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

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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 due to its reported Notch-sparing activity for the inhibition of Aβ production over Notch cleavage. We now report that BMS-708,163 binds directly to PS1-NTF, and that binding can be competed by other pan-GSIs, but not by γ-secretase modulators (GSMs). 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 non-selective 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], development of Aβ-based therapies has been a formidable challenge. Clinical trials of small molecules that target γ-secretase, the enzyme responsible for the final step of APP proteolysis to generate Aβ peptides, have not achieved satisfactory outcomes. The non-selective potent GSI LY450139 (Semagacestat) recently failed phase III clinical trials, and was terminated partly due to Notch-mediated skin tumor progression [2,3] and worsening of cognitive measures [4]. These outcomes have led to significant concerns as to whether γ-secretase is a viable target for AD treatment. 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

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Supporting Information

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

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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] (Fig. 1).

We determined the IC50s for both BMS-708,163 and 163-BPyne in our cell-free *in vitro* γ-secretase activity assays that have successfully assessed GSMs [6] and, much to our surprise, found that BMS-708,163 had only a 3-fold selectivity for cleavage of an APP substrate compared with a Notch substrate (Table 1).

The photoactivatable probe, 163-BPyne also exhibited a ~3-fold selectivity for cleavage of the APP substrate compared with the Notch substrate, without any loss of potency compared with the parent compound. Anticipating that the discrepancy in our results from previously published data must have been due to the cell-free *in vitro* format of our assays, we determined the EC50 of BMS-708,163 in our cell-based assays for NICD and Aβ generation. Again, we found only a ~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 γ-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 presently unclear how these in vitro and indirect reporter assays translate to efficacy and side-effects from long-term clinical studies.

In order 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 (Fig. 2b and Supplementary Fig. 1). Importantly, the 163-BPyne labeling of PS1 is completely blocked by 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 TAMRA-azide, followed by fractionation of labeled proteins by SDS-PAGE (Fig. 2a). This procedure allows for visualization of all labeled proteins. However, we only observed one specific band of ~30 kDa, corresponding to the predicted mass of PS1-NTF. In order 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, a ~30 kDa band is not labeled by 163-BPyne in membranes prepared from HEK293- PS1ΔE9 cells, but instead, labeling of a specific ~ 45 kDa band is apparent (Supplementary Fig. 2). 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 other established γ-secretase inhibitors and modulators, we performed competition studies of 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[12-14]: GSM-1 [6] and E2012 [15]. GSMs shift the cleavage preference of γ-secretase, resulting in increased 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, 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 (Fig. 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[6,1617]. Here, we found that all four probes were completely inhibited by BMS-708,163, indicative of non-selective pan-GSIs (Fig. 2c and 2d). 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, only the dose of 800 mg showed some inhibition of Notch-related plasma biomarkers in humans, such as *Hes1* or *DUSP6* expression[18]. However, doses at 100 mg or above were associated with higher discontinuation rates due to gastrointestinal adverse events as well as skin-related adverse events including non-melanoma skin cancer[19] which could likely be a result of the inhibition of Notch cleavage. These studies raise a question whether the level of *HES1* and *DUSP6* expression is a suitable marker of Notch side effects. Moreover, clinical studies indicate that higher doses (100-mg and 125-mg) of BMS-708,163 also showed negative cognitive effects in a mid-stage trial, similar to that reported for Semagacestat.[19] The mechanism of this side effect remains to be investigated.

It is noteworthy that clinical evaluation of lower 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AD	Alzheimers disease
APP	amyloid precursor protein
GSI	γ-secretase inhibitor
GSM	γ-secretase modulator
PS1	Presenilin 1
PS1-NTF	PS1 N-terminal fragment
NICD	Notch intracellular domain
TAMRA	tetramethyl rhodamine

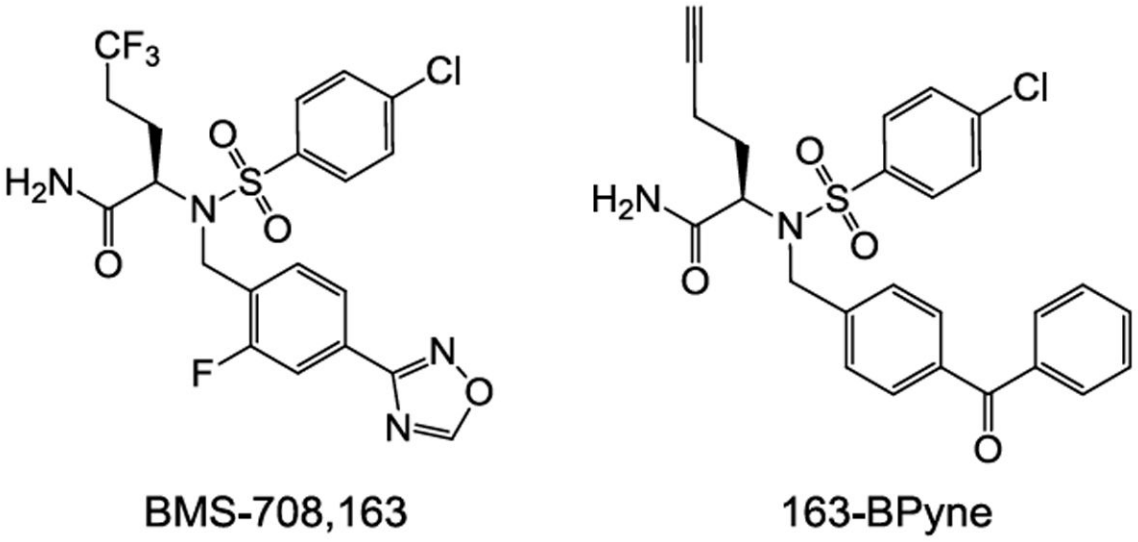

Figure 1.
Chemical structures of the GSI BMS-708,163 and the inhibitor-based probe 163-BPyne used this study.

