EMBO reports

scientific report

A γ-secretase inhibitor blocks Notch signaling *in vivo* and causes a severe neurogenic phenotype in zebrafish

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Received February 5, 2002; revised April 4, 2002; accepted May 7, 2002

Inhibition of amyloid β-peptide (Aβ) production by blocking γ-secretase activity is at present one of the most promising therapeutic strategies to slow progression of Alzheimer's disease pathology. γ-secretase inhibitors apparently block Aβ generation via interference with presenilin (PS) function. Besides being an essential component of the γ-secretase complex, PS itself may be an aspartyl protease with γ-secretase activity, which is not only required for Aβ production but also for a similar proteolytic process involved in Notch signaling. Here we demonstrate that treatment of zebrafish embryos with a known γ-secretase inhibitor affects embryonic development in a manner indistinguishable from Notch signaling deficiencies at morphological, molecular and biochemical levels. This indicates severe side-effects of γ-secretase inhibitors in any Notch-dependent cell fate decision and demonstrates that the zebrafish is an ideal vertebrate system to validate compounds that selectively affect Aβ production, but not Notch signaling, under *in vivo* conditions.

INTRODUCTION

Accumulation of amyloid β-peptide (Aβ) is an invariant feature associated with Alzheimer's disease (AD) pathology. Aβ is generated by the consecutive cuts of two proteases, known as β- and γ-secretase (Esler and Wolfe, 2001), which liberate the amyloidogenic peptide from its precursor, the β-amyloid precursor protein (APP). Subsequent aggregation is thought to result in the formation of neurotoxic protofibrils (Walsh *et al.*, 1997; Nilsberth *et al.*, 2001) and the deposition of amyloid plaques. While β-secretase has been identified (for a review, see Vassar and Citron, 2000), the nature of γ-secretase is still unclear (De Strooper and Annaert, 2001). The two homologous presenilin (PS) proteins, PS1 and PS2, which are critically required for the intramembranous γ-secretase cut, may be aspartyl proteases with γ-secretase activity (for a review, see Esler and Wolfe, 2001). This is supported by the identification of critical aspartates within the putative transmembrane domains 6 and 7 of PSs (Wolfe *et al.*, 1999c), which may be part of a catalytically active center of an intrinsic aspartyl protease activity (Wolfe *et al.*, 1999a). Moreover, γ-secretase inhibitors, which block Aβ generation, have been found to bind to PS1 (Esler *et al.*, 2000; Li *et al.*, 2000b) and PSs share considerable homology around the critical aspartate in transmembrane domain 7 with bacterial aspartyl proteases (Steiner *et al.*, 2000). However, additional co-factors are required to allow formation of the biologically active PS complex (Capell *et al.*, 1998; Li *et al.*, 2000a), and the cellular distribution of PS does not necessarily reflect the location of γ-secretase activity (Cupers *et al.*, 2001). Thus, it is currently unclear whether PSs are identical to the γ-secretase or just support its targeting to its cellular sites of action (Cupers *et al.*, 2001).

PSs are not only essential for the γ-secretase cut of the Aβ domain, but also for the highly similar site 3 (S3) cleavage of Notch (De Strooper *et al.*, 1999; for a review, see Mumm and

