Synthesis of (+)-*cis*-*N*-(4-Isothiocyanatobenzyl)-*N*-normetazocine, an Isothiocyanate Derivative of *N*-Benzylnormetazocine as Acylant Agent for the σ_1 Receptor

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Received October 29, 2001

(+)-*cis*-*N*-(4-Isothiocyanatobenzyl)-*N*-normetazocine (BNIT) (+)-(**4**) was designed and synthesized as a derivative of the potent and selective σ_1 receptor ligand (+)-*cis*-*N*-benzyl-*N*-normetazocine for irreversibly blocking σ_1 binding sites. Pretreatment of guinea pig brain membranes with BNIT (0.1, 1, and 5 μM) caused a concentration-dependent loss of binding of the selective σ_1 ligand [^{3}H]-(+)-pentazocine. Binding experiments with [^{3}H]-1,3-di(2-tolyl)-guanidine ([^{3}H]-DTG), a ligand of σ_1 and σ_2 receptors, showed that pretreatment with BNIT blocked only the σ_1 component of [^{3}H]-DTG binding.

Introduction

In recent years, the σ receptor sites have been extensively investigated and several compounds have been synthesized and tested in radioligand binding assays and in cellular and animal models.[1] However, although this effort has helped clarify numerous biological and pharmacological properties unrelated to other known receptors, some fundamental points remain obscure; examples are the identification of an endogenous ligand and their neural function.

The σ receptors can be divided into at least two subtypes: σ_1 and σ_2.[2−4] On the basis of this classification, (+)-pentazocine and (+)-*N*-allylnormetazocine ((+)-SK&F 10,047) are classified as specific σ_1 receptor ligands, and 1,3-di(2-tolyl)guanidine (DTG), a nonbenzomorphan-type σ receptor ligand, has equally high affinity for the σ_1 and σ_2 receptors. Thus, each receptor can be identified solely by ligand binding experiments using [^{3}H]-(+)-pentazocine or [^{3}H]-DTG with (+)-pentazocine to mask the labeling of σ_1 receptors.

σ_1 sites have been cloned in various tissues of guinea pig, mouse, rat, and human brain, but their structures are unlike those of other known mammalian proteins.[5−7] The σ_1 protein has homology with $\Delta^{8,7}$-isomerase, an enzyme of fungal sterol biosynthesis. This suggests a role in sterol metabolism, and since progesterone showed moderate affinity for these receptors, it has been assumed that certain neurosteroids exert modulatory effects through the σ receptors.[8]

Interest in the σ receptors is aroused by their involvement in psychosis, neuroprotection, motor disorders, cocaine abuse, learning processes, and tumors.[9] σ radioligands might therefore be used for positron emission tomography (PET) in cancer diagnosis, since several human tumoral cell lines overexpress σ receptors.[10]

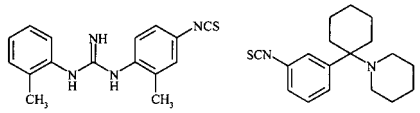

Figure 1. Isothiocyanate probes for σ receptor.

The design and synthesis of selective irreversible ligands have proved very useful in the isolation, purification, and characterization of many receptor systems. Portoghese et al.[11] postulated that an irreversible ligand first recognizes a specific binding site and then forms a covalent bond on or near that recognition site, resulting in irreversible attachment. These compounds are generally derivatives of selected ligands that may be chemically modified to retain high receptor affinity and selectivity while permitting covalent binding to the receptor protein.

