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Featured Article

Multiple BACE1 inhibitors abnormally increase the BACE1 protein level in neurons by prolonging its half-life

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Abstract

Introduction: There is keen interest in elucidating the biological mechanisms underlying recent failures of β-site amyloid precursor protein–cleaving enzyme-1 (BACE1) inhibitors in Alzheimer's disease trials.

Methods: We developed a highly sensitive and specific immunoassay for BACE1 in cell lines and iPSC-derived human neurons to systematically analyze the effects of eight clinically relevant BACE1 inhibitors.

Results: Seven of 8 inhibitors elevated BACE1 protein levels. Among protease inhibitors tested, the elevation was specific to BACE1 inhibitors. The inhibitors did not increase BACE1 transcription but extended the protein's half-life. BACE1 became elevated at concentrations below the IC_{50} for amyloid β (Aβ).

Discussion: Elevation of BACE1 by 7 of 8 BACE1 inhibitors raises new concerns about advancing such β-secretase inhibitors for AD. Chronic elevation could lead to intermittently uninhibited BACE1 when orally dosed inhibitors reach trough levels, abnormally increasing substrate processing. Compounds such as roburic acid that lower Aβ by dissociating β/γ secretase complexes are better candidates because they neither inhibit β- and γ-secretase nor increase BACE1 levels.

Keywords: Alzheimer's disease; β-Secretase; BACE1 inhibitor; Amyloid β protein; Protein homeostasis

1. Introduction

The devastating personal impact and growing societal burden of Alzheimer's disease (AD) has led to intense efforts to develop small- and large-molecule therapeutics to modify the disease course. Large-molecule approaches focus principally on monoclonal antibodies intended to clear the amyloid β protein (Aβ) or the tau protein, accumulation of which can compromise neuronal form and function [1]. Antibody infusions can result in cerebral toxicity, the resultant need for close patient monitoring, and ultimately the logistical challenge of infusing a global AD population. Oral small-molecule approaches are therefore highly desirable, and these have focused to date on inhibiting the β and γ secretases that cleave amyloid precursor protein (APP) to generate Aβ. Most current efforts center on inhibitors of β-site APP-cleaving enzyme-1 (BACE1), which is highly expressed in neurons and is the rate-limiting step in Aβ production. Heterogeneous Aβ peptides are ultimately generated and secreted into interstitial fluid and can accumulate as potentially neurotoxic oligomers and fibrillar amyloid plaques in the brain [2,3]. Pharmacological inhibition of BACE1 has become of great interest to the scientific and medical community [4].

BACE1 is a single-transmembrane aspartyl protease mainly expressed in the central nervous system and concentrated in neuronal presynaptic terminals. The luminal active site of BACE1 cleaves the distal ectodomain of APP, resulting in secretion of a large soluble extracellular fragment

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called sAPPβ. Subsequent intramembrane cleavage of the remaining C-terminal stub of APP (C99) by the presenilin/γ-secretase complex releases the APP intracellular domain into the cytoplasm and Aβ peptides of varying length into the extracellular fluid. Aβ1-42 and Aβ1-43 peptides are highly prone to oligomerization and amyloid formation. Since the initial identification of BACE1 in 1999 [2,3,5,6], it has become the prime drug target for chronically reducing Aβ production in the brain. However, despite achieving strong target engagement and up to 80% cerebrospinal fluid Aβ reductions, BACE1 inhibitors used in clinical trials on patients with mild-to-moderate AD have failed to demonstrate significant slowing of cognitive decline [7]. Moreover, recent detailed analysis of such trials has revealed modest but significant cognitive worsening in patients receiving certain BACE1 inhibitors [7–9]. Considering these disappointing results, it is important to understand BACE1 inhibitor action and its adverse effects in much more detail.

Here, we systematically analyzed the properties of eight BACE inhibitors, 7 of which have already been used in human trials. Unexpectedly, 7 of the 8 inhibitors substantially increased BACE1 protein levels in cells. The most potent compound, AZD3293, was shown to increase the level of BACE1 by prolonging its half-life in a mammalian cell line, in rat primary cortical neurons, and in iPSC-derived human neurons (iNs). In addition, 5 other BACE inhibitors were similarly shown to prolong the half-life of BACE1 in cells. We find that a significant elevation in BACE1 levels consistently accompanies the lowering of Aβ production by several BACE1 inhibitors used in clinical trials. Our results suggest that prolonged increases in the total BACE1 protein level during chronic dosing could contribute to the observed neurological side effects by intermittently augmenting BACE1 proteolytic processing of numerous single-transmembrane substrates important for proper neuronal signaling, in particular when brain levels of the inhibitor fall to trough levels during intermittent oral dosing.

2. Materials and methods

2.1. Reagents

LY2886721, LY2811376, AZD3293, AZD3839, MK-8931, pepstatin A, TAPI-1, Batimastat, and *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester (DAPT) were from Selleckchem; E2609 was from Sunshine Chemical; PF-06751979 was from MedChemExpress; β-secretase inhibitor IV was from Millipore; GI 254023X was from Tocris Bioscience; RA was from Aobious; synthetic Aβ peptides are all from AnaSpec; and recombinant human BACEl and BACE2 protein was from R&D System.

2.2. Cell culture

HEK-293 cells were maintained in standard medium: Dulbecco's modified Eagle's medium plus 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. For our HEK-293/sw-APP cell line, which overexpresses familial Alzheimer's disease–mutant human swAPP, standard medium was supplemented with G418. For the neurogenin 2 iN differentiation, iPSCs were differentiated following a published protocol [10]. Cultures were treated with doxycycline (2 mg/mL) on day 1 to induce differentiation, fed with a series of medium changes, treated at day 23 and harvested at day 24. Primary cortical neuronal cultures were prepared from Wistar rat embryos (E18).

2.3. Microsome preparations from cultured cells

For microsomes from cultured cells, the cells were first Dounce-homogenized with a tight pestle in Tris-buffered saline containing no detergent with 15 strokes, followed by passage through a 27.5-gauge needle four times. Samples were then centrifuged at 1000 × *g* followed by a 100,000 × *g* ultracentrifuge spin to pellet microsomes, which were solubilized in 50-mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid buffer, 150-mM NaCl containing 1% CHAPSO for 60 min, followed by another 100,000 × *g* spin to collect the final supernatant.

2.4. Electrophoresis and western blotting

Samples were loaded onto 4-12% Bis-Tris gels using 4-morpholineethane sulfonic acid-Sodium dodecyl sulfate running buffer (Invitrogen), transferred to Polyvinylidene fluoride membranes, and probed for various proteins using standard western blotting. The resultant blots were detected by enhanced chemiluminescence and signals were captured by the iBright system (Invitrogen) or film. The antibodies used to detect specific antigens were as follows: for mouse/rat BACE1, EPR19523 (rabbit, Abcam); for human BACE1, MAB5308 (mouse, Millipore, RRID: AB_95207); for SNAP-25, 610366 (mouse, BD, RRID: AB_397752); and for α-tubulin, T5168 (mouse, Sigma, RRID: AB_477579).