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Kopan, 2000). This cut produces the Notch intracellular domain (NICD), which translocates to the nucleus to regulate target gene transcription (Mumm and Kopan, 2000). Ablation of the PS1 and PS2 genes therefore results in a phenotype indistinguishable from that caused by a Notch knockout (Donoviel *et al.*, 1999), and totally blocks Aβ and NICD production (Herreman *et al.*, 2000; Zhang *et al.*, 2000). Moreover, mutagenesis of the critical aspartates also blocks the function of human PS in Notch signaling (Steiner *et al.*, 1999). Therefore, inhibition of PS activity not only blocks Aβ production, but also interferes with NICD generation and the Notch pathway. Indeed, pharmacological inhibition of PS1 activity blocks Notch signaling in cultured cells (De Strooper *et al.*, 1999; Berezovska *et al.*, 2000; Martys-Zage *et al.*, 2000; Doerfler *et al.*, 2001; Hadland *et al.*, 2001). From a therapeutic point of view, this suggests that drugs developed to lower Aβ production by interfering with γ-secretase activity might affect PS function and therefore also block Notch signaling in vertebrates. To prove whether a known γ-secretase inhibitor (DAPT; Dovey *et al.*, 2001) produces phenotypic side-effects in a living vertebrate, we used zebrafish (*Danio rerio*) as a model system. Our data not only demonstrate that a γ-secretase inhibitor fully blocks Notch signaling in a living vertebrate, but also suggest zebrafish as a suitable system to evaluate the effects of Aβ-lowering drugs on Notch signaling *in vivo*.

RESULTS

As a prototype γ-secretase inhibitor, we investigated the highly specific γ-secretase inhibitor DAPT (Dovey *et al.*, 2001) for its capacity to block Notch endoproteolysis. With this aim, HEK293 cells expressing endogenous PSs were stably transfected with the NotchΔE cDNA [encoding a tagged version of the transmembrane and intracellular domains of Notch (Schroeter *et al.*, 1998)] and treated with or without DAPT. As shown in Figure 1A, the NICD fragment is readily visible in untreated cells, but its generation is inhibited by DAPT treatment. Next, before testing the effects of DAPT in the zebrafish *in vivo*, we verified that DAPT was also active on a zebrafish PS1 (zfPS1)-controlled γ-secretase activity. We stably transfected HEK293 cells expressing Swedish mutant APP (HEK293/sw) (Citron *et al.*, 1992) with cDNA encoding wild-type (wt) zfPS1 or the non-functional zfPS1 D374N mutant (Leimer *et al.*, 1999). As expected (Leimer *et al.*, 1999), zfPS1 was endoproteolytically processed, while endoproteolysis of zfPS1 D374N was blocked and the full-length protein accumulated (Figure 1B). Endogenous human PS1 and PS2 were replaced (Thinakaran *et al.*, 1997) by wt and mutant zfPS1, demonstrating that zfPS1 is incorporated into the PS complex, the formation of which is required for γ-secretase activity (Li *et al.*, 2000a) (Figure 1B). Expression of zfPS1 D374N caused a dramatic accumulation of the substrates of γ-secretase, the APP C-terminal fragments (CTFs), which was accompanied by an almost complete inhibition of total Aβ (Aβ40 and Aβ42) generation (Figure 1B). In contrast, expression of wt zfPS1 did not cause APP CTF accumulation and allowed normal total Aβ production (Figure 1B). We next analyzed Notch endoproteolysis in HEK293/sw cells stably co-expressing NotchΔE and wt or D374N mutant zfPS1. Expression of wt zfPS1 allowed robust NICD production, which was strongly inhibited by the zfPS1 D374N mutant (Figure 1C). Taken together, these results demonstrate that zfPS1 is capable of controlling γ-secretase and S3 protease activity in human cells. We therefore next investigated the effects of DAPT on APP and Notch endoproteolysis in the presence of zfPS1-controlled γ-secretase and S3 protease activity. DAPT caused a strong accumulation of APP CTFs with concomitant inhibition of Aβ generation (Figure 1D). DAPT also inhibited S3 cleavage of Notch in the presence of zfPS1. As shown in Figure 1E, NICD production was inhibited by DAPT in a dose-dependent manner. Thus, these experiments demonstrate that DAPT efficiently blocks a zfPS1-dependent γ-secretase and S3 protease activity.

