γ-Secretase seems to cleave first at the C-terminal end of the transmembrane domain of C99 and then proceed in a stepwise fashion toward the middle of the transmembrane domain, until the Aβ peptide is short enough to slip out of the membrane. A recent study suggested that the negatively charged free carboxylic acid of sGSMs binds and neutralizes the positively charged lysine 28, which forms the luminal, membrane-anchoring residue in the C99 sequence (Kukar et al., 2008). This neutralization may allow C99 to insert more deeply into the membrane. As a consequence, γ-secretase may proceed up to more *N*-terminally located residues, resulting in more Aβ38 and less Aβ42. Such a mechanism is in agreement with previous findings by us and others, which showed that a change in the length of the C99 transmembrane domain has a profound effect on the cleavage specificity of γ-secretase (Murphy et al., 1999; Lichtenthaler et al., 2002). Based on our results, we conclude that the hydrophobic core of amiodarone and bepridil is sufficient for modulation of γ-secretase and that the presence or absence of a free carboxylic group—or potentially another acidic group—determines the sGSM or iGSM activity, respectively.

Together, our work serves as a proof-of-principle showing that it is possible to identify dual modulators, which simultaneously target β- and γ-secretase cleavage of APP. This indicates that drugs developed against one of the two enzymes should always be inspected for potential additional effects on the other protease cleavage. Because the dual modulatory effect of amiodarone and bepridil occurs at concentrations that are therapeutically reached in the plasma of patients, both compounds may be used for future drug development. We envisage the generation of four different classes of compounds. First, modification of the hydrophobic core may yield drugs that lack GSM activity and selectively inhibit β-secretase cleavage. Second, AMI-AcOH provides a new structural scaffold for sGSMs, which do not affect β-secretase. The development of new sGSMs is needed because a previously identified sGSM failed in a recent phase III clinical trial for lack of efficacy (Rafii and Aisen, 2009). Third, dual modulators may be generated that simultaneously inhibit β-secretase cleavage and act as sGSMs on γ-secretase. An ideal dual modulator should comprise of the following three structural elements: (1) a hydrophobic core with aromatic rings for membrane-targeting, (2) an acidic group to allow for sGSM activity, and (3) an amino group and a net positive charge to guarantee the inhibitory effect on β-secretase by raising the membrane-proximal endosomal pH. Such compounds would allow the lowering of total Aβ levels and the additional specific lowering of Aβ42, which should result in an enhanced total reduction of Aβ42 levels. Fourth, given the accumulation of weak bases in acidic organelles, we consider that the addition of an amino group may be used as a more general strategy for endosomal and

lysosomal targeting of hydrophobic compounds, which may be developed against other membrane protein targets in both acidic compartments.

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