Research paper

Reduction BACE1 expression via suppressing NF-κB mediated signaling by Tamibarotene in a mouse model of Alzheimer’s disease

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\textbf{A B S T R A C T}

This present study examined the effect of Tamibarotene (AM80) in APP/PS1 mice, a well-established AD mouse model. AM80 was intraperitoneal administered to 3-month-old APP/PS1 mice at a dose of 5 mg/kg/day for 16 weeks. The results clearly showed that AM80 could reduce amyloid-β peptides through impact on APP processing and reduce microglia and astrocyte activation in APP/PS1 mice. The most notable finding in the present study was that inhibitory effect on BACE1 mediated by NF-κB pathway underlies the anti-inflammatory action of AM80. Moreover, AM80 could significantly decrease synaptic loss and enhance the expressions of Synapsin and Drebrin. Therefore, AM80 treatment may have the preclinical prevention of AD with new therapeutic strategies.

\textbf{Introduction}

Alzheimer’s disease (AD) is a kind of neurological disorder which is involved in plaques and tangles forming in the brain and it will become more severe in the elder over time. Its onset and progression correlate with memory loss and neuroinflammatory processes (Fidelis et al., 2019; Claudio et al., 2016; Qiao et al., 2015).

A growing body of evidence showed that smaller, soluble oligomers of Aβ, rather than mature amyloid plaques, are the pathogenic species in AD. Aβ originates from sequential proteolysis of amyloid-β protein precursor (APP) by BACE1 and γ-secretase (Hardy and Selkoe, 2002; Kapoor et al., 2013). In AD brains, elevated levels of BACE1 expression are related with Aβ production and deposition (Chen et al., 2012).

Additionally, increasing evidences suggest that exacerbated Aβ production can modulate BACE1 promoter transactivation through an NF-κB-dependent pathway (Virginie et al., 2008; Wang et al., 2013). Also, activated NF-κB can affect AD pathology through enhanced neuro-inflammatory cytokines release characterized by activated microglia and astrocyte.

Vitamin A and its derivatives (Retinols) can modulate a great number of gene expressions which involved in the development of CNS via retinoic acid (RA) receptors and retinoid X receptors. A deficit in RA signaling may be associated with AD (Ding et al., 2008). AM80 is a RA receptor agonist with high specificity for RAR\textalpha and RAR\textbeta over RAR\textgamma and is inactive towards RXR\textalpha, \textbeta, and \textgamma; this selectivity is practically advantageous from the viewpoints of low adverse effects (Fukasawa et al., 2012). Moreover, it is also used to treat acute promyelocytic leukemia (APL) in Japan. However, all trans retinoic acid regulates gene expression through its nuclear receptors: the RA receptors (RARs) and retinoid X receptors (RXRs). As a drug, there will be a lot of adverse effects.

AM80 is known to be involved in cell growth arrest in antitumor therapy (Jiang et al., 2016), Bronchiolitis Obliterans (Watanabe et al., 2019) and AM80 is also considered to be a promising candidate drug for treatment of AD. Although AM80 has been reported to attenuate insoluble Aβ42 levels in APP23 mice brain (Kohichi et al., 2009) and rescue the memory loss made by scopolamine, as a traditional model of AD (Kohichi et al., 2004); Co-administration of AM80 with HX630 could make a great contribution for improvement in the memory deficits in APP23 mice (Kohichi et al., 2014). However, all those detailed mechanisms remained largely unknown.

Therefore, other animal model will be needed to further confirm the new indication of AM80 in AD. Here, we tested whether AM80 treatment in the early phase of AD, could prevent the delay AD onset in APP/PS1 double transgenic mice, a well-established AD mouse model. Interestingly, our observations provided a novel mechanistic explanation for the

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efficacy of AM80.

**Experimental procedure**

**Materials**

Tamibarotene (also known as AM80) bought from Medchem Express (Monmouth Junction, NJ, USA) were dispersed in 0.5% sodium carboxymethylcellulose solution. BCA protein assay reagent kit was purchased from Pierce Chemical (Rockford, IL). Rabbit polyclonal anti-APP-C-terminal fragments (CTFs) were purchased from Sigma. Rabbit polyclonal anti BACE1, p-NF-kB, NF-kB, Drebrin, Synapsin, IDE, anti-goat NEP were purchased from Cell Signaling. Monoclonal anti-beta amyloid 6E10 was purchased from Signet Laboratories. Rabbit polyclonal Iba1 antibody was purchased from Wako Chemicals. Rabbit polyclonal GFP antibody was purchased from Dako Cytomation. AlexaFluorTM 594 and AlexaFluorTM 488 were purchased from Invitrogen.