We subsequently used the zebrafish system to assess the effect of DAPT on Notch signaling at the morphological, molecular and biochemical levels *in vivo*. A number of zebrafish mutants

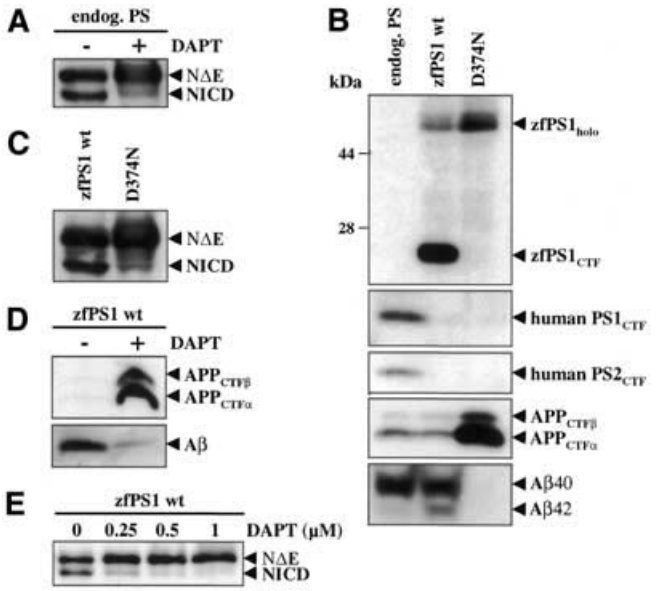

Fig. 1. DAPT blocks Notch endoproteolysis and inhibits a zfPS1-dependent γ-secretase and S3 protease activity in HEK293 cells. (**A**) HEK293 cells stably transfected with the NotchΔE cDNA (Schroeter *et al.*, 1998) were treated with or without 1 μM DAPT for 4 h. Cell lysates were analyzed for NotchΔE (NΔE; uncleaved form of Notch) and NICD (cleaved form of Notch) by immunoblotting using antibody 9E10 to the myc tag at the C-terminus of these Notch variants. (**B**) Panels 1–3: cell lysates from HEK293/sw cells stably transfected with the indicated zfPS1 cDNAs were analyzed by immunoblotting with antibody $zfPS1_{loop}$, 3027 to human PS1 or 3711 to human PS2. Note that endogenous human PS1 and PS2 are replaced by zfPS1 variants. Replacement of endogenous PSs by overexpressed PS variants is an important indication for incorporation of the ectopic PS into a biologically functional PS complex (Thinakaran *et al.*, 1997). Panel 4: cell lysates were analyzed for APP CTFs by immunoblotting with antibody 6687. Panel 5: conditioned media were analyzed for total Aβ (Aβ40 and Aβ42) species by immunoprecipitation/immunoblotting with antibodies 3926/6E10. Immunoprecipitates were separated on a Tris–bicine–urea gel that allows the identification of Aβ40 and Aβ42 (Wiltfang *et al.*, 1997). Note that expression of wt zfPS1 not only allows robust Aβ (Aβ40 and Aβ42) production, but also leads to increased production of Aβ42 (Leimer *et al.*, 1999). (**C**) HEK293/sw cells expressing either wt zfPS1 or mutant zfPS1 D374N were stably transfected with the NotchΔE cDNA (Schroeter *et al.*, 1998) and cell lysates were analyzed for NotchΔE (NΔE) and NICD as in (A). (**D**) HEK293/sw cells stably expressing wt zfPS1 were treated with or without 1 μM DAPT for 4 h and analyzed for APP CTFs as in (B) and for Aβ by direct immunoblotting with antibody 3926. (**E**) HEK293/sw cells stably expressing wt zfPS1 were treated with increasing amounts of DAPT for 4 h and cell lysates were analyzed for NotchΔE (NΔE) and NICD as in (A).