σ receptors have been examined with isothiocyanate probes. Isothiocyanate derivatives were synthesized starting from DTG (DIGIT) and phencyclidine (PCP) (methaphit) (Figure 1). DIGIT inhibits the specific binding of ^{3}H-labeled ligands such as [^{3}H]-DTG and [^{3}H]-(+)-3-PPP. This effect is selective for the σ site because binding of ligands for PCP, dopamine D_2, benzodiazepine, and μ-opioid receptors is unaffected. However there are no data for benzomorphan ligands.[12] Metaphit had different effects on the binding of various radiolabeled σ ligands.[13] The order of sensitivity to metaphit was [^{3}H]-DTG > [^{3}H]-(+)-3PPP ≫ [^{3}H]-(+)-SK&F 10,047, half-maximal loss of binding occurring at 2, 10, and 50 μM, respectively. This order of sensitivity seems to reflect the different modes of interaction of benzomorphan and non-benzomorphan ligands with σ receptors. SAR studies reported by Carroll et al. showed that certain substitutions can be made at the para position of the aromatic ring of the *N*-benzyl substituent of the potent, selective σ_1 ligand (+)-cis-*N*-benzyl-*N*-normetazocine without any appreciable loss of

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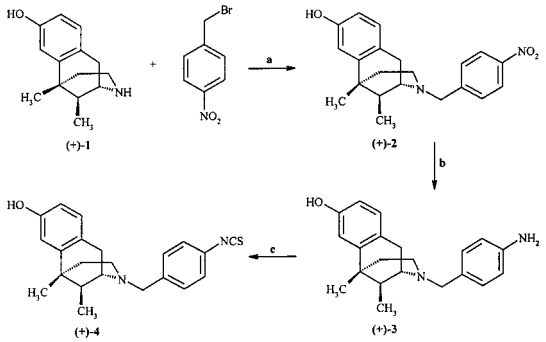
10.1021/jm010501a CCC: $22.00
Published on Web 05/02/2002

Scheme 1[a]



[a] (a) DMF, $NaHCO_3$; (b) $SnCl_2$, EtOH/EtOAc; (c) 2-pyridyl-thiocarbonate, CH_2Cl_2.

affinity.[14,15] These results indicated that the relatively small substituents retain nanomolar σ_1 binding affinity.

Here, we describe the synthesis and receptor binding profile of (+)-cis-*N*-(4-isothiocyanatobenzyl)-*N*-normetazocine (+)-(**4**), (BNIT), a selective σ_1 acylant.

Experimental Section

Chemistry. Alkylation of (+)-(2*S*,6*S*,11*S*)-6,11-Dimethyl-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol (+)-**1** with 1-(bromomethyl)-4-nitrobenzene in DMF using $NaHCO_3$ as the proton acceptor gave (+)-**2** (Scheme 1). The (2*S*,6*S*,11*S*)-3-(4-aminobenzyl)-6,11-dimethyl-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol (+)-**3** was prepared by reduction of the *N*-4-nitrobenzyl derivative (+)-**2** with $SnCl_2$ dihydrate in ethanol/EtOAc (7:3). Compound (+)-**3** was converted to (+)-(2*S*,6*S*,11*S*)-3-(4-isothiocyanatobenzyl)-6,11-dimethyl-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol (+)-**4** (BNIT) by reaction with 2-pyridyl-thio-carbonate in dichloromethane: mp 85 °C dec; NMR ($CDCl_3$) δ 0.80 (d, J = 7.2 Hz, 3H), 1.20−1.26 (m, 2H), 1.30 (s, 3H), 1.67−2.00 (m, 2H), 2.02−2.20 (m, 1H), 2.34−2.48 (m, 1H), 2.56−2.74 (m, 2H), 2.80−3.04 (m, 2H), 3.57 (d, J = 13.8 Hz, 1H), 3.70 (d, J = 13.8 Hz, 1H), 6.57−7.36 (m, 7H); FTIR (KBr) 2101 (N=C=S) cm^{-1}. Anal. ($C_{22}H_{24}N_2OS$) C, H, N.