2.5. RNA extraction and qPCR

Total RNA was extracted with a TRIzol reagent. mRNA was reverse-transcribed to cDNA using WarmStart® RTx Reverse Transcriptase. qPCR was performed with PowerUp SYBR Green Master Mix. Two sets of primers for BACE1 were used: set (1) 5′-TGATCATTGTGCGGGTGGAGA-3′ and 5′-TGATGCGGAAGGACTGGTTGGTAA-3'; set (2) 5′-ACTCCCTGGAGCCTTTCTTTG-3′ and 5′-ACTTTCTTGGGCAAACGAAGGTTGGTG-3'. Primers for actin were used for normalization of mRNA amount: 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′.

2.6. Size-exclusion chromatography

Microsomes isolated from cultured cells were solubilized in 1% CHAPSO (350 µl total volume), injected onto a Superose 6 Increase 10/300 column (24-ml bed volume), and run on a fast protein liquid chromatography system (AKTA; GE Healthcare) in 50-mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid buffer, 150-mM NaCl with 0.25% CHAPSO, pH 7.4. Fractions of 500 µl were collected for downstream experiments. Columns were calibrated with Gel Filtration Standard (BioRad), which ranges from 1350 to 670,000 Da.

2.7. Enzyme-linked immunosorbent assay

Conditioned culture medium and cell lysate were diluted with 1% bovine serum albumin (BSA) in wash buffer (Tris-buffered saline supplemented with 0.05% Tween). For our home-made assays on the MSD electrochemiluminescence platform, each well of an uncoated 96-well multiarray plate (Meso Scale Discovery, #L15XA-3) was coated with 30 µL of a phosphate-buffered saline (PBS) solution containing capture antibody (3 µg/ml 266 for all human Aβ ELISAs, 3 µg/ml M3.2 for rodent Aβ ELISAs, or 7.2 µg/ml MAB931 for BACE1 ELISA) and incubated at room temperature (RT) overnight followed by blocking with 5% BSA in wash buffer for 1 h at RT with shaking at >300 rpm. A detection antibody solution was prepared with biotinylated detection antibody, 100 ng/mL Streptavidin Sulfo-TAG (Meso Scale Discovery, #R32AD-5), and 1% BSA diluted in wash buffer. After the blocking step, 50 µL/well of the sample, followed by 25 µL/well of detection antibody solution, were incubated for 2 h at RT with shaking at >300 rpm, washing wells with wash buffer between incubations. The plate was read and analyzed according to the manufacturer's protocol. The antibodies used to detect specific antigens were D2A6H (rabbit, CST) for Aβ (1-37 specific); 67B8 for Aβ (mouse, SYSY, RRID: AB_11043334) (1-38 specific); HJ-2 for hAβ (mouse, homemade) (l-40 specific); 805901 for rodent Aβ (rabbit, Biolegend) (l-40 specific); 21Fl2 for hAβ (mouse, Elan) (1-42 specific); 18,583 for hAβ (rabbit, IBL, RRID: AB_2341377) (1-43 specific); and BAF931 (goat, R&D system) for BACE1. For TACE, cathepsin B, and ADAM9, commercial ELISAs from R&D Systems were used according to the manufacturer's protocols.

2.8. Quantification and statistical analysis

All statistical analysis was performed using the GraphPad Prism 7 software. Statistical details of experiments are described in the figure legends for Figs. 1–5, S1, and S2.

3. Results

3.1. The β-secretase inhibitor AZD3293 increases BACE1 protein levels in rat cortical neurons

BACE1 has emerged as a prime drug target for chronically reducing the levels of Aβ in human brain. AZD3293 (Lanabecestat, AstraZeneca) is a potent BACE1 inhibitor (IC_{50} = 0.6 ± 0.04 nM) [11,12]. We developed a highly sensitive sandwich immunoassay for rodent Aβ1-40 with a linear standard curve and a lower limit of quantification on a synthetic Aβ1-40 peptide of 2.92 pg/ml (0.7 pM) (Supplementary Fig. 1A). Treatment of rat primary cortical neurons (DIV15) for 24 hr with AZD3293 at increasing doses (9.7 nM to 10 µM) suppressed up to 80% of endogenous rat Aβ secretion by the cells already at the lowest dose tested (Fig. 1A). We examined, by immunoblot, BACE1 protein levels in 1% NP40 lysates of the same neurons treated with increasing doses from 625 nM to 10 µM versus vehicle-only control (dimethyl sulfoxide [DMSO]), using SNAP25 as an unchanged neuronal protein control. AZD3293 caused a dose-dependent increase in BACE1 levels (Fig. 1B). To confirm this unexpected finding and further investigate the mechanism behind the robust elevation of BACE1 protein upon AZD3293 treatment, we developed a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) on the MesoScale (MSD) platform that quantifies human BACE1 protein (Supplementary Fig. 1B). The lower limit of quantification of this assay is ∼190 pg/ml, and it does not recognize recombinant BACE2 at all (Supplementary Fig. 1B). We proceeded to use human cells to study the relationship between BACE1 inhibition and BACE1 protein level.

3.2. Seven BACE1 inhibitors upregulate BACE1 protein levels in a human cell line

Numerous inhibitors of BACE1, including AZD3293, have been considered for human use, and seven have progressed as far as phase 2 or 3 clinical trials [13,14]. We tested seven clinical candidates, along with the first reported nonpeptidic BACE1 inhibitor, called inhibitor IV. Their structures, IC_{50}s for Aβ1-x, and recent clinical trial phases are provided in Fig. 1C. Human embryonic kidney (HEK)-293 cells stably expressing the "Swedish" familial Alzheimer's disease–mutant KM670/671NL (sw) APP but only endogenous β- and γ-secretases were treated for 24 h with each of the eight BACE1 inhibitors over a wide range of concentrations (Fig. 1D). We observed robust, dose-dependent reductions of Aβ1-x levels (a surrogate for total Aβ) in the conditioned media, demonstrating the potency of these inhibitors. Unexpectedly, we found that seven out of eight inhibitors significantly elevated cellular BACE1 protein levels to varying degrees, as quantified by our home-brew

