

A specific acylating agent for the [^3H]phencyclidine receptors in rat brain

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A derivative of phencyclidine (PCP, 1 in fig. 1) bearing an isothiocyanate moiety on the *meta* position of the aromatic ring (Metaphit, 3 in fig. 1) has been synthesized and identified as a rapid and specific site-directed acylating agent of the [^3H]phencyclidine binding site in rat brain homogenates. The percentage of sites irreversibly inactivated by Metaphit was found to be the same in the hippocampus and striatum and the remaining sites were unaffected by Metaphit treatment under any conditions, suggesting that at least two distinct binding sites are present. An isomeric isothiocyanate derivative did not irreversibly inhibit [^3H]phencyclidine receptors, indicating structural specificity for Metaphit in the inhibition of these receptors. The availability of Metaphit should greatly facilitate study of the structure and function of the phencyclidine receptors.

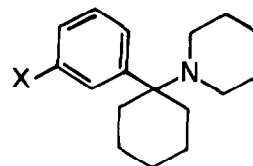
<i>Phencyclidine (PCP)</i>	<i>Metaphit</i>	<i>1-[1-(3-Isothiocyanatophenyl)cyclohexyl]piperidine</i>
<i>Phencyclidine receptor affinity ligand</i>	<i>Phencyclidine receptor</i>	<i>Irreversible inhibitor</i>

1. INTRODUCTION

The illicit use of phencyclidine (PCP, 1 in fig. 1) is a major problem in the USA that has rivaled the abuse of heroin and cocaine. The bizarre dissociative behavioral effects produced in humans by PCP range from schizophrenia-like states to violently aggressive behavior and self-mutilation. These effects have stimulated interest in the biochemical pharmacology of PCP and recent studies have described stereoselective, saturable binding sites in rat brain [1–3] and other species [4]. Despite some initial controversy concerning the pharmacological relevance of the site, a high degree of correlation between binding affinity and in vivo activity of several PCP-mimetics support the contention that the site is indeed a pharmacological receptor ([1,2] and unpublished). The recent finding of an endogenous peptide with a binding affinity much greater than PCP for this

site [5] raises questions concerning the physiological function of these sites in the CNS, and their possible involvement in neuropsychiatric states.

Recent efforts in our laboratory have resulted in the synthesis of compounds which specifically acylate mu and delta opioid receptor subtypes [6–9]. In view of the proposed relation between the



- 1 X = H
- 2 X = NH₂
- 3 X = NCS

Fig. 1. Structure of PCP (1), *m*-amino-PCP (2), and Metaphit (3).

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[^3H]PCP binding site and the putative sigma opiate receptor [10,11], we have synthesized PCP derivatives bearing acylating functional groups in an attempt to develop an affinity label for this recognition site which could prove to be of value in probing its role in the CNS, and possibly aid in isolation and characterization of the binding site proteins. We now report synthesis and biochemical characterization of the first electrophilic acylating PCP derivative 1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine, which we have termed 'Metaphit' (3 in fig.1).

2. MATERIALS AND METHODS

2.1. Synthesis

Metaphit (3) was prepared from the known [12] PCP analogue 2 (in fig.1) using standard procedures [9,13] and was fully characterized by spectral data and combustion analysis. The hydrochloride salt was prepared in isopropanol and recrystallized from methanol-ether, m.p. 214–216°C.

2.2. Receptor binding assays

Binding experiments were conducted with adult male Sprague-Dawley rats (Taconic Farms, Germantown, NY), which were decapitated and the brains quickly removed and dissected on ice. Hippocampus and striatum were separated and immediately homogenized in 70 vols ice cold Tris-HCl (5 mM, pH 7.4), or quick-frozen on dry ice for later use. For homogenization, a Brinkman Polytron (setting 6) was employed for a period of 20 s. The homogenates were centrifuged at $20000 \times g$ for 20 min at 5°C, and the pellet resuspended in fresh buffer; this washing procedure was repeated a total of three times. The final washed pellet was suspended in 35 vols fresh buffer and kept on ice prior to the experiment. Homogenates were treated with 1–10 μM Metaphit at 0°C for varying time periods, followed by three additional consecutive washings to remove unreacted Metaphit. The binding assay procedure was adapted from Zukin et al. [14]. Incubations were performed in a total volume of 1 ml consisting of 0.9 ml tissue homogenate (containing 0.7–1.0 mg protein), 0.05 ml of 8 nM [^3H]PCP (48 Ci/mmol, New England Nuclear), and 0.05 ml of buffer or

PCP (final concentration 10 μM for nonspecific binding). The total number of counts bound with 8 nM [^3H]PCP was about 12000 cpm and the number of nonspecific counts was about 3000 cpm. The mixture was incubated for 60 min at 5°C, and filtered through Whatman GF-B filters that had been soaked in 0.03% poly-L-lysine (Sigma, M_r 150000–300000) for 2 h at 5°C. Filtrations were performed on a Brandell Cell Harvester. The filters were washed with two 5-ml aliquots of ice-cold fresh buffer, and transferred to counting vials. Hydrofluor scintillation cocktail (National