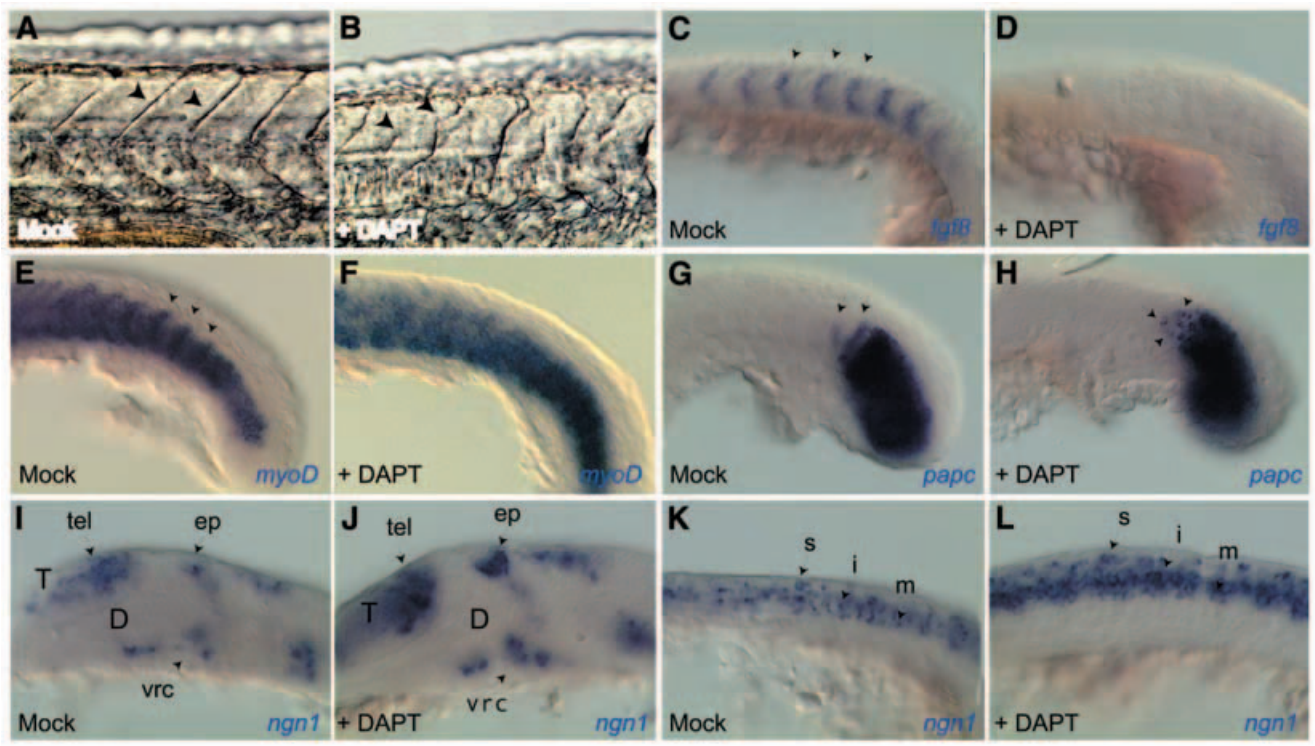

Fig. 2. DAPT affects somitogenesis (**A**–**H**) and neurogenesis (**I**–**L**) *in vivo* in a manner indistinguishable from Notch signaling deficiencies. Mock-treated (A, C, E, G, I, K) and DAPT-treated (B, D, F, H, J, L) embryos visualized at 24 h [(A) and (B), live views] or at 15 somites (C–L), flat mounts following whole-mount *in situ* hybridization with the probes indicated, anterior to the left and dorsal up except in (E) and (F) (dorsal views). (A–H), (K) and (L) are close-up views of the trunk and tail; (I) and (J) are close-up views of the brain. DAPT treatment alters somitic borders [arrows in (A) and (B)]. It affects AP polarization of the somitic mesoderm, normally revealed by the complementary expression of *fgf8* and *myoD* [arrows in (C) and (E)], and randomizes expression of cycling-dependent genes such as *papc* in nascent somites [arrows in (G) and (H)]. In the embryonic nervous system, DAPT triggers a neurogenic phenotype, with an increased number of *ngn1*-positive neuroblasts in every proneural cluster [compare the clusters identified by arrows in (I) and (J), (K) and (L)]. D, presumptive diencephalon; ep, epiphyseal cluster; i, intermediate neurons; m, motoneurons; s, sensory neurons; T, presumptive telencephalon; tel, telencephalic cluster; vrc, ventro-rostral cluster.

affecting Notch signaling have been identified and display characteristic early phenotypes, such as impaired segmentation of the somites (van Eeden *et al.*, 1996), and exacerbated primary neurogenesis (Jiang *et al.*, 1996). Their phenotypes constitute an experimental counterpart with which the *in vivo* effects of DAPT can be compared.