Pharmacology. Materials and Methods. Membranes from guinea pig brain were prepared according to Mach et al.[16] For σ_1 binding assay, membranes (1 mg/mL) were incubated in Tris-HCl, pH 7.4, with different concentrations of BNIT (0.1, 1, and 5 μM) for 10 min at 4 °C, as reported by Bluth et al.[13] The suspension was centrifuged (18 000 rpm, 6 min), the supernatant was discarded, and the pellet was resuspended in the same volume of buffer. This procedure was repeated twice. After resuspension in the original buffer volume, membranes were incubated for 60 min at 25 °C in order to dissociate any noncovalently bound ligand. The suspension was centrifuged at 18 000 rpm, the pellet was resuspended in half the original volume, and the σ_1 binding assay was done. For σ_2 binding assay, membranes (720 μg/mL) were incubated in Tris-HCl, pH 8, and treated as above.

For σ_1 saturation binding assays, guinea pig brain membranes (500 μg of protein/assay tube) were incubated for 90 min at 25 °C in 1 mL of incubation buffer (50 mM Tris-HCl, pH 7.4 at 37 °C) containing one of six concentrations of [^{3}H]-(+)-pentazocine (1−6 nM; specific activity (sa), 28 Ci/mmol). Nonspecific binding was defined in the presence of 10 μM haloperidol and accounted at most for ∼20% of the total radioactivity retained in the filters. For σ_2 saturation studies, guinea pig brain membranes (360 μg of protein/assay tube) were incubated for 90 min at 25 °C in 0.5 mL of incubation buffer (50 mM Tris-HCl, pH 8) containing one of six concentrations of [^{3}H]-DTG (1−6 nM; sa 31 Ci/mmol) and (+)-pentazocine (200 nM) to mask σ_1 sites.[4] Nonspecific binding was defined in the presence of 5 μM DTG and accounted at most for ∼20% of the total radioactivity retained in the filters.

Table 1. Effect of BNIT on Binding Parameters (K_d and B_{max}) of [^{3}H]-(+)-Pentazocine to σ_1 Sites in Guinea Pig Brain Membranes[a]

treatment	K_d (nM)	B_{max} (fmol/mg protein)
control	5.2 ± 0.3	987 ± 78.5
0.1 μM BNIT	34.5 ± 18.6*	601 ± 139.8*
1 μM BNIT	nd	41.3 ± 11.3*
5 μM BNIT	nd	nd

[a] Scatchard analysis was carried out in guinea pig brain membranes pretreated with BNIT. Control refers to membranes not exposed to BNIT, as described in Materials and Methods. Values are the mean ± SEM of three experiments, each carried out in triplicate. nd = not detectable (no accurate K_d could be calculated). *represents $P < 0.01$ vs control (Dunnet's test after ANOVA).

All experiments were run in triplicate, and at least three independent experiments were done. Incubations were terminated by rapid filtration through glass-fiber filters (Schleicher & Schuell, Dassel, Germany) presoaked in 0.5% polyethylenimine for at least 60 min before use. Radioactivity retained on the filters was measured by liquid scintillation spectometry using a 1414 Winspectral Perkin-Elmer Wallac (after overnight incubation in 4 mL of Filter Count Cocktail (Packard)) with a counting efficiency of 60%. The apparent dissociation constants (K_d), and the maximum number of binding sites (B_{max}) were calculated using the LIGAND or EBDA programs (Elsevier-BIOSOFT, Cambridge, U.K.).

Data were fit to both a one-site and two-site model using LIGAND program in order to determine which gave the best fit. In all experiments, the best fits were determined by a run test showing a nonserial correlation ($P < 0.05$) plus the presence of a significant F test ($P < 0.05$) between the more complex and the next simplest fit.

Data are expressed as mean ± SEM. Statistical comparisons were made by ANOVA for repeated measures and the post hoc Dunnett's multiple comparison test, with differences of P less than 0.05 being considered significant (GraphPAD Software, San Diego, CA).