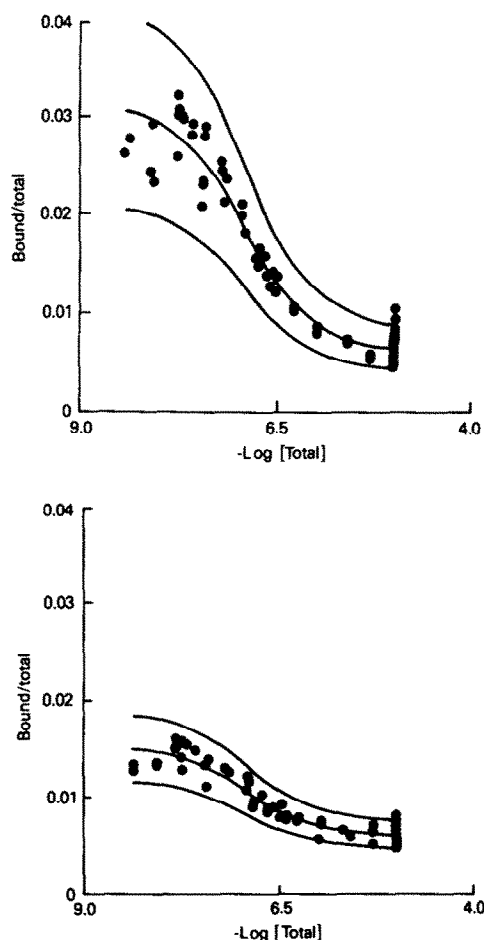


Fig.2. Effect of Metaphit treatment on [^3H]PCP binding to rat brain homogenates. Results shown are composite plots of 5 separate experiments. (A) Displacement curve for PCP using control (washed, untreated) tissue. (B) Displacement curve on a portion of the same tissue which had been pretreated with 10 μM Metaphit.

Diagnostics) was added, and the vials allowed to stand overnight prior to counting. All assays were performed in triplicate. Data from these experiments were analyzed using the LIGAND program [15]. Binding methodologies for other receptors were taken from the literature [16–19].

3. RESULTS

Comparison of displacement curves generated with untreated control tissue (fig.2A) and tissue treated with 10 μ M Metaphit (fig.2B) revealed a significant loss of binding capacity in the treated tissue. Scatchard plots of the data (fig.3) clearly show that Metaphit treatment results in a significant loss of sites without altering the affinity of the remaining sites (K_d for control, 1.14×10^{-7} M, for treated 1.17×10^{-7} M; B_{max} for control, 2.96 pmol/mg protein, for treated, 1.09 pmol/mg protein). In control experiments, substitution of Metaphit with PCP at various concentrations (10–50 μ M) did not result in a significant loss of tissue binding, indicating that the washing procedure used was sufficient to remove any unbound ligand from the tissue. The percentage of binding sites which were acylated by Metaphit treatment did not differ in the hippocampus and striatum. Tissue homogenates prepared from rat hippocam-

pus showed 45% acylation after exposure to 10 μ M Metaphit, and 50% of the sites in homogenates from striatum were Metaphit sensitive (table 1). In all cases a significant component of control [3 H]PCP binding sites remained intact following treatment with Metaphit, and no conditions were found for complete acylation of these remaining sites. These results suggest that the [3 H]PCP binding site population consists of at least two subtypes which can be differentiated on the basis of their sensitivity to Metaphit. Variation of treatment time revealed that a 5 min exposure was sufficient to label all susceptible sites, and longer treatments of up to 2 h did not result in any additional loss of binding (fig.4). Interestingly, the Metaphit isomer 1-(1-phenyl)cyclohexyl-4-isothiocyanatopiperidine (with an isothiocyanate function on the piperidine ring rather than on the aromatic ring) did not irreversibly inhibit [3 H]phencyclidine receptors under the usual conditions, indicating Metaphit structural specificity for the acylation of these receptors. The binding affinity of this Metaphit

Table 1

Ability of 10 μ M Metaphit to reversibly and irreversibly antagonize binding of various brain receptor ligands

Ligand	Maximum % acylation by 10 μ M Metaphit	IC_{50} (μ M) for Metaphit	IC_{50} (μ M) for PCP
[3 H]PCP			
Hippocampus	45 ^b	–	0.075
Striatum	50 ^b	–	0.070
[3 H]QNB	0	19	25
[3 H]Dihydro- morphine	0	11	13 ^a
[3 H]Diazepam	0	>1000	>200

^a Obtained from [18]

^b These average numbers are not statistically different

The ligand concentrations used were: 8 nM [3 H]phencyclidine, 0.5 nM [3 H]QNB, 1 nM [3 H]dihydromorphine, and 5 nM [3 H]diazepam. The binding methodology for PCP [14] was modified (figs 2A,B,3,4), the others were from the literature [16–19]. Acylation by Metaphit refers to the % of acylation in washed, treated tissue compared with similarly washed controls. IC_{50} values for Metaphit were estimated from competitive displacement curves