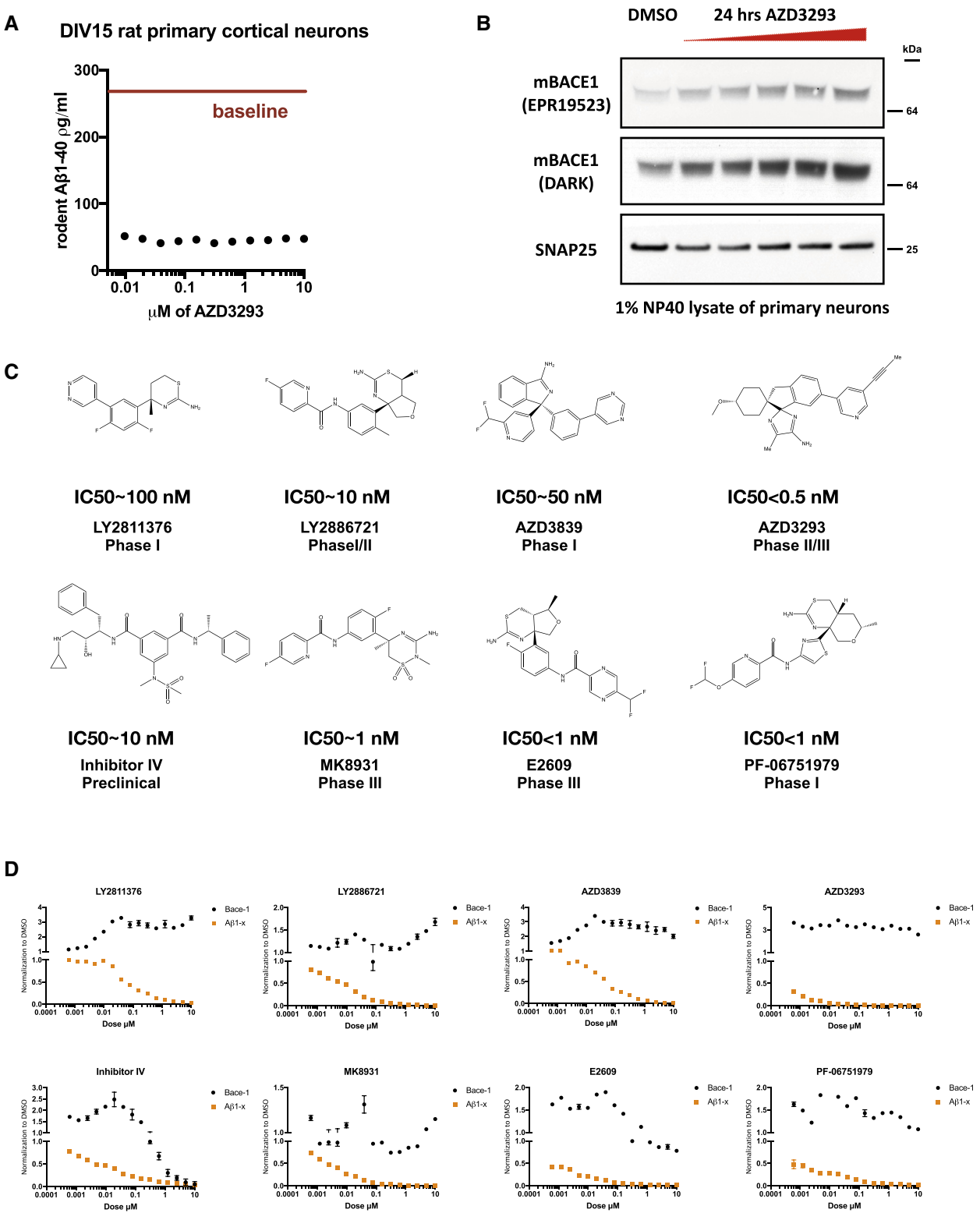

Fig. 1. BACE1 inhibitors increase cellular levels of BACE1. (A) Aβ1-40 in conditioned media measured by ELISA after 24-h treatment of DIV15 rat primary cortical neurons with AZD3293 (doses from 4.9 nM to 10 μM) (n = 3, means ± SD; error bars too small to see). Red solid line: Aβ1-40 level with plain DMSO treatment. (B) Intracellular BACE1 was measured by immunoblot after 24-h treatment of DIV15 rat primary cortical neurons with AZD3293 (doses from 625 nM to 10 μM); SNAP25 serves as a loading control. (C) Structures, IC50s for Aβ1-x and recent clinical trial phases for eight BACE1 inhibitors. (D) Extracellular Aβ1-x and intracellular BACE1 were quantified by ELISA after 24-h treatment of HEK-293-sw cells with the indicated BACE1 inhibitors (doses from 0.6 nM to 10 μM). All values are normalized to DMSO treatment (n = 2, means ± SD; some error bars too small to see). Abbreviations: DMSO, dimethyl sulfoxide; BACE1, β-site APP-cleaving enzyme-1; APP, amyloid precursor protein; Aβ, amyloid β-protein; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; HEK, human embryonic kidney.

