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BMS-708,163 Targets Presenilin and Lacks Notch-Sparing Activity

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Supporting Information

ABSTRACT: The "Notch-sparing" γ-secretase inhibitor (GSI) BMS-708,163 (Avagacestat) is currently in phase II clinical trials for Alzheimer's disease. Unlike previously failed GSIs, BMS-708,163 is considered to be a promising drug candidate because of its reported Notch-sparing activity for the inhibition of Aβ production over Notch cleavage. We now report that BMS-708,163 binds directly to the presenilin-1 N-terminal fragment and that binding can be challenged by other pan-GSIs, but not by γ-secretase modulators. Furthermore, BMS-708,163 blocks the binding of four different active site-directed GSI photoaffinity probes. We therefore report that this compound acts as a nonselective γ-secretase inhibitor.

Although genetic evidence from mutations in presenilin-1 (PS1), presenilin-2 (PS2), and the amyloid precursor protein (APP) supports the amyloid cascade hypothesis,[1] the development of Aβ-based therapies has been a formidable challenge. Clinical trials with small molecules that target γ-secretase, the enzyme responsible for the final step of APP proteolysis to generate Aβ peptides, have not produced satisfactory outcomes. The nonselective potent γ-secretase inhibitor (GSI) LY450139 (Semagacestat) recently failed phase III clinical trials and was terminated partly because of Notch-mediated skin tumor progression[2,3] and worsening of cognitive measures.[4] These outcomes have led to significant concerns about whether γ-secretase is a viable target for the treatment of Alzheimer's disease (AD). At a mechanistic level, the side effects of Semagacestat likely reflect the broad spectrum of the compound for APP and Notch as well as other substrates. Subsequently, a "Notch-sparing" GSI, BMS-708,163 (Avagacestat),[5] has been developed with a reported 193-fold selectivity for APP over Notch cleavage.[5] The mechanism of action of this inhibitor has not been reported.

The Notch-sparing GSI BMS-708,163 is currently undergoing phase II clinical trials and carries with it new hopes for AD therapy. In view of the significant implications for AD treatments, we sought to determine the mechanism of action and selectivity of BMS-708,163. We designed and synthesized 163-BPyne, a probe based on the BMS-708,163 scaffold that contains both a photolabile benzophenone moiety, allowing for cross-linking to binding partners, and a terminal alkyne that can be conjugated to an azide reporter tag using click chemistry[6] (Figure 1).

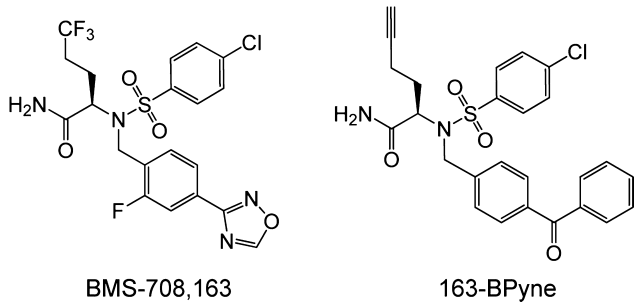

Figure 1. Chemical structures of the GSI BMS-708,163 and the inhibitor-based photoaffinity probe 163-BPyne used in this study.

We determined the IC_{50} values for both BMS-708,163 and 163-BPyne in our cell-free in vitro γ-secretase activity assays that have successfully assessed γ-secretase modulators (GSMs),[6] and much to our surprise, we found that BMS-708,163 had an only 3-fold selectivity for cleavage of an APP substrate compared with a Notch substrate (Table 1).