**Animal and AM80 treatment**

APPwe/PS1dE9 (ApdE9) mice were purchased from Jackson Laboratory, USA. All animals were housed according to standard animal care protocols approved by the local ethics committee at Guangdong Pharmaceutical University.

APP/PS1 double transgenic mice and wild-type littersmates aged 3 months were randomly allocated into four groups: treated or untreated APP/PS1 mice or wild-type mice. Treated groups received AM80 suspended in 0.5% (w/v) carboxymethylcellulose solution by intraperitoneal administration (5 mg/kg/day). Vehicle groups received the same volume of carboxymethylcellulose solution. Drug treatment was started when the mice were 3 months old before the appearance of Aβ plaques (preplaque phase) and was continued for 16 weeks. AM80 does was chosen based on pilot studies and was adjusted regularly according to the body weight of mice.

**Immunohistochemistry and confocal imaging**

Mice were anesthetized with pentobarbital and perfused with 50 mL of ice-cold PBS followed by equal volume of 4% paraformaldehyde. After perfusion, mouse brains were removed and embedded in paraffin. The blocks were sectioned horizontally into 6µM setting and the sections were immersed in 70% formic acid for 5 min for Aβ staining. After blocked, the brain sections were incubated with primary antibodies at 4 °C overnight followed by the incubation of fluorescence-conjugated secondary antibody at room temperature for 1 h. After washed, the sections were mounted and examined using confocal microscope (LSM510 Meta; Carl Zeiss, Germany). NA of the objectives is 0.5; scan speed is 2000 lines/sec, the depth of each focal plane is 2µM.

**Enzyme-linked immune sorbent assay (ELISA) sandwich**

After cortical and hippocampal tissues were homogenized and centrifuged in TBS, the supernatants (soluble fraction) were taken. The pellets were resuspended and homogenized in 70% formic acid (insoluble fraction). Following the appropriate dilution, levels of total Aβ40/42 were detected by the ELISA method, using a mAb specific for the NH2 terminus of human Aβ as capture for the 1–40 or 1–42 sequence respectively, according to the manufacturer’s instructions (Wako Pure Chemical Industries, Osaka, Japan). Sample values were expressed as nmol of Aβ40 or Aβ42 /gram brain tissue weight.

In order to detect the amounts of TNF-α, the TBS-soluble fractions of cerebral cortex and hippocampus were analyzed by means of sandwich ELISA followed the manufacturer’s instructions. The concentrations for unknown samples were calculated by the standard curve.

**Western blot analysis**

After homogenized in RIPA buffer containing a mixture of protease inhibitors, the brain homogenates were centrifuged at 15,000 g for 30 min at 4 °C. Fifteen micrograms of total protein from each samples was run on a 12% SDS-PAGE gel (BioRad) and transferred to nitrocellulose membrane. The membranes were blocked with 5% dry milk in TBST for 1 h and the membranes were incubated with primary antibodies overnight, followed by horseradish peroxidase-conjugated secondary antibodies against the corresponding species. Bands were analyzed using densitometric software.

**Electron microscopy**

Mice were perfused with PBS followed by glutaraldehyde (2%) in cacodylate buffer (pH 7.4). Brains were removed and postfixed overnight in 2% glutaraldehyde. After postfixed in 1.0% osmium peroxide buffered for 1 h, the hippocampal specimens were stained by 0.5% uranyl acetate at 50% ethanol. After dehydrated, specimens were embedded with the epoxy resin. The grids were stained in uranyl acetate and lead citrate, mounted on copper grids and finally analyzed with a Zeiss EM 10 (Oberkochen, Germany) electron microscope.

**Statistical analysis**

Data are presented as mean ±SD. Histograms were conducted by Prism 5 software. Results were analyzed using ANOVA followed by Turkey-Kramer multiple comparisons test. P < 0.05 was regarded as statistically significant.

**Results**

**Effect on Aβ level after AM80 treatment in APP/PS1 transgenic mice**

To investigate whether or not AM80 could prevent AD development in APP/PS1 mice, a typical AD model mouse, the intraperitoneal administration of AM80 for 16 weeks. We used sandwich ELISA to determine and compare the amounts of total Aβ40 and Aβ42 in the whole brain. Importantly, the total Aβ42 level of AM80-treatment showed lower than that of vehicle-group, but the total Aβ40 level didn’t show obvious difference (Fig. 1A, B). These interesting results strongly indicated AM80 could slow Aβ production in APP/PS1 mice from the preplaque phase. The effect of AM80 on Aβ levels in wild-type mice was not determined, because the level was below the detection limit in the ELISA system.