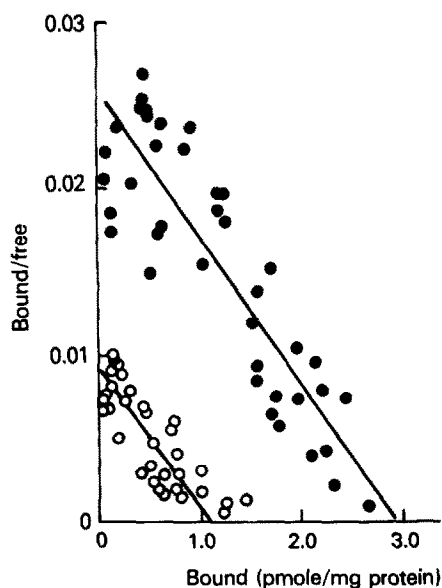


Fig.3. Scatchard plots of the data shown in fig.2A,B. Control, ●; treated, ○.

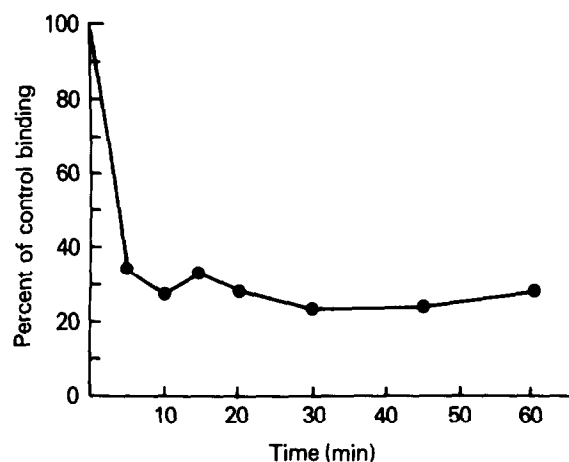


Fig.4. Time course of Metaphit acylation, plotting % of control sites remaining after $10 \mu\text{M}$ Metaphit pretreatment vs pretreatment time.

isomer was between two and three orders of magnitude less than PCP.

4. DISCUSSION

The extreme reactivity of Metaphit toward a subpopulation of sites was demonstrated by the requirement for high concentrations of added PCP to protect these sites. Only when the tissue homogenate (pooled striatum and hippocampus) was preincubated with $50 \mu\text{M}$ cold PCP prior to a brief (10 min) exposure to Metaphit ($10 \mu\text{M}$, 5°C) was an attenuation observed in the number of sites acylated (25% decrease in sites, compared with a 49% loss when PCP was not included as a pretreatment). The results suggest that susceptible binding sites may contain a suitably located lysine or cysteine residue, since isothiocyanates are particularly reactive toward amine and sulfhydryl nucleophiles [9,20]. The reactivity of the isothiocyanate moiety was also a concern with respect to solution stability, since the Tris-HCl buffer used contains a primary amino group. The stability of Metaphit in Tris buffer was established experimentally, as was its rapid reaction with methylamine under the same conditions. The UV spectrum of Metaphit taken in methanol or water showed absorption maxima at 269 and 278 nm. Comparison of UV spectra taken at zero time and after 2 h at room temperature, of

a $10 \mu\text{M}$ solution of Metaphit in 5 mM Tris-HCl (pH 7.4) revealed no change in the spectrum, and only small changes were observed after 16 h at room temperature, which could be accounted for by approx. 15% hydrolysis in that time. The failure to observe reactivity between the primary amino group in Tris and Metaphit is probably due to the steric hindrance of the amino group in Tris and hydrogen bonding of this function to the hydroxyl groups. Addition of excess methylamine to a Metaphit solution resulted in the rapid disappearance of these bands, coincident with the appearance of a new absorption at 241 nm. This latter band was attributed to the thiourea derivative of Metaphit produced by reaction with the methylamine. Thus, any reaction with the Tris component of the buffer would be negligible in the brief tissue exposure time employed.

The acylating specificity of Metaphit for the [^3H]PCP site was determined because PCP and its congeners have been reported to interact at cholinergic receptors [18] and with opioid receptors, and possess analgesic activity [19]. Metaphit was found to displace [^3H]QNB (quinuclidinyl benzilate) from muscarinic receptors in rat brain homogenates with a potency equivalent to PCP, without a significant loss of QNB binding compared with control tissues (table 1). Likewise, Metaphit competitively inhibited [^3H]dihydromorphine binding, but no irreversible blockade of opioid receptors could be observed. Metaphit was inactive in all respects toward the [^3H]diazepam site. These data, coupled with the insensitivity of a portion of the [^3H]PCP binding sites, indicate that Metaphit is highly specific as a covalent labelling reagent.

In summary, the PCP congener Metaphit has been found to covalently modify a significant portion of the [^3H]PCP binding sites found in rat brain. The insensitivity of some of these sites to acylation leaves open the possibility that the binding site pool is comprised of at least two different sites, perhaps with different pharmacological roles. Biochemical and pharmacological studies addressing this question and others concerning the structure and function of phencyclidine recognition sites are in progress using Metaphit and congeners. Metaphit has been noted to antagonize PCP receptor binding and behavioral actions in the rat after icv introduction (unpublished).

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