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A photoreactive probe that differentiates the binding sites of noncompetitive GABA receptor antagonists

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ABSTRACT

γ-Aminobutyric acid (GABA) receptors are postsynaptic membrane protein complexes that are important not only in the regulation of the nervous system but also as targets of drugs and insecticides. We synthesized a photoreactive straight-chain noncompetitive antagonist (NCA), 2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenyl 4-(4-methoxycarbonyl-1-butynyl)benzoate (NMB), to probe the NCA binding site. Our data show that this probe labels the NCA site and demonstrate that the NCA insecticide fipronil binds at a site distinct from that of other NCAs, such as picrotoxinin and 4′-ethynyl-4-*n*-propylbicycloorthobenzoate. The unique molecule NMB will be useful in identifying the cross-linking site of straight-chain NCAs in GABA receptors and mapping allosteric binding sites. Such studies should provide invaluable information in designing novel NCAs.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system of vertebrates and invertebrates. The receptors mediating its inhibitory effects are pentameric ligand-gated ion channels, and each subunit is a four-transmembrane domain (4-TM) protein. The receptor's integral channels open upon GABA binding and enhance chloride permeability into neurons, thereby suppressing the depolarization induced by excitatory neurotransmitters. GABA receptors are important not only physiologically but also as targets of a variety of anxiolytic/anticonvulsant drugs, insecticides, and toxic substances.[1,2]

Noncompetitive antagonists (NCAs) inhibit the channel function by binding to a site(s) distinct from the orthosteric binding site. The best studied NCA is picrotoxinin (PTX), a sesquiterpene isolated from poisonous *Menispermaceae* plants (Fig. 1A).[3] Since the discovery of PTX as an NCA, many structurally diverse natural and synthetic compounds have been reported to exhibit antagonist activity.[2] Site-directed mutagenesis and homology modeling studies have shown that the pore-lining amino acids at the 2′-, 6′-, and 9′-positions of the second TM are involved in the interaction of the receptor with a wide array of NCAs.[4–9] However, the binding modes of NCAs remain largely unclear, and several lines of evidence suggest the existence of distinct binding sites for different NCAs.[6,10–12] Chemical approaches to clarify the binding mode(s) and site(s) of NCAs are needed to facilitate rational design and development of chemicals targeting these important receptors.

We previously found that the straight-chain compound 5-[4-(3,3-dimethylbutoxycarbonyl)phenyl]-4-pentynoic acid (DBCPP) acts as an NCA at GABA receptors,[13] and we suggested that this unique NCA might serve as a tool to probe the NCA binding site. To show the utility of this class of NCAs, we synthesized a photoreactive analogue of DBCPP and examined its competition with two known antagonists for the NCA binding site of rat GABA receptors.

To examine the reactivity of ligands with the NCA site, competition assays were performed using [^{3}H]4′-ethynyl-4-*n*-propylbicycloorthobenzoate ([^{3}H]EBOB), a high affinity NCA.[14] DBCPP is the most potent competitive inhibitor of [^{3}H]EBOB binding in the

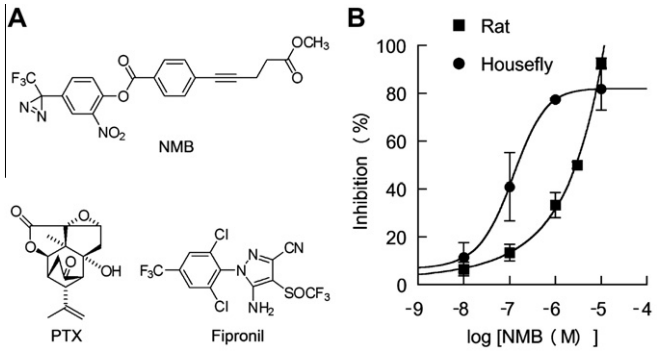

Figure 1. (A) Structures of NMB and two representative NCAs of GABA receptors. (B) Concentration-inhibition curves of NMB on specific [^{3}H]EBOB binding to rat brain and housefly head membranes. Error bars represent SD ($n = 3$).