Treating zebrafish embryos with DAPT during the first 24 h of development did not trigger gross morphological abnormalities, but reproducibly impaired somite formation (91%, $n = 54$). At 24 h, somitic boundaries were misshapen, and delimited somites of irregular size (Figure 2A and B). Morphological observations at earlier stages indicated that, upon DAPT treatment, the first 4–8 somites formed normally while most posterior somites did not (data not shown). This phenotype strikingly resembles zebrafish Notch pathway mutants such as *beamter* (*bea*), *deadly-seven* (*des*), *after-eight* (*aei*) and *white-tail* (*wit*) (Jiang *et al.*, 1996; van Eeden *et al.*, 1996). At the molecular level, Notch activity normally controls somite anteroposterior (AP) polarity, as well as the cycling expression of somite prepatterning genes in the presomitic mesoderm (Pourquie, 2000). To confirm our morphological analysis, we assessed the effect of DAPT on these two Notch-dependent processes. Probing DAPT-treated embryos for *fgf8* and *myoD* expression, which respectively label the anterior and posterior halves of each presumptive somite in the unsegmented mesoderm (Figure 2C and E), confirmed that DAPT, like Notch deficiencies, affected somite AP polarity: *fgf8* expression was abolished upon DAPT treatment, while *myoD* expression became ubiquitous (100%, $n = 18$ and 23) (Figure 2D and F). The latter phenotype is similar to that reported in *bea*, *des*, *aei* and *wit* mutants [see figure 7 in van Eeden *et al.* (1996), figure 5 in Jiang *et al.* (1996) and figure 5 in Holley *et al.* (2000)]. In the region of nascent somites, Notch-dependent synchronized gene cycling normally lays down a banded pattern of *paraxial protocadherin* (*papc*) expression, which highlights the cells most recently arrested in a somitic state (Yamamoto *et al.*, 1998; Jiang *et al.*, 2000) (Figure 2G). *papc* expression is modified to a randomized pattern in Notch signaling mutants [figure 3 in Jiang *et al.* (2000)]. Similarly, we observed a random mixture of *papc*-positive and -negative cells in the anterior presomitic mesoderm of DAPT-treated embryos (Figure 2H) (100%, $n = 12$). Thus, DAPT treatment affects somitogenesis *in vivo* in a manner similar to deficiencies in Notch signaling.

We subsequently tested whether DAPT treatment also mimics Notch signaling impairments during neurogenesis. Primary neurogenesis in lower vertebrates involves the selection of individual neuroblasts from proneural clusters by a Notch-dependent lateral inhibition process (Lewis, 1998; Chitnis, 1999; Blader and Strahle, 2000). Specifically, analysis of zebrafish mutants (Jiang *et al.*, 1996) and *in vivo* misexpression experiments (Dornseifer *et al.*, 1997; Haddon *et al.*, 1998; Takke