**Influence of AM80 on APP processing in a mouse model of AD**

To demonstrate the AM80-induced amyloidogenic APP processing in vivo, APP-CTF was detected after drug treatment. Interestingly , we found a 50% reduction in CTFs (C99 and C83) (Fig. 2A, B) after AM80 treatment in APP/PS1 mice. However, the full-length APP was no significant change between the two groups of AD mice. Moreover, we also found a dramatically reduction in the levels of Aβ oligomers after AM80 treatment in APP/PS1 mice (Fig. 2C, D). These results obviously indicated that AM80 specifically inhibited amyloidogenic processing of APP in APP/PS1 mice.

**Effect of AM80 administration on BACE1 expression and inflammatory NF-kB inhibition**

It was reported that all-trans-retinoic acid could suppress BACE1 expression in Tg2576 mice. We further examined whether AM80 could reduce amyloidogenic processing of APP by affecting the rate-limiting β-secretase. After continuous AM80 treatment, we observed a significant reduction of BACE1 protein in the brain of APP/PS1 mice compared
Fig. 1. Effect on Aβ level after AM80 treatment in AD mice. (A-B) Levels of total Aβ40 and Aβ42 are analyzed using the ELISA kits (n = 6). *P < 0.05 vs. APP/PS1-vehicle mice.

Fig. 2. AM80 reduced the production of APP-CTFs (C99 and C83) and Aβ oligomers. (A, C) Western blots of APP, APP-CTFs and Aβ oligomers (a mouse anti-Aβ monoclonal antibody 6E10) in the hippocampus and cerebral lysates of different groups of mice are shown. (B, D) Quantitative results of APP-CTFs and Aβ oligomers are shown (n = 8). *P < 0.05 vs. APP/PS1-vehicle mice, **P < 0.01 vs. APP/PS1-vehicle mice.
with the APP/PS1-vehicle mice as expected (Fig. 3A, B). This novel finding suggested that AM80 might influence APP processing via a mechanism by modulating the expression of BACE1.

Several studies reported that the expression of BACE1 was transcriptionally regulated by NF-κB. Moreover, AM80 could inhibit CXCL2 production via attenuation NF-κB signaling in microglia BV2 cells. To determine whether AM80 could play a role in transcriptional regulation of BACE1 through NF-κB signaling in APP/PS1 mice, we examined the effect of AM80 on the expression of p-NF-κB. Interestingly, the result strongly implied that p-NF-κB protein was remarkably reduced after AM80 treatment in APP/PS1 mice. However, the total NF-κB almost kept unchanged (Fig. 3C, D).

**AM80 treatment attenuated the activation of microglia and astrocyte**

Activated microglia has been considered a dangerous feature of AD pathology. Senior plaques are often closely associated with activated microglia and astrocytes. We hypothesized that AM80 could inhibit glial cells activation in this animal model. Brain sections were co-immunostained with an antibody mixture containing anti-Iba1 and 6E10. Compared with the wildtype mice, distinct microgliosis, characterized by clustering of hypertrophic Iba1-positive microglia around Aβ deposits, was observed in the brains of APP/PS1 vehicle mice. However, there was a significant decrease in the number of hypertrophic microglia in AM80 treated APP/PS1 mice compared with untreated APP/PS1 controls (Fig. 4A–E).

Astrogliosis was also observed by co-immunostained with an antibody mixture containing anti-GFAP and 6E10. In the neocortex, there is a marked increase of activated astrocyte in the APP/PS1 vehicle mice. However, significant decreased in the GFAP immunoreactivity was observed after AM80 treatment (Fig. 5A–C).

**AM80 treatment attenuated the production of TNFα**

Activated microglia produce and secrete several proinflammatory mediators, including TNFα, IL-6, and NO, all of which can confer neurotoxicity. Astrocytes also participate in and propagate the neuroinflammatory environment in AD. Here, we wondered if AM80 could attenuate the production of proinflammatory factor TNFα owing to the reduction of activated glia cells. As expected, the result revealed an obvious reduction of the TNFα in AM80 treated APP/PS1 mice compared with untreated APP/PS1 controls (Fig. 6). Therefore, this finding strongly suggested that AM80 could suppress the production of inflammatory cytokines through neurotoxic microglial activity in the early phase of AD. Possibility, its anti-inflammatory role might also be correlated with the suppressive effect on BACE1.