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class of straight-chain antagonists, with IC_{50} values of 88 nM and 3.41 μM for rat and housefly GABA receptors, respectively.[13] 2-Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenyl 4-(4-methoxycarbonyl-1-butynyl)benzoate (NMB) (Fig. 1A) was synthesized as a carbene-generating photoreactive analogue of DBCPP in 5.3% yield over 8 steps from *p*-bromoanisole (Supplementary data). This analogue inhibited [^{3}H]EBOB binding to rat and housefly GABA receptors in a concentration-dependent manner, with IC_{50} values of 1.49 (1.07–2.08) μM and 0.24 (0.16–0.37) μM, respectively (95% confidence intervals in parentheses) (Fig. 1B). Although the affinity of NMB for housefly GABA receptors was found to be higher than that for rat GABA receptors, membranes from housefly heads were not used in photoaffinity labeling (PAL) experiments due to their liability under the experimental conditions. Rat brain membranes were used as a rich source of GABA receptors.

PAL experiments were conducted as shown in Figure 2. Stirred suspensions containing rat brain P_2 membranes and NMB were irradiated at 365 nm in the absence and presence of PTX or fipronil at 4 °C for 10 h after preincubation for 90 min at 37 °C in a dark room. The optimum photolabeling condition was determined after preliminary experiments performed in terms of UV intensity, irradiation time, incubation temperature, protein concentration, probe concentration, ligand concentration, etc. The photolabeled product was also identified in a model reaction with methanol (data not shown). The preincubation before irradiation was necessary as a process to establish an equilibrium. After washing out membrane-bound ligands, the membranes were examined for the ability to bind [^{3}H]EBOB (Supplementary data).

Figure 3 compares the results of PAL of membranes with NMB in the absence and presence of PTX. Without irradiation, [^{3}H]EBOB specifically bound to rat brain membranes at 82.2% of the control level after incubation with NMB followed by washing. The membranes also specifically bound [^{3}H]EBOB after incubation in the presence of both NMB and PTX, although the levels were slightly lower than those found in the presence of NMB alone. With irradiation, specific binding of [^{3}H]EBOB was significantly reduced after incubation with NMB in the absence of PTX. This result indicates that [^{3}H]EBOB binding was hindered by PAL of the EBOB binding site with NMB. In contrast, [^{3}H]EBOB binding was not significantly reduced when similar experiments were performed in the presence of PTX. This result indicates that noncovalent binding of PTX protected the EBOB binding site from covalent binding with NMB.

Similar PAL experiments were conducted with fipronil, an insecticide that acts as an NCA (Fig. 4). Without irradiation, incubation of membranes in the presence of both NMB and fipronil resulted in slightly reduced levels of [^{3}H]EBOB binding compared with those in the presence of NMB alone, most likely because fipronil was not completely washed away from membranes due to its hydrophobicity. With irradiation, incubation of membranes with NMB resulted in reduced levels of [^{3}H]EBOB binding due to PAL, and, in contrast to the results with PTX, these levels were unchanged in the presence of both NMB and fipronil. This result indicates that fipronil did not protect the EBOB site from access by NMB and that NMB was capable of forming a covalent bond with the EBOB binding site even in the presence of fipronil.

Despite efforts by several groups, the location of the fipronil binding site on GABA receptors remains elusive, whereas numerous reports indicate that PTX interacts with the 2′ and 6′ amino acid residues of the second TM of each subunit.[4,5,15–20] Because GABA receptors with a mutation at the 2′-position were less sensitive to fipronil, fipronil is thought to interact with the 2′ residue of fruit flies (*Drosophila melanogaster*) and whitebacked planthoppers (*Sogatella furcifera*).[21,22] Housefly GABA receptors bearing a mutation at the same position, however, bound fipronil with an affinity similar to that of the wild-type receptor.[23] Furthermore, Le Goff et al. reported that a mutation in the third TM of GABA receptors of fipronil-resistant fruit flies also reduced the sensitivity of GABA receptors to fipronil, suggesting a different mode of binding for fipronil.[11] From the results of experiments using chemically reactive fipronil analogues and cysteine mutants of rat GABA receptors, it was proposed that fipronil interacts not only with the 2′ residue but also with the 17′ residue, both of which are located within the