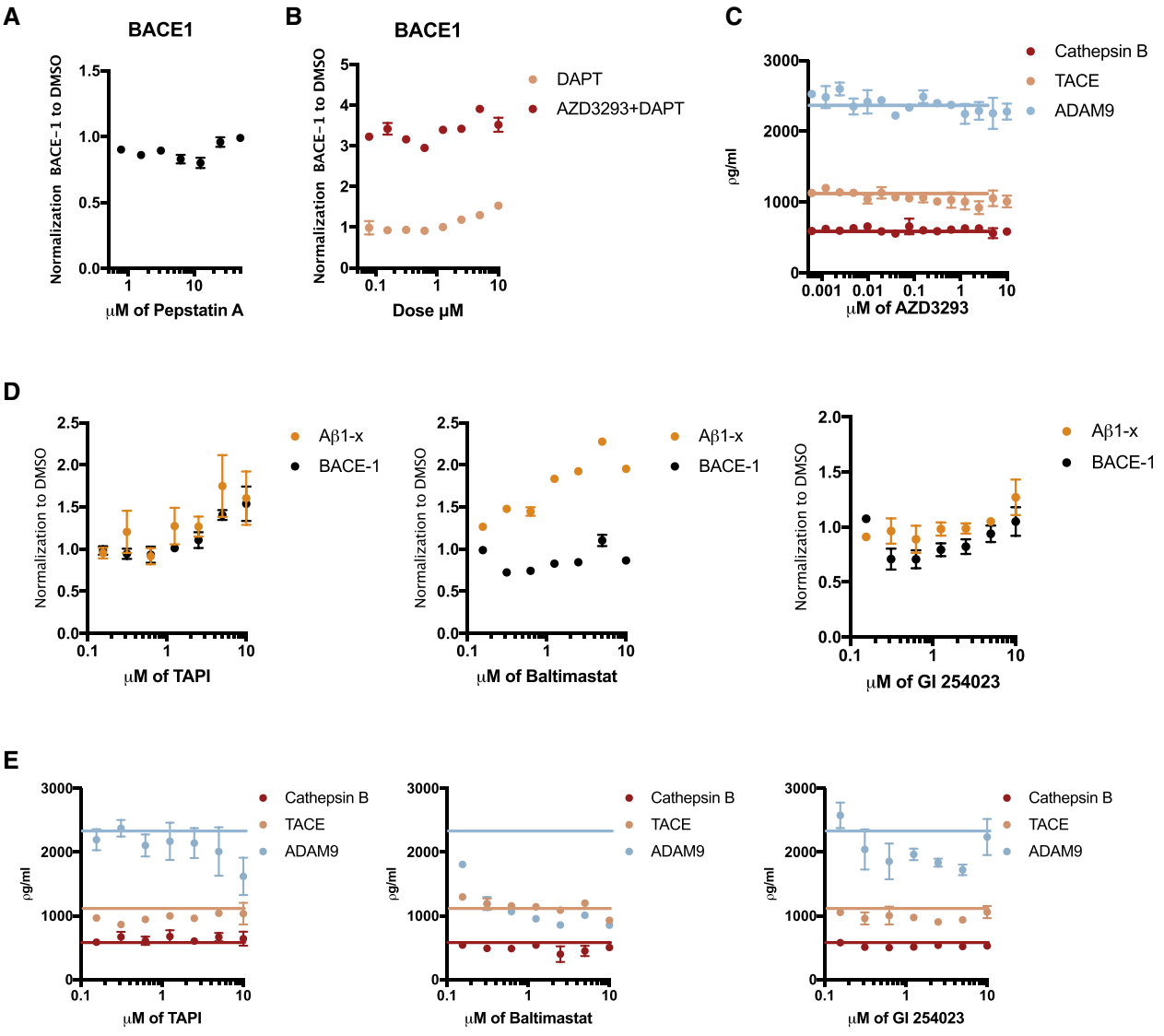

Fig. 2. The elevated cellular level of BACE1 is specific to BACE1 inhibitor treatment. (A) Intracellular BACE1 were measured by ELISA after 24-h treatment of HEK-293-sw cells with pepstatin A (doses from 790 nM to 50 μM). All values are normalized to DMSO treatment (n = 3, means ± SD; some error bars too small to see). (B) Cellular BACE1 was measured by ELISA after 24-h treatment of HEK-293-sw cells with DAPT or else DAPT plus AZD3293 (doses from 78 nM to 10 μM). All values are normalized to DMSO treatment (n = 2, means ± SD; some error bars too small to see). (C) Cellular cathepsin B, TACE, and ADAM9 were measured by ELISA after 24-h treatment of HEK-293-sw cells with AZD3293 (doses from 0.6 nM to 10 μM) (n = 2, means ± SD; some error bars too small to see). Blue, orange, and red lines: cathepsin B, TACE, and ADAM9 levels with plain DMSO treatment, respectively. (D) Extracellular Aβ1-x and intracellular BACE1 were measured by ELISA after 24-h treatment of HEK-293-sw cells with different compounds; all values normalized to DMSO treatment (n = 2, means ± SD; some error bars too small to see). (E) Cellular cathepsin B, TACE, and ADAM9 were measured by ELISA after 24-h treatment of HEK-293-sw cells with different compounds. Blue, orange, and red lines: cathepsin B, TACE, and ADAM9 levels with plain DMSO treatment, respectively. Abbreviations: DMSO, dimethyl sulfoxide; BACE1, β-site APP-cleaving enzyme-1; DAPT, *N*-[*N*-(3,5-Difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; HEK, human embryonic kidney.

BACE1 ELISA that we optimized on the MSD platform. While seven out of eight compounds variably elevated BACE1 protein levels, one of them, AZD3293, markedly increased BACE1 to 360% of vehicle at the lowest dose tested (0.6 nM), and the high elevation persisted virtually throughout the dose curve, revealing a particularly sensitive response of cellular BACE1 to this compound. Moreover, we tried to use even lower doses for AZD3293, as 0.6 nM is already higher than its IC_{50} for Aβ1-x in our cells. As shown in Supplementary Fig. 1C, we tested AZD3293 from 0.58 fM to 10 μM, and we observed that 0.9 pM could barely inhibit Aβ generation (10% inhibition) but already caused a clear increase in intracellular BACE1 levels (50% elevation). Inhibitor IV is noteworthy as it showed a bell-shaped curve for cellular BACE1 levels as its dosage increased (Fig. 1D). A similarly striking finding was that at a 10-nM dose, LY2811376 could barely inhibit Aβ generation but already caused a clear increase in intracellular BACE1 (Fig. 1D), which suggested nonsynchronized effects of

this BACE1 inhibitor. To assess whether an inhibitor, selective for BACE1 would also elevate the protease, we examined the recently developed PF-06751979 that is reported to have good BACE1 selectivity (BACE1 IC50 = 7.3 nM; BACE2 IC50 = 193 nM) [15]. We observed a similar elevation of BACE1 protein level. MK8931 had little or no effect on BACE1 levels despite its dose-dependent inhibition of Aβ production. To confirm the BACE1 ELISA findings, we immunoblotted cell lysates for BACE1 and found a similar increase in protein level upon treatment with AZD3293 (Supplementary Fig. 1D). As expected, no BACE1 protein was detected in the conditioned media of cells treated with BACE1 inhibitors versus vehicle (DMSO) alone (data not shown), ruling against gross cytotoxicity under our experimental conditions.

3.3. Upregulation of BACE1 protein levels upon inhibition of BACE1 is unique and specific

We asked whether the cellular BACE1 elevation might result from aspartyl protease inhibition *per se*. We tested a broad-spectrum aspartyl protease inhibitor, pepstatin A, as to whether general inhibition of aspartyl proteases leads to a similar effect. Doses ranging from 780 nM to 50 μM did not alter BACE1 cellular levels (Fig. 2A). Then, we tested DAPT, a potent inhibitor of the intramembrane aspartyl protease, γ-secretase, to test whether its inhibition led to a similar effect. Only the highest dosage tested (10 μM) elevated BACE1 protein level by ∼50% versus plain DMSO; doses <5 μM had no significant effect (Fig. 2B). In accordance, there was no difference between cotreating cells with DAPT plus AZD3293 versus with AZD3293 alone (Fig. 2B). We then asked whether the elevation of BACE1 by AZD3293 was specific to the BACE1 protease: could AZD3293 change the cellular levels of two matrix metalloproteinases (ADAM9, TACE) or the cysteine protease cathepsin B. AZD3293 had no effect on the three proteases tested (Fig. 2C). Conversely, we examined three matrix metalloproteinase inhibitors, TAPI, GI 254023X, and Batimastat, for any effects on BACE1. GI-254023X and Batimastat had no effect on BACE1 levels, but TAPI increased the BACE1 level at high doses of 2.5, 5, and 10 μM (Fig. 2D). However, the IC_{50}s of TAPI for endogenous and overexpressed sAPPα generation are reported to be 1.2 and 0.92 μM, respectively [16], so TAPI's elevation of BACE1 levels occurs well above its IC_{50}. Then, we investigated whether inhibitors of matrix metalloproteinases (TAPI, GI 254023X, and Batimastat) could change the cellular levels of two matrix metalloproteinases or the cysteine protease cathepsin B. We quantified ADAM-9, TACE, and cathepsin B protein levels in HEK cell lysates by ELISA (Fig. 2E). The three metalloprotease inhibitors did not significantly change the cellular levels of TACE or cathepsin B. Regarding ADAM-9, Batimastat significantly reduced ADAM-9 protein levels at all doses beginning as low as 156 nM, while GI 254023X caused lowering of ADAM9 only at certain doses (Fig. 2E). Therefore, 7 out of 8 BACE1 inhibitors substantially elevate BACE1 protein levels, and among the other classes of protease inhibitors we tested, only TAPI did this but only modestly and at doses well above its IC50. These data support the specificity of the effect of BACE1 inhibitors in elevating BACE1 levels.