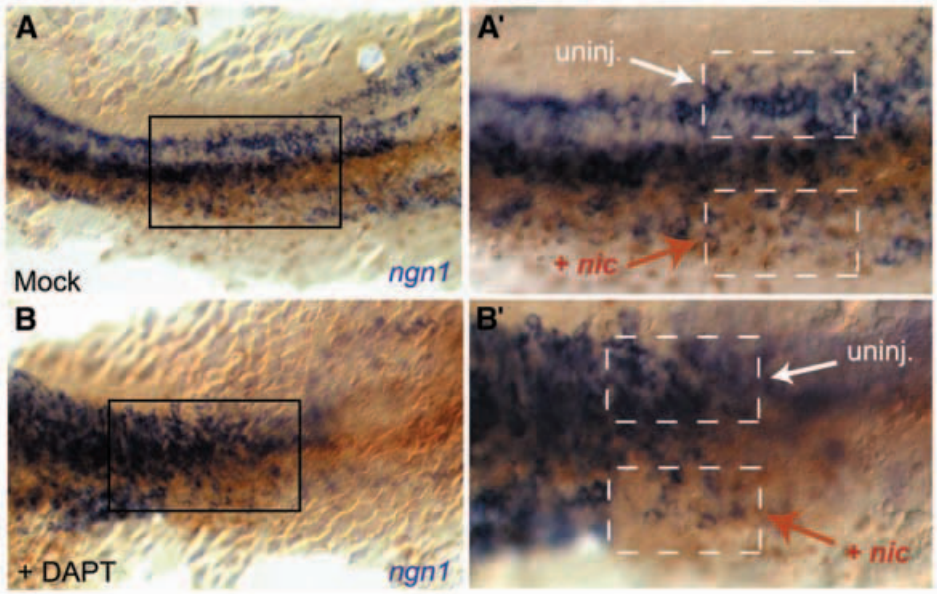

Fig. 3. Expression of the soluble cytoplasmic domain of Notch prevents DAPT-mediated inhibition of Notch signaling. Flat-mounted, mock-treated (**A** and **A′**) and DAPT-treated (**B** and **B′**) embryos visualized at 10 somites following *in situ* hybridization for *ngn1* expression (blue staining) (dorsal view, anterior to the left). All embryos were injected into one blastomere at 4 cells with the NICD-encoding *nic* RNA and *nlslacZ* RNA as lineage tracer (brown nuclei identify the progeny of the injected blastomere). (A′) and (B′) (same magnification) are enlarged views of the boxed areas in (A) and (B), respectively. Injected (+ *nic*) and non-injected (uninj.) territories are indicated on either side of the embryo midline and boxed with a dotted line in (A′) and (B′). DAPT strongly increases the number of primary neurons within the neural plate (Figure 2L and non-injected territory in Figure 3B′ compared with 3A′), while *nic* has the opposite effect (compare injected and non-injected territories in A′). Note that NICD activity overrides the neurogenic effect of DAPT, as a similarly reduced number of neurons follows *nic* expression with or without DAPT [compare injected sides in (A′) and (B′)].

et al., 1999) demonstrated that Notch signaling maintains its expressing cells in an undifferentiated state, while neighboring Delta-positive cells express the neuronal specification factor neurogenin (Ngn1) (Blader *et al.*, 1997) and generate neuroblasts. We observed that DAPT treatment strongly and reproducibly increased the number of *ngn1*-positive cells within each proneural cluster at all levels of the body axis during primary neurogenesis (Figure 3I–L) (100%, *n* = 13), triggering a neurogenic phenotype similar to zebrafish mutants deficient in Notch signaling, such as *wit* (Jiang *et al.*, 1996), and to zebrafish embryos rendered insensitive to Notch by misexpression of the extracellular form of Delta (Haddon *et al.*, 1998). Thus, like an absence of Notch signaling, DAPT prevents the lateral inhibition process of neuroblast selection *in vivo*.