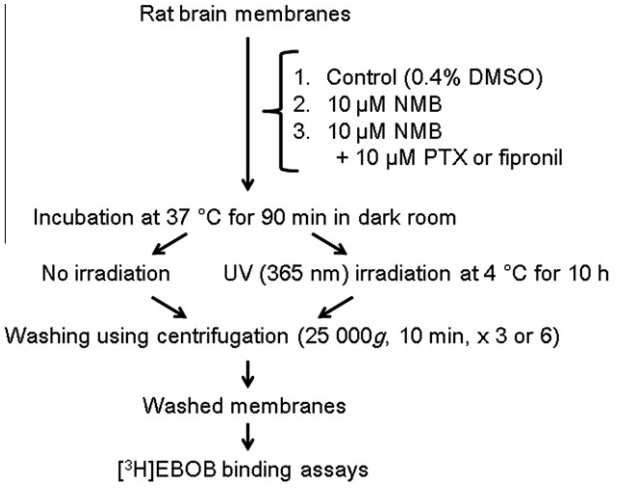

Figure 2. Protocol for photoaffinity labeling with NMB.

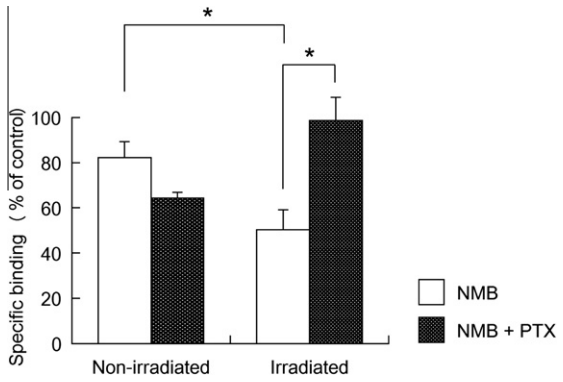

Figure 3. Photoaffinity labeling of rat GABA receptors with 10 μM NMB (6.7 K_i) in the absence and presence of 10 μM PTX (19.6 K_i).[24] Treated membranes were examined for their ability to specifically bind [^{3}H]EBOB. Error bars represent SD (n = 3). *P <0.01 (unpaired t-test).

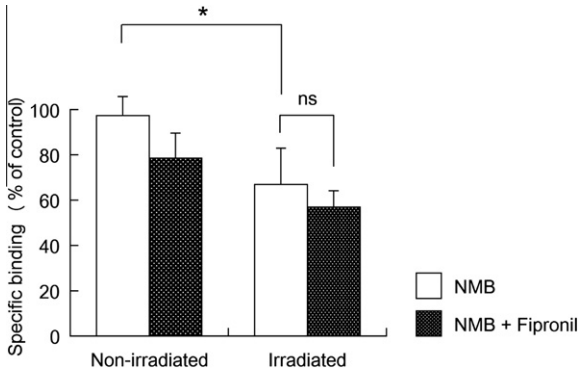

Figure 4. Photoaffinity labeling of rat GABA receptors with 10 μM NMB (6.7 K_i) in the absence and presence of 10 μM fipronil (16.4 K_i).[24] Treated membranes were examined for their ability to specifically bind [^{3}H]EBOB. Error bars represent SD (n = 6). *P <0.01 (unpaired t-test). ns, not significantly different (P >0.05).

channel.[6] The presence of these two binding sites was supported by recent homology modeling and docking studies.[12] Our present data indicate that fipronil has a binding site distinct from that of EBOB and NMB in rat GABA receptors. However, as it is impossible to readily extrapolate the results obtained with rats to insects in view of the differences between insect and vertebrate GABA receptors,[2] similar studies using insect preparations need to be performed when the sites of action of insecticides are considered.

In conclusion, the unique molecule NMB differentiates the binding sites of the insecticide fipronil and other NCAs in rat GABA receptors. This probe will be useful in identifying the cross-linking site of straight-chain NCAs in GABA receptors and mapping allosteric binding sites. Such studies should provide invaluable information for the design of novel NCAs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.118.

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24. The inhibition constants (K_is) were calculated using the K_d of EBOB = 2.45 nM, the IC_{50} of PTX = 0.62 μM, the IC_{50} of fipronil = 0.728 nM, and the concentration of [^{3}H]EBOB = 0.5 nM. For calculations see Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099. For experimental details on binding assays see Supplementary data.