3.4. AZD3293 does not change BACE1 transcription

To further explore the clear evidence that BACE1 inhibitors can increase intracellular BACE1 protein levels, we investigated the mechanism of this unexpected finding. First, we suspected that AZD3293 would increase the transcription of BACE1 mRNA, as BACE1 was known to be regulated by diverse transcription factors [17], including Specificity Protein 1, hypoxia-inducible factors-1α, NF-kappaB, and cdk5/P25. Taking into consideration that a change in the mRNA might occur rapidly, we treated HEK-293-sw cells with AZD3293 at 2.5 μM, a dose that markedly increases cellular BACE1 protein, for 3, 6, or 12 hr. Total RNA was extracted from cells treated with AZD3293 versus just DMSO, followed by reverse transcription. Quantitative polymerized chain reaction (qPCR) was used to quantify mRNA of BACE1, and β-actin served as a control for normalization. AZD3293 treatment at all time points did not change levels of BACE1 mRNA after carefully testing two independent sets of qPCR probes for BACE1 (Fig. 3A). To confirm this finding, we constructed and transfected into HEK-293 cells a plasmid containing the open reading frame of green fluorescent protein (GFP) under the regulation of the 5′UTR of the human BACE1 gene (−1471 to +152 bp; transcription start site is designated +1, which is 457 bp from the translation start site) and monitored the promoter activity (GFP reporter) in real time using a live-cell automated imaging system (IncuCyte by Sartorius). To validate this assay, we first confirmed that two compounds, YC-1 and BMS-345541, which target hypoxia-inducible factors-1α and NF-kappaB signaling, respectively, dose-dependently reduced BACE1 protein levels, with PHA-793887 (a potent CDK inhibitor) as a negative control (Fig. 3B). Then, we transiently expressed the BACE1 5′UTR-GFP reporter construct in HEK-293-sw cells in a 96-well format and followed treatment over time with increasing doses of YC-1, BMS-345541, or AZD3293 versus vehicle (DMSO) alone. GFP signals from the cells were captured every hour up to 20 hr, and the normalized GFP intensity was calculated from each well. As expected, we observed increasing GFP expression over time in just DMSO, and YC-1 or BMS-345541 treatments could each dose-dependently suppress this time-dependent rise in GFP intensity (Fig. 3C, upper panels). In contrast, AZD3293 treatment did not change the BACE1-driven GFP expression versus DMSO alone (Fig. 3C, lower panels), confirming the negative qPCR results.

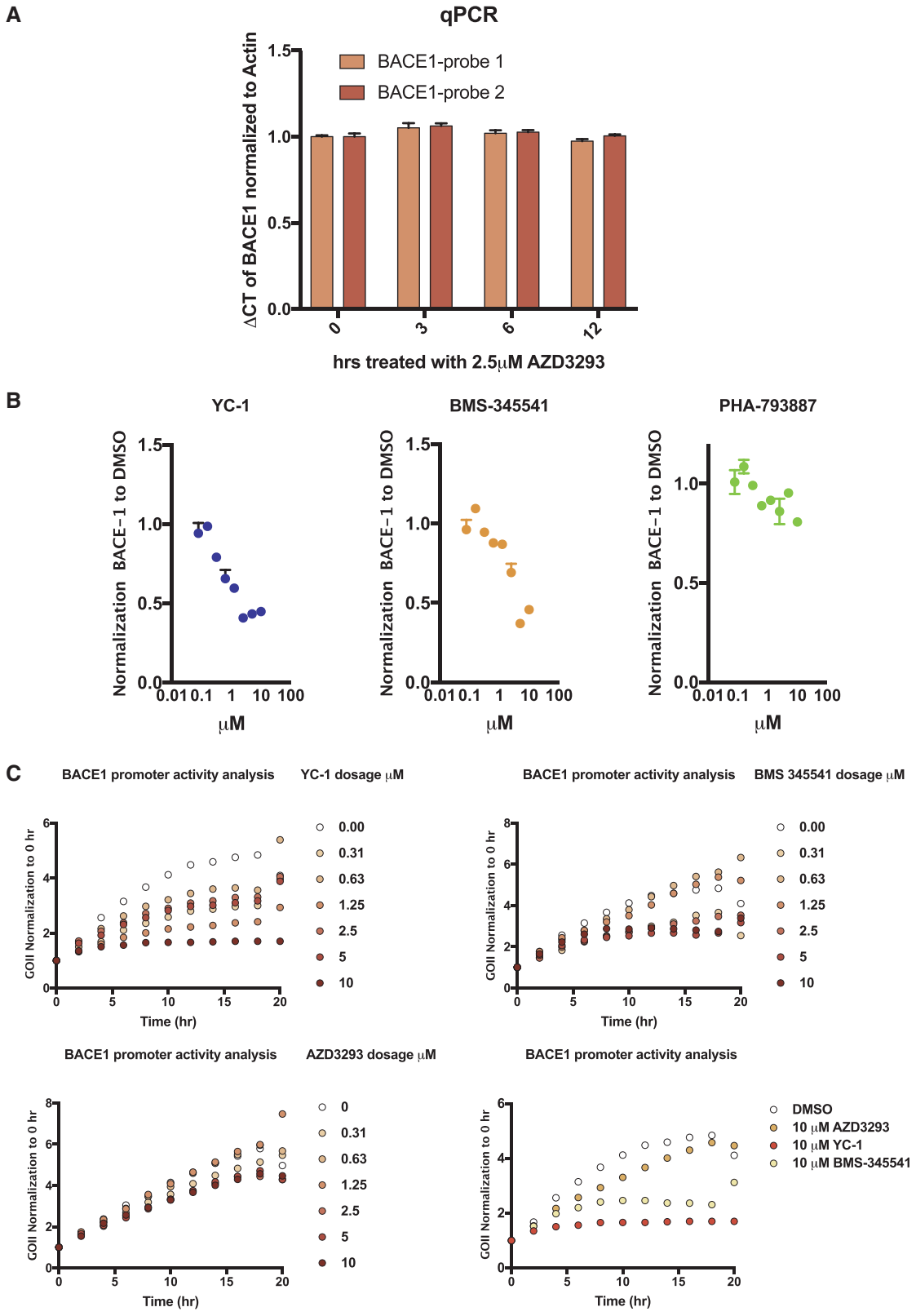

Fig. 3. BACE1 inhibitors do not change BACE1 transcription level. (A) BACE1 mRNA levels were measured by qPCR using two different probes after 0, 3, 6, and 12 h treatment of HEK-293-sw cells with 2.5-μM AZD3293. (B) Cellular BACE1 was quantified by ELISA after 24-h treatment of HEK-293-sw cells with the indicated compounds (doses from 78 nM to 10 μM). All values are normalized to DMSO treatment (n = 2, means ± SD; some error bars too small to see). (C) BACE1 promoter activity was measured by a GFP reporter during 20-h treatment of HEK-293-sw cells with different compounds (IncuCyte automated live-cell imaging). Abbreviations: BACE1, β-site APP-cleaving enzyme-1; DMSO, dimethyl sulfoxide; APP, amyloid precursor protein; qPCR, quantitative polymerized chain reaction; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; GOII, Green Object Integrated Intensity; GFP, green fluorescent protein.