Results and Discussion

We first investigated the effect of BNIT on [^{3}H]-(+)-pentazocine binding in guinea pig brain membranes exposed to various concentrations of BNIT for 10 min at 4 °C. This was followed by extensive washing, as described under Materials and Methods, and determination of the saturation binding of [^{3}H]-(+)-pentazocine. These values were compared to controls not exposed to BNIT. After 90 min of incubation at 25 °C, [^{3}H]-(+)-pentazocine bound saturably to a single site with a K_d of 5.2 ± 0.3 nM and a B_{max} of 987 ± 78.5 fmol/mg protein. BNIT (0.1, 1, and 5 μM) caused a concentration-dependent loss of binding of the radioligand. Scatchard analysis indicated that the maximum binding capacity (B_{max}) of [^{3}H]-(+)-pentazocine was ∼40% lower in membranes exposed to 0.1 μM BNIT and 96% lower in those exposed to 1 μM BNIT (Table 1). The K_d value was significantly higher in membranes pretreated with 0.1 μM BNIT (Table 1). After pretreatment with 1 μM BNIT, there was extensive loss of receptor binding labeled by [^{3}H]-(+)-pentazocine such that we could not calculate the K_d.

To determine the selectivity of inhibition by BNIT at σ_1 binding sites, saturation binding assays were carried out in the presence of [^{3}H]-DTG. In agreement with previous studies,[3,4] [^{3}H]-DTG binding was best described by a two-site model. Conceivably, the two sites may correspond to σ_1 and σ_2 binding sites. The binding parameters were K_d = 7.5 and 15.6 nM for σ_1 and σ_2,

Table 2. K_d (nM) and B_{max} (fmol/mg protein) of [^{3}H]-(+)DTG Binding to Guinea Pig Brain Membranes Exposed or Not Exposed (Control) to BNIT As Described in Materials and Methods[a]

treatment	K_d	K_{d1} (σ_1)	K_{d2} (σ_2)	B_{max}	B_{max1}(σ_1)	B_{max2}(σ_2)
control		7.5 ± 1.4[b]	15.6 ± 3.5[b]		987 ± 78.5[b]	1280 ± 189.4[b]
0.1 μM BNIT		12.8 ± 1.6*[b]	21.4 ± 7.8[b]		674 ± 35.8**[b]	1330 ± 210.5[b]
1 μM BNIT	13.6 ± 0.3[c]			1013 ± 148.4[c]		
5 μM BNIT	11.6 ± 0.5[c]			957 ± 58.9[c]		
(+)-pentazocine (200 nM)	11.6 ± 2.8[c]			1120 ± 98.5[c]		

[a] Values are the mean ± SEM of three experiments, each carried out in triplicate. *represents $P < 0.01$. **represents $P < 0.05$ vs control (Dunnet's test after ANOVA). [b] Two-site fit model was better than one-site fit. [c] One-site fit model was better than two-site fit.

respectively; the corresponding B_{max} values were 987 and 1280 fmol/mg protein ($n = 3$) (Table 2). The lower concentration of BNIT (0.1 μM) caused a significant elevation of K_d and a reduction of the B_{max} values limited to the σ_1 component of the [^{3}H]-DTG binding to guinea pig brain membranes, whereas binding to σ_2 sites was not modified (Table 2). The higher concentrations of BNIT (1 and 5 μM) abolished [^{3}H]-DTG binding to σ_1 sites; under these conditions, [^{3}H]-DTG bound to a single site (Table 2). BNIT 1 and 5 μM reduced the maximum binding capacity of [^{3}H]-DTG by approximately 45%, with no concentration-dependent effect, whereas the K_d values related to σ_2 binding sites of [^{3}H]-DTG were not modified by pretreatment with the higher concentrations of this compound (Table 2). Exposure of guinea pig brain membranes to BNIT irreversibly blocked σ_1 binding sites, whereas σ_2 sites were not sensitive to this compound, since it blocked only the σ_1 component of [^{3}H]-DTG binding. This is further supported by saturation binding experiments of [^{3}H]-DTG on guinea pig brain membranes in the presence of (+)-pentazocine (200 nM). This latter is a selective σ_1 ligand[17] that blocks only σ_1 sites and under these conditions; thus, [^{3}H]-DTG interacts only with σ_2 sites. In these experiments, the B_{max} of [^{3}H]-DTG was reduced to 1120 ± 98.5 fmol/mg protein, similar to that in [^{3}H]-DTG binding assays on guinea pig membranes pretreated with BNIT (Table 2). K_d values of [^{3}H]-DTG were not modified by exposure to BNIT or the presence of (+)-pentazocine (Table 2).

