J. Pestic. Sci., **34**(2), 96–99 (2009)
DOI: 10.1584/jpestics.G08-38

Note

3-(4-Hydroxybenzylidene)anabaseine: High-affinity ligand for *Periplaneta americana* nicotinic acetylcholine receptors

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(Received October 6, 2008; Accepted December 12, 2008)

3-Benzylideneanabaseine analogues with electron-donating *ortho*- and/or *para*-substituents on the benzene ring were synthesized. The affinity of the analogues for nicotinic acetylcholine receptors in the nerve cord of the American cockroach (*Periplaneta americana* L.) was determined by the radioligand binding assay using [^{3}H]epibatidine. Among the compounds tested, 3-(4-hydroxybenzylidene)anabaseine (**6**) displayed the highest potency, with IC_{50} values of 3.6 nM. In contrast, 3-(4-methoxybenzylidene)anabaseine (**5**) showed the lowest potency (IC_{50}= 188.3 nM). Compound **6** was found to have 52-fold higher affinity than **5**. 3-(2,4-Dimethoxybenzylidene)anabaseine (**1**) and 3-(2-hydroxy-4-methoxybenzylidene)anabaseine (**2**) showed higher potency than **5**, with IC_{50} values of 41.6 and 63.5 nM, respectively. Compound **6** might prove to be a good lead compound for the development of novel insecticides.

Keywords: nicotinic acetylcholine receptor, 3-benzylideneanabaseine, American cockroach.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop superfamily of ligand-gated ion channels, which includes serotonin type-3, γ-aminobutyric acid, and glycine receptors. nAChRs play an important role in mediating excitatory neurotransmission in the nervous systems of vertebrates and invertebrates. Human nAChRs have also been identified as promising targets for the treatment of neurological disorders, such as Alzheimer's disease, Parkinson's disease, attention deficit disorder, and anxiety.[1,2] Meanwhile, insect nAChRs have drawn much attention as effective targets for insecticides, since selective toxicity between insects and mammals was achieved with neonicotinoids such as imidacloprid.[3]

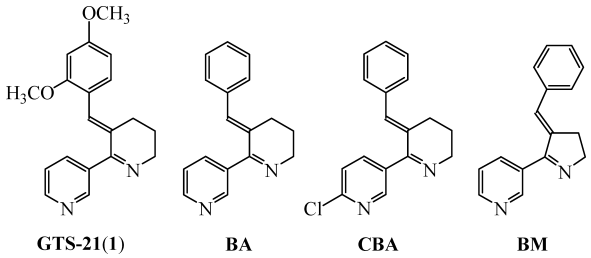

Fig. 1. Structures of GTS-21, 3-benzylideneanabaseine (BA), 6′-chloro-3-benzylideneanabaseine (CBA), and 3-benzylidenemyosmine (BM).

3-(2,4-Dimethoxybenzylidene)anabaseine (GTS-21; **1**), a synthetic benzylidene derivative of the marine worm toxin anabaseine, selectively stimulates vertebrate α7-nAChRs (Fig. 1).[4] This compound is a drug candidate for the treatment of Alzheimer's disease and schizophrenia, based on its behavioral actions and ability to protect rat neurons from various apoptotic and necrotic insults, both *in vivo* and *in vitro*.[5] We previously reported that 3-benzylideneanabaseine (BA) acts as an agonist in nAChRs of the American cockroach (*Periplaneta americana* L.) by neurophysiological experiments,[6] and that 6′-chloro-3-benzylideneanabaseine (CBA) analogues, which have a chlorine atom at the 6′-position of the pyridine ring, have insecticidal activity against German cockroaches.[7] Recently, we have also reported the affinity of a series of 3-benzylidenemyosmine (BM) and 3-cinnamylidenemyosmine analogues for the nAChRs of *P. americana*, using [^{3}H]epibatidine binding assays.[8] The results showed that (*i*) a BM analogue with both *para*- and *ortho*-hydroxyl groups on the benzene ring of the benzylidene moiety displayed the highest affinity, followed by a BM analogue with a *para*-hydroxyl group, (*ii*) a BM analogue with an *ortho*-hydroxyl group on the benzene ring of BM displayed moderate affinity, and (*iii*) an analogue with a *meta*-hydroxyl group on the benzene ring showed little affinity. These findings prompted us to further synthesize the analogues and examine their affinity for *P. americana* nAChRs (*Pa*-nAChRs) to understand the interactions between ligands and the receptor in more detail.