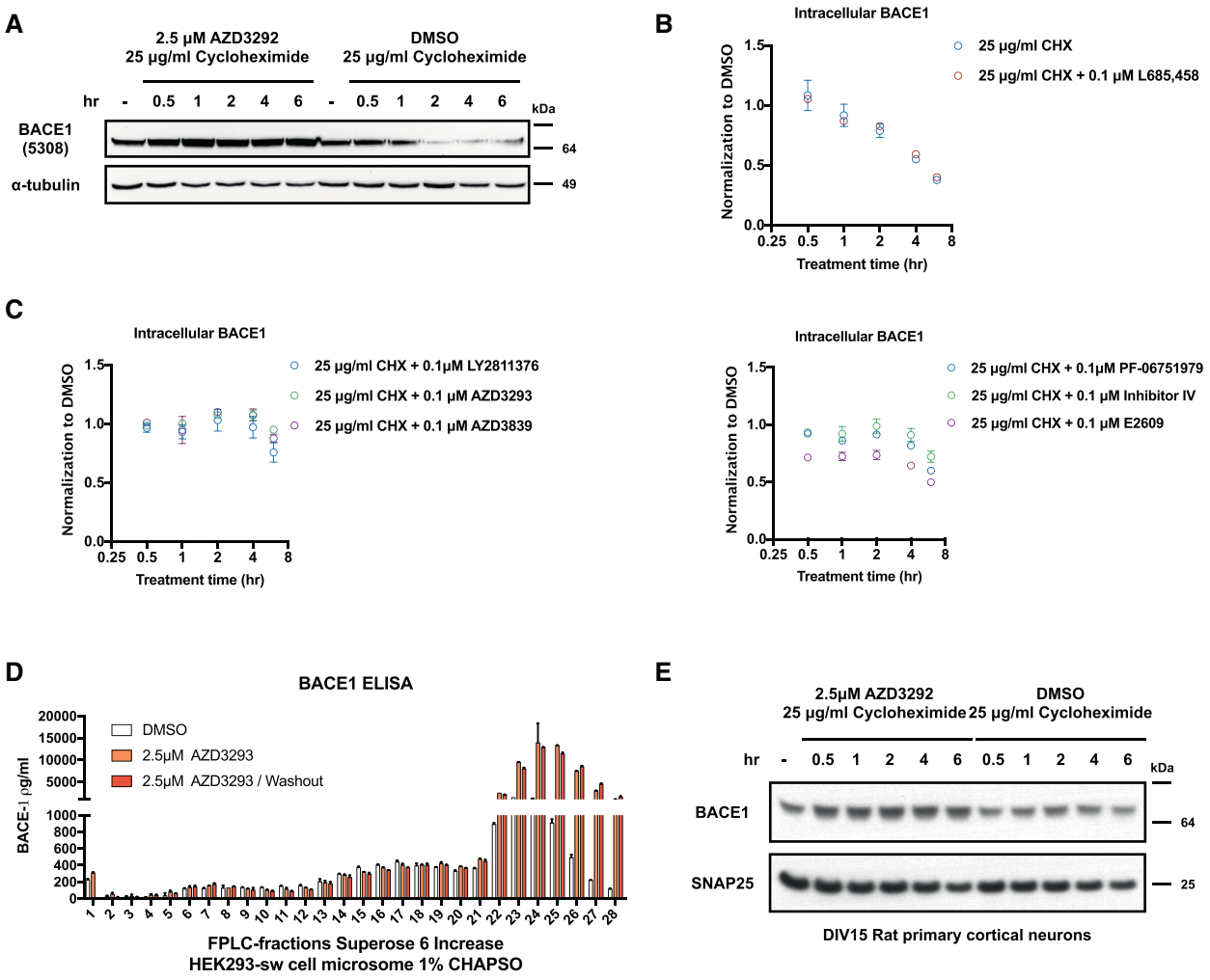

Fig. 4. BACE1 inhibitors extend BACE1 half-life. (A) Cellular BACE1 levels were measured by immunoblot after treatment of HEK-293-sw cells with 25 μg/ml cycloheximide together with either DMSO or 2.5 μM AZD3293 for 0.5, 1, 2, 4, and 6 h. α-Tubulin serves as loading control. (B-C) Cellular BACE1 levels were measured by ELISA after treatment of HEK-293-sw cells with 25 μg/ml cycloheximide together with either DMSO or 0.1 μM L685,458 (negative control) or else 0.1 μM of six different BACE1 inhibitors (LY2811376, AZD3839, AZD3293, PF-06751979, Inhibitor IV, E2609) for 0.5, 1, 2, 4, and 6 h (n = 3; means ± SD). (D) BACE1 ELISA of FPLC fractions from HEK-293-sw cells treated with AZD3293 for 24 h or else AZD3293 for 24 h followed by another 24-h washout with medium only (n = 2; means ± SD). (E) Cellular BACE1 levels were measured by immunoblot after treatment of rat primary cortical neurons (DIV15) with 25 μg/ml cycloheximide plus DMSO or 2.5 μM AZD3293 for 0.5, 1, 2, 4, and 6 h. SNAP25 serves as loading control. Abbreviations: DMSO, dimethyl sulfoxide; BACE1, β-site APP-cleaving enzyme-1; APP, amyloid precursor protein; HEK, human embryonic kidney; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

3.5. AZD3293 and other inhibitors extend the half-life of the BACE1 protein

Then, we asked whether AZD3293 acted by stabilizing BACE1 protein from degradation. A cycloheximide (CHX) chase assay (used to block new protein synthesis) indicated that (1) the half-life of endogenous BACE1 is around 2-4 hr, as estimated in HEK-293 cells conditioned in DMSO versus CHX (25 μg/ml); and (2) cotreatment with CHX and 2.5-μM AZD3293 revealed stabilization of the endogenous BACE1 protein from degradation, as assessed by Western blot (Fig. 4A). To better quantify the effects of BACE1 inhibitors on BACE1 protein stability, we used our BACE1 ELISA and treated HEK-293 cells with CHX plus one of six BACE1 inhibitors, each at the low dose of 0.1 μM, or else with L685,458 (a γ-secretase inhibitor) as a negative control. We observed similar declines of BACE1 levels with CHX alone and CHX plus L685,458 over 6 hours of treatment (Fig. 4B), confirming the ∼4-hr half-life of BACE1 and showing that another aspartyl protease inhibitor (L685,458) had no effect on it. Cotreating the cells with CHX plus each of the six BACE1 inhibitors showed that BACE1 was strongly stabilized by LY2811376, AZD3293, and AZD3839 compared with CHX alone (Fig. 4C, left panel), and it was moderately stabilized by PF-06751979, inhibitor IV, and E2609 (Fig. 4C, right panel). We conclude that all BACE inhibitors tested increased cellular BACE1 levels by stabilizing the protein from degradation. To further validate this finding, we tested whether AZD3293 could