This study indicates that BNIT promises to be a useful pharmacological tool for irreversibly blocking σ_1 binding sites. This binding activity is not recoverable even after extensive washing of guinea pig membranes exposed to BNIT. However, BNIT did not cause any loss of σ_2 receptors. These findings add more evidence for the existence of physically separate σ_1 and σ_2 binding sites. Interestingly, at the lowest concentration employed (0.1 μM), BNIT drastically reduced the B_{max} of [^{3}H]-(+)-pentazocine and the σ_1 component of [^{3}H]-DTG binding to guinea pig brain membranes; moreover, it lowered the affinity of these radioligands for σ_1 sites.

BNIT may interact differently with these sites. First, it may irreversibly bind to the protein receptor, and second, it may affect the binding of [^{3}H]-(+)-pentazocine to the residual σ_1 sites. Bluth et al.[13] reported that pretreatment of guinea pig brain membranes with 1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine (metaphit) caused irreversible loss of PCP receptors labeled by [^{3}H]-TCP while decreasing the affinity of [^{3}H]-DTG to σ sites. They suggested that metaphit may induce acylation of PCP receptors. It is not able to acylate the σ sites, but it may simply partition in the lipid environment, producing high local concentrations of ligand, or it may acylate a nucleophile at a site adjacent to the σ_1 receptor. Adams et al.[12] found that di-*o*-tolylguanidine isothiocyanate (DIGIT) was an irreversible inhibitor at σ receptors. They suggested that DIGIT may acylate or form covalent bonds with amino groups of the σ binding protein. However, it could not discriminate between σ_1 and σ_2 receptors. We propose BNIT as a novel selective agent capable of irreversibly blocking only the σ_1 binding sites. Further research efforts are necessary to decide whether this compound acylates or forms covalent bonds with the σ_1 receptor or with any adjacent protein(s).

References

(1) Itzhak, Y., Ed. *Sigma receptors*; Academic Press: San Diego, CA, 1994.

(2) Hellewel, S. B.; Bowen, W. D. A Sigma-Like Binding-Site in Rat Pheochromocytoma (PC12) Cells: Decreased Affinity for (+)-Benzomorphans and Lower Molecular Weight Suggest a Different Sigma Receptor Form from That of Guinea Pig Brain. *Brain Res.* **1990**, *527*, 244−253.

(3) Walker, J. M.; Bowen, W. D.; Walker, F. O.; Matsumoto, R. R.; De Costa, B.; Rice, K. C. Sigma Receptor: Biology and Functions. *Pharmacol. Rev.* **1990**, *42*, 355−402.

(4) Quiron, R.; Bowen, W. D.; Itzhak, Y.; Junien, J. L.; Musacchio, J. M.; Rothman, R. B.; Su, T.-P.; Tam, S. W.; Taylor, D. P. A Proposal for the Classification of Sigma-Sites. *Trends Pharmacol. Sci.* **1992**, *13*, 85−86.

(5) Hanner, M.; Moebius, F. F.; Flandorfer, A.; Kanus, H.-G.; Streisseng, J.; Kempner, E.; Glossmann, H. Purification, Molecular Cloning and Expression of the Mammalian σ_1-Binding Site. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8072−8077.