In this paper, we report the affinity of BA analogues for native *Pa*-nAChRs, and show that the 4-hydroxybenzylidene analogue has the highest affinity among the series of BM, BA, and CBA analogues synthesized to date.

Materials and Methods

1. Materials

Anabaseine (ANA) was prepared according to the method de-

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Published online April 28, 2009

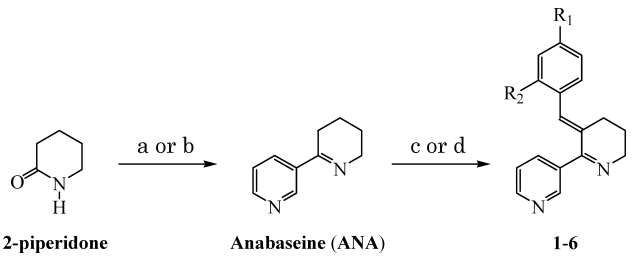

Fig. 2. Synthesis of 3-benzylideneanabaseine analogues.
Reagents and conditions: a) i) 2-piperidone, benzoic anhydride, 180°C, 4 h, ii) NaH, ethyl nicotinate, 110°C, 48 h, then conc. HCl, 130°C, 7 h; b) i) LDA, 2-piperidone, TMSCl, abs.THF/hexane, −78°C, 15 min, then ethyl nicotinate, 20 h, iii) conc. HCl, reflux,1 day; c) benzaldehyde, conc. HCl , abs. EtOH, 60°C; d) benzaldehyde, 0.6 M acetic acid, 0.2 M sodium acetate, abs. MeOH, 60°C

scribed by Späth and Mamoli[9] or Leete[10] (Fig. 2). BA analogues were synthesized by the reaction of ANA with the appropriate benzaldehyde, as previously described.[11] Physical and spectral data (^{1}H NMR spectra and mass spectra) of each compound were in good agreement with those reported in the literature.[4]

(±)-Epibatidine was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Radioligand [^{3}H](±)-epibatidine (1.96 TBq/mmol) was purchased from Amersham Biosciences UK Limited (Little Chalfont, UK). All other materials were of the highest quality commercially available.

2. *Biological Assays*

2.1. *Preparation of membranes from P. americana nerve cords*

Membrane homogenates of *P. americana* nerve cords were prepared as previously described by modification of the method of Orr *et al*.[6,12]

2.2. *Binding assays with [^{3}H]epibatidine*

Binding assays were performed by modification of the method of Orr *et al*.[6,12]

Results and Discussion

On the basis of our previous findings that the introduction of a *para*-hydroxyl group into the benzene ring of BM increased the binding affinity for *Pa*-nAChRs, whereas BM analogues with a *meta*-hydroxyl group or a *meta*-methoxy group showed little affinity,[8] we synthesized BA analogues (**1**–**6**) with electron-donating *ortho*- and/or *para*-substituents on the benzene ring, and examined the affinity for *Pa*-nAChRs. All test compounds inhibited the specific binding of [^{3}H]epibatidine to *P. americana* nerve cord membranes in the nanomolar range. Table 1 lists IC_{50} values of BA and its analogues (**1**–**6**), and Fig. 3 shows the dose-response curves of these compounds.