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**Fig. 3.** AM80 reduced the expressions of BACE1 and p-NF-kB. (A, C) Western blots of BACE1, NEP, IDE, p-NF-kB and NF-kB in the whole brain lysates of four groups of mice are shown. (B, D) Quantification of p-NF-kB and BACE1 immunoblots are shown. Data are mean ±SD (n = 8). *P < 0.05 vs. APP/PS1-vehicle mice.
AM80 prevents loss of synaptic vesicles and enhances synapses in APP/PS1 mice

Synapsin (SYN), a marker protein of synaptic vesicles which is abundant in presynaptic boutons, has been shown to be decreased and correlated with cognitive deficits in AD brain. Here, we are eager to know whether AM80 treatment can make a great contribution for rescuing the loss of synaptic vesicles. Immunohistochemistry revealed the expression

Fig. 4. AM80 attenuated microglial activation. (A-D) High-magnification (20×) images are showing Iba1-immunopositive microglia (Green) around Aβ (Red) in neocortex. Scale bars, 100 µm. (E) Quantification of microglial number in neocortex is shown (n = 8). *P < 0.05 vs. APP/PS1-vehicle mice. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

Fig. 5. AM80 attenuated astrocyte activation. (A) Representative images (63×) of GFAP (Green) and Aβ plaques (Red) are shown in neocortex. Scale bar, 100 µm. (B) Low magnification (4×) of GFAP (Green) and Aβ plaques (Red) are shown. (C) Quantification of astrocyte is shown. Data were mean ±SD (n = 8). **P < 0.01 vs. APP/PS1-vehicle mice. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

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of SYN in the CA3 zone of mouse brain. Compared with the APP/PS1-vehicle control groups, the SYN immuno-reactivity were remarkable increased in the APP/PS1 mice treated by AM80 (Fig. 7A).

In support of above finding, we also performed Western blot analysis for Drebrin A, an F-actin binding protein that is particularly concentrated in dendritic spines. Reduced Drebrin levels were observed in the hippocampus of APP/PS1 mice compared with wildtype mice. In contrast, a significant increase (P < 0.05) in the levels of Drebrin was observed in the hippocampus of APP/PS1 mice after AM80 treatment (Fig. 7C). Thus, AM80 may slow dendritic spine loss in APP/PS1 mice.

Next, we analyzed the synapse number in the CA1 region by transmission electron microscopy after AM80 treatment. Normally, the presence of the electron-dense postsynaptic density juxta-opposed to the presynaptic terminal containing synaptic vesicles is defined as a synapse. According to this criterion, we found that the number of synapses was significantly lower in APP/PS1 vehicle mice compared with APP/PS1-AM80 treated mice (Fig. 7B, D). These results demonstrated that AM80 may play an important role in rescuing cognitive deficits through modulating the synapse function in APP/PS1 mice.

Discussion

Previous works have indicated that a deficit in RA signaling may be associated with neurodegeneration in AD. Thereof, RAR agonists should be a satisfied therapeutic stratagem to reduce the production and counteract the untoward effects of excess Aβ (Goncalves et al., 2013). Though AM80 is regarded as a promising candidate drug because of its action in the pathology of AD using several mouse models (Kohichi et al., 2004; Kohichi et al., 2014), the therapeutic mechanism of AM80 in AD remained unascertainable. Still no clinical studies have been carried out yet. The main purpose of this study was to clarify the effects of AM80 in APP/PS1 double transgenic mice from the early stage of AD.

Our trials indicated that AM80 treatment for 16 weeks, the brain level of total Aβ 42 and Aβ oligomers were remarkably decreased after AM80 treatment in APP/PS1 mice. Therefore, it was considered that the beneficial effect of AM80 might possible due to affect the production and accumulation of Aβ in APP/PS1 mice.