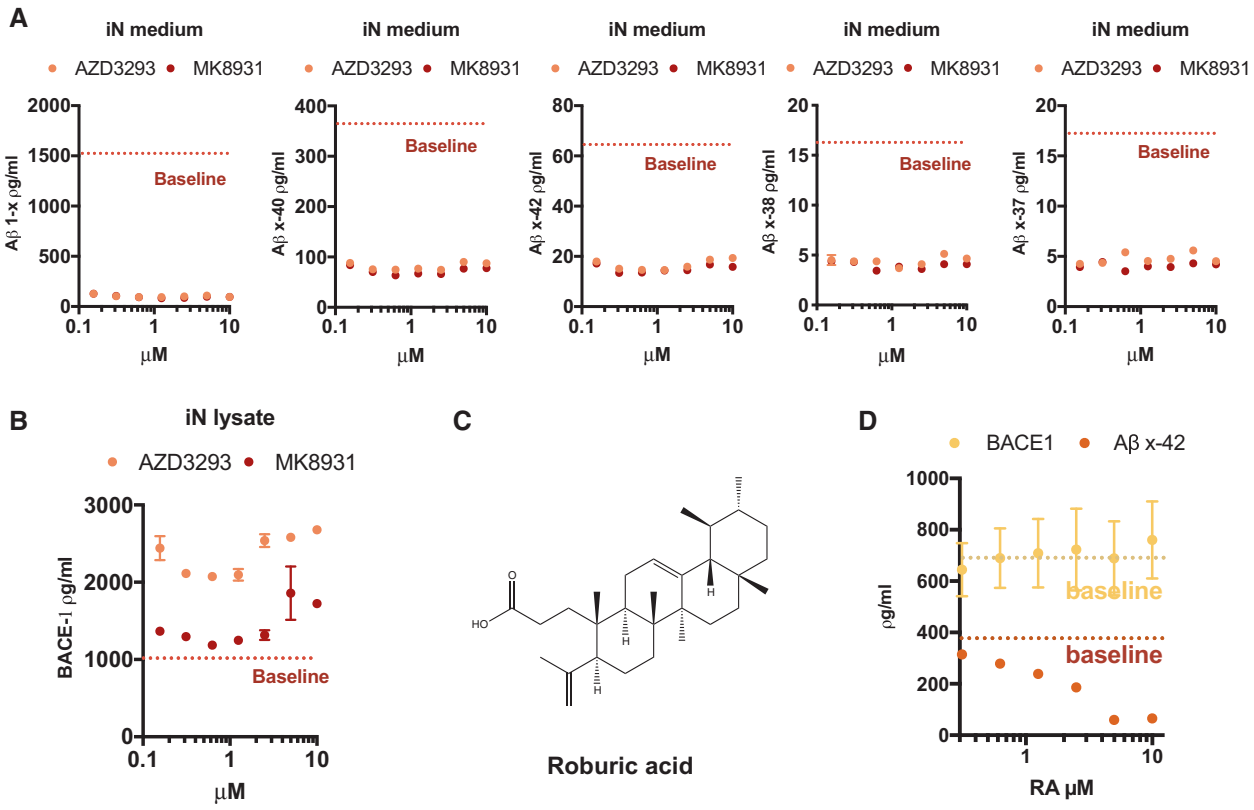

Fig. 5. Roburic acid decreases Aβ generation from human neurons without alteration of BACE1 levels. Extracellular Aβs (A) and intracellular BACE1 (B) were measured by ELISA after 24-h treatment of neurogenin-induced human neurons with AZD3293 or MK8931 (dosages from 150 nM to 10 μM; n = 2, means ± SD; some error bars too small to see). Red dotted line: Aβ1-x level with plain DMSO treatment. (C) Structure of roburic acid. (D) Extracellular Aβx-42 and intracellular BACE1 were measure by ELISA after 24-h treatment of iN cells with roburic acid (dosages from 312 nM to 10 μM) or DMSO (n = 2, means ± SD; some error bars too small to see). Yellow and red dotted lines: BACE1 and Aβx-42 levels with plain DMSO treatment, respectively. Abbreviations: BACE1, β-site APP-cleaving enzyme-1; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; Aβ, amyloid β.

enhance the stability of exogenous BACE1. Here, we overexpressed wild-type BACE1 transiently in HEK-293 cells and treated the cells with increasing doses of AZD3293. As shown in Supplementary Fig. 2A, all doses elevated the BACE1 protein levels 1.5- to 2-fold. The ELISA-measured absolute level of this exogenously expressed BACE1 (∼1.2 μg/ml) was ∼2000 times that of endogenous BACE1 (∼500 pg/ml) in HEK-293 cells, so it is surprising that AZD3293 could still stabilize an already very high cellular level of BACE1. To further explore the nature of this increase in BACE1 protein, we fractionated microsomes of 1% CHAPSO-solubilized HEK-293 cell lysates by size-exclusion chromatography (SEC) after treating the cells with AZD3293 (2.5 μM) for 24 hr (vs. just DMSO vehicle) using a Superose 6 increase gel filtration column. Each SEC fraction was lyophilized and used to measure BACE1 levels by ELISA (Fig. 4D). Treating the cells with AZD3293 for 24 hr increased BACE1 protein levels by a striking 4- to 15-fold in the low molecular weight (MW) (<150 kDa) SEC fractions #23-28, without changing them in the high MW (>670 kDa) fractions #6-13 (all as compared to treating with vehicle alone). The BACE1-elevating effect of AZD3293 remained even after removing and washing out the compound for 24 hr (Fig. 4D). Then, we performed subcellular fractionation of cell homogenates by ultracentrifugation on a discontinuous iodixanol gradient (Supplementary Fig. 2B). We found a high AZD3293-mediated accumulation of BACE1 in EEA1+ and Rab11a + fractions, which thus are enriched in endosomes. Placing these last two findings in context, we recently documented in cultured cells, mouse brain, and human brain the existence of an endogenous, proteolytically active high molecular weight β/γ secretase complex of ∼5 MDa that can mediate sequential cleavages of holo-APP substrate to generate cellular Aβ [18]. We also documented a low MW (<150 kDa) pool of BACE1 that lacks γ-secretase and therefore cannot contribute to Aβ generation [18]. The AZD3293-induced increase in BACE1 occurred selectively in this low molecular weight pool of the protease (Fig. 4D). Moreover, AZD3293 did not noticeably alter the subcellular distribution of BACE1 (Supplementary Fig. 2B). Thus, a BACE1 inhibitor appears to increase the pool of non–Aβ-generating BACE1 localized in part to endosomes. Then, we tested AZD3293 effects on BACE1 stability in rat primary cortical neurons (DIV15) using the same CHX chase assay. The half-life of BACE1 was longer in the primary neurons, where 6 hr

of CHX treatment alone decreased cellular BACE1 levels by only ~20%. However, AZD3923 cotreatment with CHX did stabilize BACE1 protein levels in the primary neurons (Fig. 4E).

3.6. β-Secretase inhibitors upregulate BACE1 protein levels in iPSc-derived human neurons

Then, we asked whether the elevation of BACE1 by its inhibitors could be observed in human neurons. We treated iPSC-derived, neurogenin-induced wild-type human neurons (iNs) at DIV 23 with AZD3293 or MK8931; these two compounds had shown similar potency for decreasing Aβ production in HEK-293 cells (Fig. 1D). In the human neurons, we assayed six different Aβ peptides, using novel Aβ assays that we developed for this study. Both inhibitors decreased the peptides Aβ1-x, Aβx-37, Aβx-38, Aβx-40, and Aβx-42 by >90%, beginning at even the lowest dose tested (Fig. 5A). Aβx-43 could only be detected in the medium of the DMSO-treated cells (12.0 ± 2.6 pg/ml, mean ± SD, n = 3) and became undetectable after treatment with either inhibitor (not shown). AZD3293 elevated human neuronal BACE1 protein levels robustly, while MK8931 only had an effect at high doses (Fig. 5B). Thus, augmentation of BACE1 protein levels by β-secretase inhibitors is shared by rat primary cortical neurons, human HEK cells, and wild-type human neurons. Recently, we examined a group of natural triterpenoids, including roburic acid (RA), that can serve as novel Aβ-reducing reagents by disrupting the newly identified high molecular weight β/γ secretase complex, without inhibiting either β or γ secretase proteolytic activity [18]. In accordance with this report, increasing doses of RA from 312.5 nM to 10 μM decreased Aβx-42 secretion from iNs, without changing the neuronal BACE1 level (Fig. 5D). Further screening of compounds such as RA will be an alternative approach to bypass the known liabilities of secretase inhibition, including BACE1 inhibitors and γ-secretase inhibitors.