The neurogenic phenotype resulting from Notch signaling deficiencies can be reverted *in vivo* by overproduction of the NICD fragment (see Haddon *et al.*, 1998; Takke *et al.*, 1999). To further confirm that the neurogenic phenotype triggered by DAPT *in vivo* resulted from impaired Notch processing, we therefore tested whether it could be reverted by injection of *nic*, an mRNA encoding the NICD fragment of zebrafish Notch1 (Takke *et al.*, 1999). As concluded previously (Haddon *et al.*, 1998; Takke *et al.*, 1999), local misexpression of NICD reduced the number of *ngn1*-positive primary neurons in the embryonic neural plate (Figure 3A and A′). This effect is epistatic to the action of DAPT, as the number of primary neurons in *nic*-injected areas was similarly reduced in DAPT-treated and control embryos (compare Figure 3B and B′ and A and A′). Elsewhere in the neural plate, and as documented above (Figure 2I–L), neurogenesis was prominently enhanced by DAPT (Figure 3B and B′). Thus, the neurogenic effect of DAPT can be reverted by NICD, confirming that DAPT acts by interfering with Notch signaling and upstream of NICD activity. Taken together, our results demonstrate that DAPT affects zebrafish embryonic development in a manner indistinguishable from Notch signaling deficiencies, both at the morphological and molecular levels.

DISCUSSION

Our results demonstrate that DAPT, a known and carefully characterized γ-secretase inhibitor (Dovey *et al.*, 2001; Sastre *et al.*, 2001), severely interferes with Notch signaling in zebrafish embryos. DAPT and other γ-secretase inhibitors were developed as Aβ-lowering drugs thought to be used for long-term treatment in human patients (Wolfe *et al.*, 1999b; Dovey *et al.*, 2001). However, concerns about such a strategy were raised because it could apparently interfere with the biological function of PS. Based on numerous previous findings, PS clearly plays a role in Notch signaling by facilitating NICD generation (for a review, see Mumm and Kopan, 2000; Steiner and Haass, 2000). One may therefore expect that such inhibitors not only have beneficial effects with regard to Aβ production, but also unwanted side-effects on the control of cellular differentiation via interference with the Notch signaling pathway. Along this line, Hadland *et al.* (2001) recently demonstrated that a distinct γ-secretase inhibitor (Cpd.11) (Wolfe *et al.*, 1999b) added to fetal organ cultures represses thymocyte development, probably by reducing Notch signaling. However, in this study, direct evidence that proteolytic generation of NICD generation was indeed affected in the $CD4^{-}/CD8^{-}$ precursor cells was lacking.

We now demonstrate for the first time that a γ-secretase inhibitor interferes with Notch signaling in vertebrates *in vivo*, directly suggesting that rather significant putative side-effects are to be expected from such drugs during long-term treatment in humans.

The detrimental effects of DAPT were observed during embryogenesis of zebrafish. However, they are likely to occur in adults as well, as Notch signaling is active at all stages and in multiple tissues. For example, hematopoiesis is required throughout life and thymocyte differentiation requires Notch signaling (Hadland *et al.*, 2001). All Notch factors are also expressed in the adult brain (Weinmaster *et al.*, 1992; Higuchi *et al.*, 1995; Berezovska *et al.*, 1998; Irvin *et al.*, 2001), where they are likely to play pivotal roles in terminally differentiated neurons, as well as in the control of gliogenesis and neural stem cell differentiation. However, a conditional knockout of the PS1 gene had no obvious effects on Notch signaling in mice (Yu *et al.*, 2001). The lack of effects on Notch signaling is likely to be due to the abundant expression of PS2 (Yu *et al.*, 2001), which supports Notch signaling like PS1 (Steiner *et al.*, 1999). In contrast to the conditional PS1 depletion, γ-secretase inhibitors will affect both PS1 and PS2 function (Esler *et al.*, 2000; Li *et al.*, 2000b). Besides putative side-effects on Notch signaling, PSs bind β-catenin, thus independently interacting with yet another signaling pathway potentially controlling cell proliferation in adults. Indeed, loss of PS1 in mice also results in enhanced β-catenin signaling, which causes skin tumors in adult mice (Xia *et al.*, 2001).

In summary, our data not only demonstrate that a γ-secretase inhibitor blocks Notch signaling in a living vertebrate, but also provide a novel model system for the validation of drugs that differentially affect Aβ production and NICD formation (Petit *et al.*, 2001). After only 24 h, numerous zebrafish embryos can be investigated for deficits in somitogenesis or neurogenesis, which provide a precise and reliable read-out of Notch signaling activity. Therefore, our results identify the zebrafish as a valuable test system for the validation of Aβ-lowering drugs that do not interfere with other physiologically important signaling pathways.