(6) Kekuda, R.; Prasad, P. D.; Fei, Y. J.; Leibach, F. H.; Ganapathy, V. Cloning and Functional Expression of the Human Type 1 Sigma Receptor (hSigmaR1). *Biochem. Biophys. Res. Commun.* **1996**, *229*, 553−558.

(7) Mei, J.; Pasternak, G. W. Molecular Cloning and Pharmacological Characterization of the Rat Sigma$_1$ Receptor. *Biochem. Pharmacol.* **2001**, *62*, 349−355.

(8) Moebius, F. F.; Reiter, R. J.; Hanner, M.; Glossmann, H. High Affinity of σ_1-Binding Site for Sterol Isomerization Inhibitors: Evidence for a Pharmacological Relationship with the Yeast Sterol C_8−C_7 Isomerase. *Br. J. Pharmacol.* **1997**, *121*, 1−6.

(9) Bowen, W. D. Sigma Receptors: Recent Advances and New Clinical Potentials. *Pharm. Acta Helv.* **2000**, *74*, 211−218.

(10) Wheeler, K. T.; Wang, L. M.; Wallen, C. A.; Childers, S. R.; Cline, J. M.; Keng, P. C.; Mach, R. H. Sigma-2 Receptors as a Biomarker of Proliferation in Solid Tumours. *Br. J. Cancer* **2000**, *82* (6), 1223−1232.

(11) Portoghese, P. S.; Takemori, A. E. Affinity labels as probes for opioid receptor types and subtypes. In *Opioid Peptides: Medicinal Chemistry*; Rapaka, R. S., Barnett, G., Hawks, R. L., Eds.; National Institute on Drug Abuse Research Monograph 69. DHHS Publication No. (ADM)87-1454; National Institute on Drug Abuse: Washington, DC, 1986; pp 157−168.

(12) Adams, J. T.; Teal, P. M.; Sonders, M. S.; Tester, B.; Esherick, J. S.; Scherz, M. W.; Keana, J. F. W.; Weber, E. Synthesis and Characterization of an Affinity Label for Brain Receptors to Psychotomimetic Benzomorphans: Differentiation of σ-Type and Phencyclidine Receptors. *Eur. J. Pharmacol.* **1987**, *142*, 61−71.

(13) Bluth, L. S.; Rice, K. C.; Jacobson, A. E.; Bowen, W. D. Acylation of σ Receptors by Metaphit, an Isothiocyanate Derivate of Phencyclidine. *Eur. J. Pharmacol.* **1989**, *161*, 273−277.

(14) Mascarella, W. S.; Bai, X.; Williams, W.; Sine, B.; Bowen, W. D.; Carrol, F. I. (+)-*cis*-*N*-(Para-, meta-, and ortho-substituted benzyl)-*N*-normetazocines: Synthesis and Binding Affinity at the [^{3}H]-(+)-Pentazocine-Labeled (σ_1) Site and Quantitative Structure−Affinity Relationship Studies. *J. Med. Chem.* **1995**, *38*, 565−569.

(15) Danso-Danquah, R.; Bai, X.; Zhang, X.; Mascarella, S. W.; Williams, W.; Bethel, S.; Bowen, W. D.; Carroll, I. F. Synthesis and σ Binding Properties of 1′- and 3′-Halo- and 1′,3′-Dihalo-*N*-normetazocine Analogues. *J. Med. Chem.* **1995**, *38*, 2986−2989.
(16) Mach, R. H.; Smith, C. R.; Childers, S. R. Ibogaine Possesses a Selective Affinity for Sigma 2 Receptors. *Life Sci.* **1995**, *57* (4), 57−62.
(17) De Haven-Hudkins, D. L.; Fleissner, L. C.; Ford-Rice, F. Y. Characterization of the Binding of [^{3}H](+)-pentazocine to σ Recognition Sites in Guinea Pig Brain. *Eur. J. Pharmacol.* **1992**, *227*, 371−378.

JM010501A