The photoactivatable probe, 163-BPyne, also exhibited an ∼3-fold selectivity for cleavage of the APP substrate compared with the Notch substrate, without any loss of potency compared with that of the parent compound. Anticipating that the discrepancy in our results from previously published data might have been due to the cell-free in vitro format of our assays, we determined the EC_{50} of BMS-708,163 in our cell-based assays for Notch intracellular domain (NICD) and Aβ generation. Again, we found an only ∼7-fold selectivity for inhibition of Aβ40 versus NICD production. Notably, our assay quantitatively measures the amount of NICD protein released from the membrane and therefore directly measures the γ-

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Table 1. In Vitro IC_{50} Values of Compounds Used in This Study

compound	IC_{50} (nM)			
	Aβ40	Aβ42	NICD[a]	NICD/Aβ40
		Cell-Free Assay		
BMS-708,163	0.26 ± 0.07	0.35 ± 0.3	0.84 ± 0.3	3
163-BPyne	0.20 ± 0.05	0.40 ± 0.2	0.61 ± 0.08	3
		Cell-Based Assay		
BMS-708,163	1.2 ± 0.5	–	7.9 ± 1.4	7

[a]Notch intracellular domain.

secretase activity for Notch1 cleavage, while Gillman et al.[5] used a reporter-based assay that relies on NICD-mediated activation of *CBF1*, a Notch target gene. In addition, two other groups recently showed that BMS-708,163 exhibited only 26-fold selectivity against Aβ40 over Notch when either a *RBP-Jk* luciferase[7] or a *HES-1* secreted-alkaline phosphatase reporter[8] was used. It should be noted that our studies also suggest a slight preference for proteolysis of APP over Notch, but further studies will be required to determine whether these subtle differences reflect effects on substrate binding and/or catalysis. Moreover, it is at present unclear how these in vitro and indirect reporter assays translate to efficacy and side effects from long-term clinical studies.

To determine the cellular target of BMS-708,163, we exploited the clickable probe, 163-BPyne. We show that 163-BPyne specifically labels only PS-1 NTF in HeLa cell membrane preparations (Figure 2b and Figure 1 of the Supporting Information). Importantly, the 163-BPyne labeling of PS1 is completely blocked by an excess of BMS-708,163, suggesting that the chemical derivation of the parent compound did not alter the target specificity. To identify possible binding partners in a more unbiased fashion, we labeled HeLa membranes with 163-BPyne followed by click chemistry using the fluorescent tag tetramethyl rhodamine-azide (TAMRA-azide), followed by fractionation of labeled proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 2a). This procedure allows for visualization of all labeled proteins. However, we observed only one specific ~30 kDa band, corresponding to the predicted mass of PS1-NTF. To establish the identity of the ~30 kDa band as PS1-NTF, we labeled membranes prepared from HEK293-PS1ΔE9 cells,[9] which stably express the FAD-linked PS1ΔE9 variant that is not subject to endoproteolysis into NTF and CTF fragments and thus exhibits a mobility of ~45 kDa.[10] Moreover, PS1ΔE9 replaces endogenous PS1-NTF/CTF.[10] As expected, an ~30 kDa band is not labeled by 163-BPyne in membranes prepared from HEK293-PS1ΔE9 cells; instead, labeling of a specific ~45 kDa band is apparent (Figure 2 of the Supporting Information). Thus, we conclude that the ~30 kDa band labeled by 163-BPyne in HeLa cell membranes represents PS1-NTF.

To compare the binding mechanism of BMS-708,163 with those of other established γ-secretase inhibitors and modulators, we performed competition studies with 163-BPyne in the presence of the parent compound BMS-708,163, the active site-directed GSI L-685,458,[9] the pan-GSIs[11] LY-450,139 and Compound E, and two different classes of GSMs:[12−15] GSM-1 and E2012. GSMs shift the cleavage preference of γ-secretase, resulting in an increased level of production of Aβ37 and Aβ38 peptides and reduced Aβ42 and Aβ40 levels. However, GSMs do not appear to inhibit overall APP processing or NICD production. Therefore, GSMs represent another class of molecules that selectively target γ-secretase. Interestingly,