METHODS

Cell lines and cell culture. HEK293 cell lines were generated and cultured as described previously (Steiner *et al.*, 2000).

Antibodies. The polyclonal antibodies against PS1 (3027) and PS2 (3711), against zfPS1 (zfP1$_{loop}$) (Leimer *et al.*, 1999), against the C-terminus of APP (6687) and against Aβ1–42 (3926) have been described previously (Steiner *et al.*, 2000). Monoclonal antibodies against Aβ1–17 (6E10) and the c-myc epitope (9E10) were obtained from Senetek (6E10) and from the Developmental Studies Hybridoma Bank, University of Iowa (9E10).

PS, APP and Notch endoproteolysis. Analysis of human and zebrafish PS expression was as described previously (Leimer *et al.*, 1999; Steiner *et al.*, 2000). APP CTFs were analyzed as described before (Steiner *et al.*, 2000) and Aβ production was analyzed by combined immunoprecipitation/immunoblotting of conditioned media with antibodies 3926/6E10 as described previously (Sastre *et al.*, 2001) or by direct immunoblotting of aliquots of conditioned media with antibody 6E10. Notch endoproteolysis was analyzed by immunoblotting of cell lysates with antibody 9E10.

Fish strains. Embryos were obtained from natural spawning of wild-type (AB strain) adults; they were raised and staged according to Kimmel *et al.* (1995).

DAPT treatments. A 10 mM stock of DAPT in DMSO was diluted in embryo medium and applied to dechorionated zebrafish embryos at 28°C from the sphere stage (late blastula) until the stage of analysis (see figure legends). Control embryos were mock treated with embryo medium containing the same concentration of DMSO carrier only. We first performed a dose–response analysis and established that a minimal concentration of 50 μM DAPT was required to affect somitogenesis at the morphological and molecular levels (30 and 60% of cases, respectively, $n = 34$; not shown). All the results reported here were obtained using a dose of 100 μM DAPT. HEK293 cells were treated with 1 μM DAPT for 4 h.

Capped mRNA injections in zebrafish embryos. *nic* capped RNA (encoding the NICD of zebrafish Notch1) was synthesized as described by Takke *et al.* (1999) using the mMessage mMachine kit (Ambion), and 5 pg were injected (together with 4 pg of *nlslacZ* RNA as lineage tracer) into a single blastomere of 4-celled embryos. Nucleus-localized β-galactosidase was revealed using rabbit anti-β-galactosidase (1/4000) followed by goat anti-rabbit–HRP (1/200; Jackson Laboratories) antibodies and DAB revelation.

***In situ* hybridizations and immunocytochemistry.** *In situ* hybridizations were carried out according to standard protocols (Thisse *et al.*, 1993) using the following probes: *fgf8* (Reifers *et al.*, 1998), *myoD* (Weinberg *et al.*, 1996), *papc* (Yamamoto *et al.*, 1998) and *ngn1* (Blader *et al.*, 1997).

ACKNOWLEDGEMENTS

We thank the Boehringer Ingelheim Pharma KG for the gift of DAPT and S. Amacher, P. Blader, J.A. Campos-Ortega, D. Edbauer, R. Kopan, U. Leimer, C. Thisse and E. Weinberg for providing cDNAs, antibodies and cell lines, and G. Basset for technical assistance. We thank R. Baumeister, C. Goridis and W. Wurst for critically reading this manuscript, and H. Takeda for communicating unpublished data. This work was supported by grants from the Deutsche Forschungsgemeinschaft (to C.H. and H.S.), the European Community (to C.H.) and the Volkswagen-Stiftung (to A.G. and L.B.-C.).

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DOI: 10.1093/embo-reports/kvf124