Of the compounds tested, 3-(2,4-dimethoxybenzylidene)anabaseine (**1**) and 3-(4-methoxy-2-hydroxybenzylidene)anabaseine (**2**) were slightly less potent than unsubstituted BA. Analogues with an *ortho*-methoxy or an *ortho*-hydroxyl group, **3** and **4**, were also slightly less potent than BA. An analogue (**5**) with a *para*-methoxy group showed a further drop in potency compared with these compounds. In contrast, an analogue (**6**) with a *para*-hydroxyl group displayed the highest potency among the analogues synthesized, with an IC_{50} value of 3.6 nM for *Pa*-nAChRs. The potency of **6** was 4- and 86-fold higher than those of the previously reported *para*-hydroxyl counterparts of CBA[7] and BM,[8] respectively.

Table 1. Potencies of 3-benzylideneanabaseine (BA) and BA analogues in the inhibition of [^{3}H]epibatidine binding to *P. americana* nerve cord membranes

Compound	R_1	R_2	IC_{50} (nM)
BA	H	H	35.0 (25.1–48.5)[a]
1 (GTS-21)	OCH_3	OCH_3	41.6 (33.0–52.6)
2	OCH_3	OH	63.5 (49.4–81.6)
3	H	OCH_3	71.1 (55.4–91.3)
4	H	OH	44.2 (38.4–51.0)
5	OCH_3	H	188.3 (157.4–225.3)
6	OH	H	3.6 (3.2–4.2)

[a] Values in parentheses indicate 95% confidence limit.

These results suggest that (*i*) the *para*-hydroxyl group that has a sufficient electron-donating effect increases its negative charge on the imine nitrogen atom by a resonance effect *via* the conjugated double-bond system, which eventually enhances the affinity for *Pa*-nAChRs, (*ii*) the *ortho*-hydroxyl group is not as effective as the *para*-hydroxyl group, and (*iii*) the *para*-methoxy group rather lowers the affinity of BA, although the decrease in affinity is somewhat compensated for by the introduction of an electron-

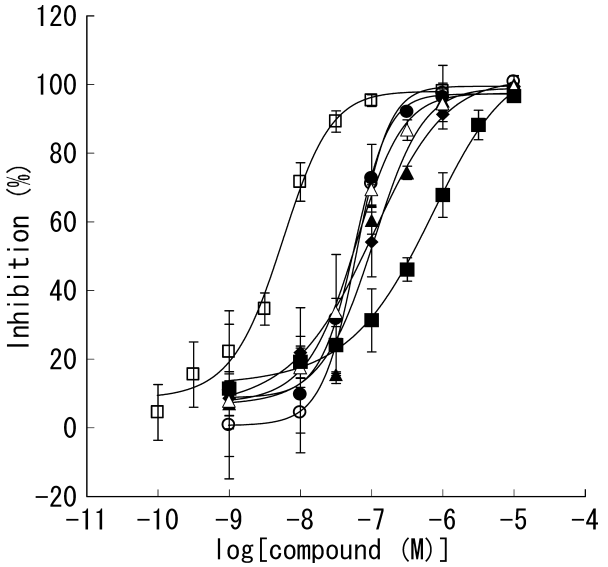

Fig. 3. Concentration-inhibition curves of 3-benzylideneanabaseine (BA) and BA analogues for specific [^{3}H]epibatidine binding to *P. americana* nerve cord membranes. Data are the mean±standard deviation of three experiments, each performed in duplicate, and fitted to the logistic equation. ◆, BA; ●, **1**; ○, **2**; ▲, **3**; △, **4**; ■, **5**; □, **6**.

donating group at the *ortho*-position.