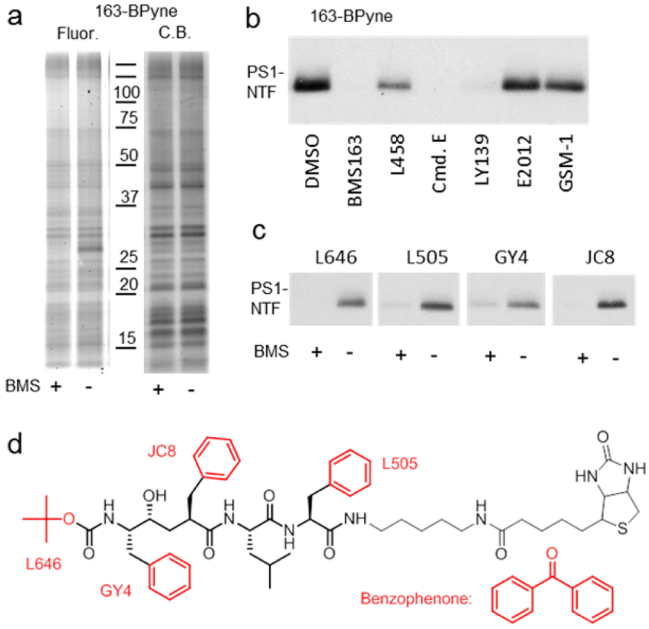

Figure 2. (a) Fluorescence labeling of HeLa membranes with 20 nM 163-BPyne with or without 1 μM BMS-708,163 (left) and Coomassie blue staining of total protein loaded (right). (b) Labeling of HeLa membranes with 20 nM 163-BPyne probe with or without 1 μM GSIs [BMS-708,163, L-685,458, Compound E, and LY-450,139 (Semagacestat)] or 25 μM GSMs (E2012 and GSM-1), followed by click chemistry with biotin azide, pull down with streptavidin resin, and Western blot with the PS1 N-terminal fragment (PS1-NTF) antibody. (c) Labeling of HeLa membranes with 20 nM biotinylated active site-directed photoprobes L646, L505, GY4, and JC8 with or without 2 μM BMS-708,163, followed by pull down with streptavidin resin and Western blot with the PS1-NTF antibody. (d) Structural representation depicting each of the four active site probes used in panel c, where each respective moiety highlighted in red is replaced with a benzophenone moiety.

163-BPyne labeling was completely blocked by the presence of both of the pan-allosteric GSIs and partially blocked by the presence of the active site GSI, L-685,458. None of the GSMs had any effect on labeling by 163-BPyne at 25 μM (Figure 2b). We then asked whether BMS-708,163 had any allosteric effects on the active site of γ-secretase by implementing our "photophore walking" approach, with which we had previously demonstrated subtle changes in the shape of the active site of γ-secretase following incubation of selective GSIs or GSMs.[6,16,17] Here, we found that all four probes were completely inhibited by BMS-708,163, indicative of nonselective pan-GSIs (Figure 2c,d). Taken together, these findings strongly suggest that BMS-708,163 functions as an allosteric GSI with poor Notch-sparing activity. In fact, in clinical studies with a single dose of BMS-708,163 at 0.3, 1.5, 5, 15, 50, 100, 200, 400, or 800 mg,

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only the 800 mg dose showed some inhibition of Notch-related plasma biomarkers in humans, such as *Hes1* or *DUSP6* expression.[18] However, ≥100 mg doses were associated with higher discontinuation rates because of gastrointestinally adverse events as well as skin-related adverse events, including non-melanoma skin cancer[19] that could likely be a result of the inhibition of Notch cleavage. These studies raise a question of whether the level of *HES1* and *DUSP6* expression is a suitable marker of Notch side effects. Moreover, clinical studies indicate that larger doses (100 and 125 mg) of BMS-708,163 also showed negative cognitive effects in a midstage trial, similar to the results reported for Semagacestat.[19] The mechanism of this side effect remains to be investigated.

It is noteworthy that clinical evaluation of smaller doses of BMS-708,163 is currently underway, with the expectation that side effects will be minimized, while still maintaining the potential for clinical efficacy. We have demonstrated that a BMS-708,163-derived probe directly interacts with PS1-NTF. Moreover, the lack of considerable Notch-sparing activity of BMS-708,163 in our biochemical and cellular studies raises questions regarding the development of BMS-708,163-based therapies. It will behoove the AD research community to coordinate efforts to develop methodologies and standards for evaluating the potency and selectivity of GSIs and GSMs, leading to the development of safe and effective therapies.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures and detailed methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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