4. Discussion

Despite efforts to address the specificity of BACE1 inhibitors in treating AD, even the most advanced compounds have not achieved optimal on-target specificity for lowering Aβ. The problem arises from the similarity between the proteolytic sites of BACE1 and many other human aspartic proteases. There has been progress in addressing the issue of nonselective inhibition of cathepsin D and E and BACE2 but not yet on the nonselective inhibition of processing of BACE1 substrates besides APP, which are known to be important for diverse biological functions [19,20]. Among the BACE1 inhibitors entered into phase III or phase II/III clinical trials, it has been shown that (1) AZD3293 inhibits BACE1 and BACE2 with equal potency; (2) MK8931 inhibits BACE2 more potently than BACE1, which is supported by the trial data on altered hair color (a BACE2 inhibition phenotype) [21]; and (3) the inhibition of APP processing does not spare other critical BACE1 substrates such as Sez6 [22]. Of great concern is the cognitive worsening revealed recently in some BACE1 inhibitor trials [7–9]. Its origin is unclear, but it may be consistent with in-depth analyses of BACE1 knock-out mice that have notable neurological phenotypes which include but are not limited to seizures, schizophreniform behavior, cognitive dysfunction, and impaired axonal organization [23–26].

Conceptually, therapeutic BACE1 inhibition attempts to decrease newly generated Aβ monomers to lower Aβ oligomer formation over time and thus lessen neuronal and glial cytotoxicity and slow cognitive decline. However, targeting the rate-limit enzyme that normally generates Aβ may not efficiently slow or reverse the growth of established plaques [27]. Moreover, even initiating treatment earlier than prodromal AD may be ineffective, as monomer lowering may not correct a decades-long dyshomeostasis of Aβ and could also lead to gradual accrual of adverse effects. Our analysis of 8 BACE1 inhibitors strongly support the latter concern, as we observed a surprising and unwanted effect with almost all BACE1 inhibitors we tested: they increase cellular BACE1 protein levels by extending the half-life of BACE1 itself. Beyond the reported side effects potentially arising from inhibition of BACE2 or decreased physiological processing of other BACE1 substrates, our new findings show that potent BACE1 inhibitors increase the stability of the protease, suggesting that chronic BACE1 inhibition could elevate the protease so that the usual peaks and valleys of tissue levels of an orally dosed inhibitor could permit intermittent overprocessing of BACE1 substrates.

Our experiments show that AZD3293 is a highly potent BACE1 inhibitor that can suppress up to 80% of endogenous Aβ1-40 production by rat primary cortical neurons (DIV15) while simultaneously increasing BACE1 protein levels in the neurons. We confirmed that upregulation of BACE1 by AZD3293 occurs in wild-type human neurons. Testing the additional 7 BACE1 inhibitors, we observed robust reductions of Aβ1-x levels (a proxy for total Aβ) in the conditioned media, confirming the potency of these inhibitors. But the inhibitors unexpectedly and significantly elevated cellular BACE1 protein levels to varying degrees. Moreover, while 7 out of 8 compounds elevated BACE1 protein levels, one inhibitor, AZD3293, markedly increased its levels to 360% of vehicle already at the lowest dose tested (0.6 nM), and this high elevation persisted virtually throughout the dose curve, revealing a particularly sensitive response of cellular BACE1 to this compound. We have confirmed this surprising phenomenon in HEK-293-sw cells, rat primary neurons, and human neurons. Mechanistically, we initially focused on AZD3293 and found it increased BACE1 protein levels by extending the half-life of BACE1, as seen by CHX chase assays. We ruled out AZD3293 elevation of BACE1 transcription through qPCR, a BACE1 promoter activity assay, and a test of AZD3293 effects on exogenously expressed BACE1 lacking

the cognate promoter and enhancer sequences. Thus, BACE1 elevation by AZD3293 occurs by stabilizing the enzyme at the protein level, and we showed this is a general class effect by quantifying the prolonged half-life of BACE1 by 5 other BACE inhibitors using CHX chase assays. Intriguingly, when cell lysates were fractioned under nondenaturing conditions, the increased BACE1 protein occurred in low molecular weight biochemical fractions, which we recently showed to lack PS/γ-secretase complexes and thus cannot contribute to Aβ production [18]. The increasing levels of BACE1 are of special concern; excess BACE1 that is intermittently uninhibited could both generate additional Aβ from APP and excessively process many BACE1 substrates involved, for example, in sodium channel function [28], synaptic formation [29], and immunity [30].

In light of the reported clinical failures of semagacestat [31], a γ-secretase inhibitor, and verubecestat [21], a β-secretase inhibitor, inhibition of the secretases for prolonged treatment of AD will be highly challenging. There remain substantial uncertainties about the enzymology during the high-affinity bindings of inhibitors to the secretases. For example, semagacestat was shown retrospectively to actually increase intracellular accumulation of the longer Aβ43, Aβ45, and Aβ46 peptides rather than inhibiting γ-secretase [32]. It is difficult to choose a safe dosage when BACE1 inhibitors act as potentially neurotoxic agents. However, we believe that other small-molecule therapies directed at Aβ are still important and attractive for prevention and treatment of AD. In particular, recent developments in the field of γ-secretase modulators (GSMs) are promising, including the oral GSMs BPN-15606 (IC_{50} ~7 nM for Aβ42) [33], PF-06442609 (IC_{50} ~6 nM) [34], and compounds 30, 44, 45 (IC_{50}s as low as 4.9 nM) [35]. Allosteric modulation of γ-secretase by GSMs will not alter the endoproteolytic activity of γ-secretase and therefore will not impair signaling mediated by the intracellular domains of many α-, β-, and γ-secretase substrates but rather will shift APP cleavage to decrease longer, more amyloidogenic Aβ peptides (Aβ43, Aβ42, and Aβ40) and increase shorter, less-aggregation-prone peptides (Aβ37 and Aβ38). This precise targeting of longer Aβ species by GSMs is likely to be both safer and more effective in lessening Aβ oligomerization and plaque formation. As a distinct new small-molecule approach, we recently discovered a natural triterpenoid, RA, that lowers Aβ42 and Aβ43 by (1) modulating γ-secretase activity similar to a GSM and (b) by decreasing the generation of all Aβ peptides by partially dissociating a high MW β/γ secretase complex that generates cellular Aβ, without inhibiting either secretase [18]. Based on these properties, we and others will now screen for additional natural compounds that have one or both of these mechanisms.

In summary, in the context of previous studies on BACE1 physiological functions and the adverse effects of BACE1 inhibitors in recent trials, our new finding that numerous BACE1 inhibitors significantly elevate cellular levels of the protease further complicates the advance of β-secretase inhibition as a chronic treatment for AD.

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Author contributions: L.L., B.M.L., L.D., and M.R. conducted the experiments; L.L., M.S.W., and D.J.S. designed the experiments, performed data analysis and interpretation, and wrote the article.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jalz.2019.06.3918.

RESEARCH IN CONTEXT

1. Systematic review: Recent research suggests that several β-site amyloid precursor protein–cleaving enzyme-1 (BACE1) inhibitors can worsen cognitive functions in patients with Alzheimer's disease. The biological mechanisms are largely unknown.
2. Interpretation: Using highly sensitive assays and systematic evaluation of 8 clinically relevant BACE1 inhibitors, we found that 7 BACE1 inhibitors elevate levels of BACE1 in cells. These BACE1 elevation effects are specific and unique for BACE1 inhibitors and act by extending BACE1 half-life.
3. Future directions: Future studies should investigate compounds such as roburic acid, which reduce amyloid β (Aβ) generation by partially dissociating a high MW β/γ-secretase complex that generates cellular Aβ without inhibiting either secretase.

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