The effect of AM80 on the accumulation of Aβ may likely attribute to its action on APP processing. Normally, APP is cleaved by α-secretase within the Aβ domain in the nonamyloidogenic pathway, thereby precluding Aβ generation. Such cleavage produces APPα (sAPPα) and C83 α-CTF). However, if APP is first cleaved by the β-site APP-cleaving enzyme 1 (BACE1) in the amyloidogenic pathway, leading to the release of APPβ (sAPPβ) and generating C99 β-CTF). Then APP-C99 is further cleaved by γ-secretase to generate Aβ and AICD (Jarvis et al., 2010). Our interesting finding provided the first direct evidence that AM80 exerted a significant inhibitory effect on APP processing. Moreover, it has been also reported that retinoic acid may modulate expression of Aβ-degrading enzyme, including Insulin-Degrading Enzyme (IDE) and Neprilysin (NEP) (Ding et al., 2008). However, these two important Aβ-degradation enzymes did not show significant difference in this kind of AD mouse model after AM80 treatment (Fig. 3A).

It has shown that RARs agonists could process APP into the non-amyloidic pathway leading to decrease Aβ production by increasing α-secretase expression and activity or alter the localization of BACE1 to impair AβPP cleavage (Jarvis et al., 2010). So α-secretase is the main BACE1 competitor for intracellular APP cleavage (Satoh and Kuroda, 2000). Therefore, our attention is focused on the BACE1 which has been shown to be involved in the generation of Aβ. Our interesting results strongly implied that AM80 could result in impaired β-secretase cleaving and lowering Aβ production.

Several studies found that both BACE1 and NF-kB levels were elevated in AD patients, and also NF-kB could facilitate BACE1 gene expression and APP processing (Chen et al., 2012). In addition, it has been reported that Aβ itself can trigger NF-kB mediated BACE1 expression in glial cells (Bourne et al., 2007). Moreover, it is also confirmed that knocking-down of p65, a key NF-kB component, significantly reduced BACE1 expression in both HEK293 and SH-SY5Y cells (Zheng et al., 2015; Wang et al., 2015). All these researches suggested that NF-kB signaling is an important molecule modulator for BACE1 regulation. Our observation also suggested that AM80 may interfere with NF-kB signaling to affect BACE1 expression since the promoters containing NF-kB binding sites. Moreover, it was also reported that AM80 could prevented LPS-induced nuclear translocation of NF-kB (Takaoka et al., 2016). Therefore, suppression of NF-kB signaling as discussed above may be responsible for the inhibitory effect of AM80 on gene transcription. In addition, suppressing NF-kB signaling is a plausible strategy since NF-kB is constantly over activated in AD brain.

Inflammatory processes, caused by activated microglia and astrocytes surround the amyloid plaques, are involved in the pathogenesis of AD. With β-amyloid deposition, however, some microglial processes become static, showing stable association with plaques. Recent evidence suggests that microglia form a protective barrier around amyloid deposits, compacting amyloid fibrils into a tightly packed and potentially less toxic form, preventing the accretion of new Aβ onto existing plaques, and reducing axonal dystrophy in the nearby neuropil. Moreover, microglia can also protect from uptake and clearance of soluble Aβ species, phagocytosis of insoluble fibrillar Aβ deposits (Streit et al., 2014). In this study, the significant decrease of activated microglia and astrocytes shown in APP/PS1 mice treated by AM80 may be attributed to reducing Aβ level.

There is growing consensus that NF-kB signaling is at the center of many inflammatory pathways and its activation is vital for releasing a cascade of damaging cytokines in brain. One hallmark of this process is the secretion of TNFs, which may lead to further degeneration and prevention of Aβ clearance (Dheen et al., 2005). Our above studies suggested that AM80 may decrease TNF-α production through reducing microglial activation and inhibition of NF-kB nuclear translocation. Therefore, the anti-inflammatory effect of AM80 in APP/PS1 double transgenic mice provides additional evidence for its therapeutic potential for AD.

It is well known that Aβ oligomers could interfere specifically with synaptic function, which ultimately causes degeneration of synapses in AD. Researchers Kohichi et al. (2014) and Koichi et al. (2004) suggested that AM80 could improve the cognitive behavior of animals, but the mechanism kept unclear. Our important findings firstly demonstrated that the beneficial effect of AM80 on cognitive improvement may likely be attributable to enhancing the expressions of SYN in synaptic vesicles.
Taken together, our findings provided further evidence that AM80 could regulate APP processing through blunting BACE1 expression and NF-κB regulated neuroinflammation which contribute to the reduction of Aβ production and deposit firstly. In addition, AM80 could improve the learning and memory through modulating the function of synapses in APP/PS1 double transgenic mice. Other mechanisms may also be possible and need to be confirmed further.

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Conflicts of interest

The authors declare no competing financial interest.

Ethical approval

The authors certify that the animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 or
the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, or the European Communities Council Directive of 24 November 1986 (86/609/EEC).

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