It is difficult to clearly explain the structure-activity relationships of BAs in light of the interactions with *Pa*-nAChRs as the subunit composition of any native insect nAChRs is not known at present; however, this benefits from atomic scale views of the BA binding site on the crystal structures of acetylcholine binding proteins (AChBPs), as well as recognition properties at the ligand binding site.[13–15] AChBP was first discovered as a modulatory protein in the cholinergic synapse of the snail *Lymnaea stagnalis* (*Ls*), and the high resolution X-ray crystal structure of the AChBP was elucidated.[16,17] The discovery and subsequent crystal structure analyses of structural and functional surrogates of the agonist binding domain of nAChRs have been of great help in promoting understanding about the ligand recognition properties of the binding site at atomic resolution,[18,19] and knowledge of the neonicotinoid binding site of insect nAChRs has remarkably advanced.[20–25]

In comparisons between ligand affinities for AChBPs from three molluskan species and those for *Pa*-nAChRs, it was observed that the addition of a hydroxyl group to the *para*-position of BA to give **6** led to 25- and 10-fold increases in affinity for *Ls*-AChBP and *Pa*-nAChRs, respectively, whereas the influence of this substitution was small for *Aplysia californica* (*Ac*)- and *Bulinus truncates* (*Bt*)-AChBP, indicating the similarity of *Pa*-nAChRs to *Ls*-AChBP.[13] *Pa*-nAChRs are also similar to *Ls*-AChBP in terms of the markedly low affinity of **5** compared to **6**; however, *Pa*-nAChRs are similar to *Bt*-AChBP in the effect of the *ortho*-methoxy group. The addition of a methoxy group to the *ortho*-position of the benzene ring of **5** to generate **1** had little effect on the affinity for *Ls*-AChBP, whereas it caused a 3-fold decrease and a 5-fold increase in affinity for *Ac*- and *Bt*-AChBP, respectively.[13] In our assays, **1** showed a 5-fold increase in affinity for *Pa*-nAChRs compared to that of **5**. These observations suggest that the binding site of BAs might structurally resemble *Ls*-AChBP at the subsite that interacts with the *para*-substituent, while it is analogous to *Bt*-AChBP at the subsite that interacts with the *ortho*-substituent.

Compound **6** with a *para*-hydroxyl group on the benzene ring showed the highest affinity for *Pa*-nAChRs. Recent spectroscopic studies of BA-AChBP complexes have indicated that the zwitterion with a protonated imine nitrogen atom and a phenolate oxygen atom is stabilized by a resonance effect through the conjugated double bond in the case of **6**.[13] The zwitterion might contribute to the binding to *Pa*-nAChRs through electrostatic and hydrogen bonding interactions. In contrast, **5** with a *para*-methoxy group in the benzene ring displayed the lowest affinity for *Pa*-nAChRs, although **5** has a methoxy group as an electron-donating group, which should increase the basicity of the imine nitrogen, and the imine nitrogen has the potential of protonation at physiological pH. Hammet σ_p^+ values for the *para*-hydroxyl and the *para*-methoxy groups are estimated to be -0.92 and -0.78, respectively.[26] These σ_p^+ values suggest that *para*-hydroxyl group possesses a larger electron-donating resonance contribution to the imine nitrogen than the *para*-methoxy group. In our previous paper,[7] the low potency of the CBA analogue of **5** was quantitatively explained using a STERIMOL parameter defined as the minimum width of the *para*-substituent; namely, the thinner the *para*-substituent, the higher the affinity. Recent computational docking studies using GTS-21 analogues showed that a BA analogue with a *para*-hydroxyl group and an *ortho*-methoxy group (4-OH-GTS-21) was more stable when docking to a homology model of the α7-nAChR subunit dimer than GTS-21(**1**).[14] In this docking, van der Waals interaction with the binding site is proposed to work preferentially rather than potential hydrogen binding. As 4-OH-GTS-21 has a smaller volume than GTS-21 by demethylation, 4-OH-GTS-21 is subjected to less repulsion. This could provide one explanation for the higher affinity of **6** than that of **5**.

In conclusion, we have shown that one of the synthesized BA analogues, **6**, has nanomolar affinity for *Pa*-nAChRs. This compound might serve as a good lead compound for the development of novel insecticides.

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