γ-Secretase/presenilin inhibitors for Alzheimer's disease phenocopy *Notch* mutations in *Drosophila*[1]

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SPECIFIC AIMS

The presenilin/γ-secretase complex processes the amyloid-β (Aβ) precursor protein and the Notch receptor. Because presenilin and the Notch signaling pathway are highly conserved in metazoans, we tested whether Aβ-lowering γ-secretase inhibitors under development for human use in the treatment of Alzheimer's disease would reduce Notch function and cause developmental defects in *Drosophila*.

PRINCIPAL FINDINGS

1. γ-Secretase inhibitor DAPT inhibits of Notch proteolysis, nuclear translocation, and signaling and blocks affinity labeling of presenilin

N-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine *t*-butyl ester (DAPT) is a γ-secretase inhibitor developed to lower Aβ levels in vivo as a potential treatment for Alzheimer's disease. We found that DAPT also inhibits proteolysis of a recombinant Notch-based substrate, N100Flag, in a solubilized γ-secretase assay with an IC_{50} of 5–10 nM. After transmembrane cleavage of Notch (N), the intracellular domain translocates to the nucleus, where it activates Lag1/HES/CBF1-mediated transcription. Transfection of CHO cells with cDNA encoding a truncated, constitutively cleaved Notch-EGFP fusion protein allows visualization of EGFP fluorescence in the nucleus, and this nuclear fluorescence was inhibited by DAPT at concentrations that also blocked proteolysis in the solubilized assay. DAPT also reduced signaling from a luciferase reporter activated by released NICD. In addition, we tested the ability of DAPT to affect presenilin (PS). Compound WPE-III-63, a transition-state analog γ-secretase inhibitor, contains a carboxyl-terminal biotin and a photoactivatable P2′ residue (benzoylphenylalanine). This compound cross-links both the NTF and CTF subunits of PS1. DAPT prevented this affinity labeling of PS1 by WPE-III-63, suggesting that this compound either directly binds to the active site of PS or alters the active site through an allosteric interaction.

2. γ-Secretase inhibitors induce wing defects in developing *Drosophila* similar to genetic mutations in the *N* signaling pathway

Several structurally distinct γ-secretase inhibitors have been found to directly bind to mammalian presenilins (PS). N signaling in *Drosophila* requires a highly conserved PS-dependent γ-secretase activity. Reductions in either *N* or *PS* function along the presumptive wing margin of *Drosophila* leads to loss of wing margin structures in the adult. We therefore tested whether treatment with the PS/γ-secretase inhibitor DAPT could cause developmental defects in flies similar to those caused by genetic reduction of *N* signaling. Adult wild-type flies were introduced into vials with food containing 1 mM of DAPT. The adults were kept at 25°C and allowed to lay eggs over the course of 5 days. The progeny displayed a dose-sensitive wing notching phenotype indistinguishable from genetic reductions in the *N* pathway (cf., **Fig. 1*a–g***). The wing effects ranged in severity from a mild to strong notching phenotype (Fig. 1*d–g*), and these effects were not seen with a structurally related compound DAT (Fig. 1*h*). The appearance of the wing effect was dose dependent, with complete penetrance at 1 mM (Fig. 1*i*). We noted that DAPT-treated flies displayed additional phenotypes consistent with defects in N signaling. These include an increased number of bristles on the notum, defects in leg segmentation, and small eyes (Fig. 1*j, k* and data not shown). However, these defects were observed only at low frequency. Other γ-secretase in-

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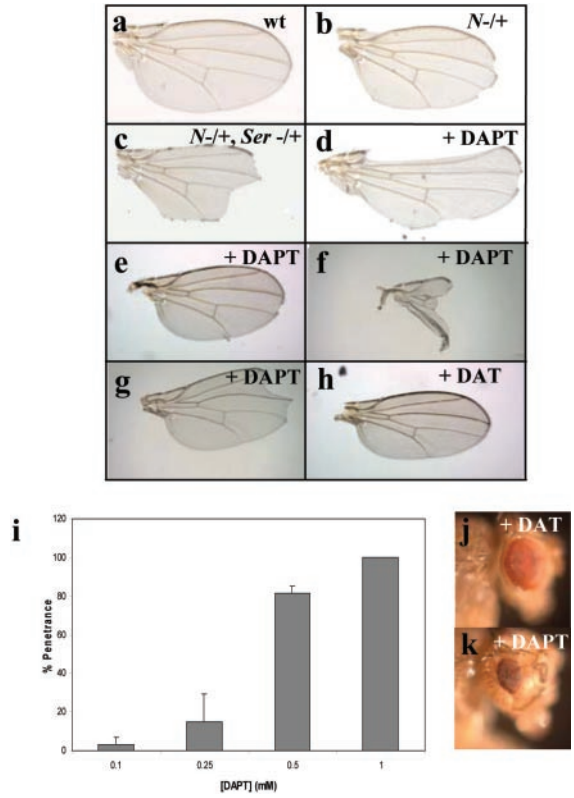

Figure 1. Effect of DAPT on *Drosophila* development. Wings from *a)* wild-type *Drosophila*, *b)* $N^{55e11}/+$, *c)* $N^{55e11}/+$, TM3 *Ser*/+, *d–g)* wild-type in the presence of 1 mM DAPT, and *h)* wild-type in the presence of 1 mM control compound DAT. *i)* Percent of enclosed flies with the *N* wing phenocopy (i.e., penetrance) after growth in different concentrations of DAPT. *j)* Eyes from wild-type *Drosophila* grown in the presence of 1 mM DAT or *k)* 1 mM DAPT. Adult wings are mounted with anterior at the top.

hibitors could also interfere with wing development, although with less penetrance and higher lethality than DAPT.