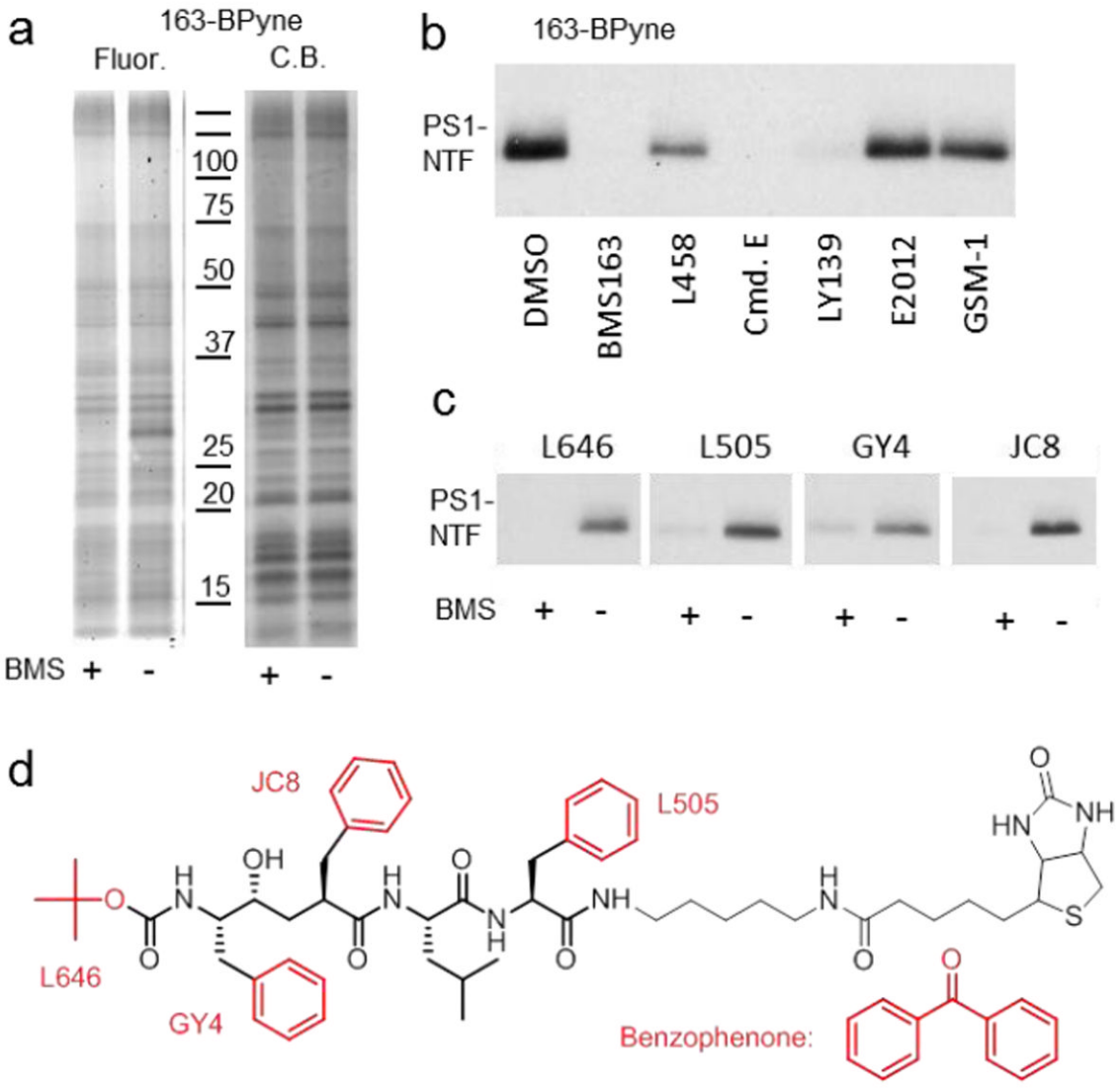

Figure 2.

a) Fluorescence labeling of HeLa membranes with 20 nM of 163-BPyne in the absence or presence of 1 μM BMS-708,163 (left panel) and Coomassie blue staining of total protein loaded (right panel). **b)** Labeling of HeLa membranes with 20 nM 163-BPyne probe in the absence or presence of 1 μM of GSIs: BMS-708,163, L-685,458, Compound E, LY-450,139 (semagacestat), or 25 μM of GSMs: E2012, and GSM-1; followed by click chemistry with biotin azide, pull down with streptavidin resin and western blot with PS1-NTF antibody. **c)** Labeling of HeLa membranes with 20 nM of the biotinylated active site-directed photoprobes L646, L505, GY4 or JC8 in the presence or absence of 2 μM BMS-708,163, followed by pull-down with streptavidin resin and western blot with PS1-NTF antibody. **d)** Structural representation depicting each of the four active site probes used in (c) where each respective moiety highlighted in red is replaced with a benzophenone moiety.

Table 1

In vitro IC50 values (nM) of compounds used in this study

Compound	IC50 Aβ40	IC50 Aβ42	IC50 NICD	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

*NICD = Notch IntraCellular Domain