3. DAPT elicits wing defects on the fourth day of *Drosophila* development

Analysis of a temperature-sensitive allele of *N* has demonstrated that *N* is required during the third larval instar for wing margin development. To determine when DAPT caused its effects on wing formation, we transferred larvae into or out of DAPT-containing food at defined intervals. Among the adult flies recovered from this experiment, no effect on wing development was detected on DAPT treatment before or after the fourth day (72–96 h) of development. However, exposing larvae to DAPT only during the fourth day (72–96 h) of development was also sufficient to induce wing defects. This corresponds closely to the period in which *N* is known to be required for wing margin formation, suggesting that DAPT treatment mimics a mild reduction of N signaling during wing development.

4. Immunostaining demonstrates that DAPT blocks N signaling in vivo

By the third larval instar, several genes are known to be expressed in a narrow stripe 3–5 cells wide that marks the presumptive wing margin. These include the homeobox-containing transcription factor encoded by *cut* (*ct*) and the secreted ligand encoded by *wingless* (*wg*). Reduction or loss of *N* along the presumptive wing margin leads to loss of both *ct* and *wg* expression and to wing notching.

To determine whether the wing notching phenocopy associated with DAPT treatment was a direct consequence of reduced N signaling, we tested the effect of DAPT on *N* target gene expression. Third instar larvae grown on food containing 1 mM of DAPT were dissected and stained with anti-Ct and anti-Wg antisera. Compared with untreated larvae, discs from treated larvae displayed variable effects on Ct and Wg protein levels. In the most extreme cases, little or no expression of either marker could be detected along the presumptive wing margin; the wing pouch size was substantially reduced, a further indication that N signaling had been affected.

We examined the effect of DAPT treatment on the expression of the *vestigal* (*vg*) intron-2-*LacZ* reporter. Like *ct* and *wg*, *vg* intron 2 is expressed along the presumptive wing margin in an *N*-dependent manner. However, unlike *ct* and *wg*, *vg* intron 2 has been shown to be a direct target of Suppressor of Hairless (Su(H)), the fly orthologue of the mammalian CBF1 protein and the primary effector of N signal transduction. Compared to untreated controls, DAPT treatment resulted in a variable reduction of *vg* intron 2 expression along the presumptive wing margin (**Fig. 2**). Thus, DAPT is sufficient to disrupt the expression of a direct transcriptional target of the N pathway.

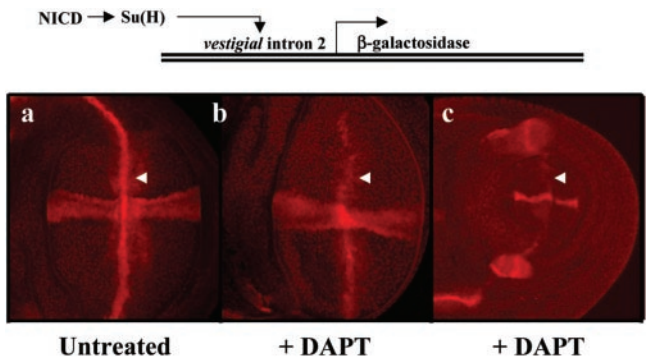

Figure 2. DAPT treatment affects *vg* intron 2, a direct transcriptional target of *N*. *a)* Untreated *vg*-intron 2 LacZ disc. Arrowhead shows *N*-dependent *vg* intron 2 LacZ expression along presumptive wing margin. *b) vg* intron 2 LacZ wing disc treated with DAPT. Arrowhead shows reduction of *vg* intron 2 LacZ expression along the wing margin. *c) vg* intron 2 LacZ wing disc treated with DAPT that shows a more severe reduction in *vg* intron 2 LacZ expression along the wing margin. Note that the size of the wing pouch in this disc is reduced, consistent with a strong reduction in N signaling. Discs are mounted with anterior at the top.

CONCLUSIONS AND SIGNIFICANCE

We have demonstrated here that the γ-secretase inhibitor DAPT blocks N signaling and induces an *N*-deficient phenocopy in *Drosophila*. Other γ-secretase inhibitors also elicit this phenocopy. These results demonstrate the remarkable conservation of the drug binding site(s) on the enzyme complex: pharmacological agents optimized to inhibit human γ-secretase also interact with the fly orthologue. The implications of these findings are threefold. First, pharmacological agents can be used in vivo as surrogates for genetic manipulations in the study of developmental biology. Drugs and drug candidates designed for human use may affect *Drosophila* development in other cases where the target protein, like PS, is well conserved and thought to play a role in development. Second, developmental biology can help elucidate the in vivo mechanism of drug action. We have illustrated this approach using developmental markers to determine whether DAPT blocks N signaling in vivo. Similar strategies could help identify the site of in vivo action of other compounds with unknown mechanisms. As a necessary criterion, the compound of interest must cause specific developmental defects in a model organism. Third, model organisms for developmental biology may aid evaluation of drug candidates for human use. γ-Secretase inhibitors that cause an *N* phenocopy in the fly apparently possess reasonable metabolic stability and the ability to cross biological barriers. Such compounds would therefore be more likely to access and inhibit γ-secretase in mammals than those compounds with no effect in the developing fly. This assay may be useful for ranking compounds for more extensive in vivo testing. FJ