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Synthesis of Some Phenothiazine Derivatives as Potential Affinity Ligands for the Central Dopamine Receptors

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Abstract—Syntheses of several phenothiazine ligands as potential affinity probes for the D_1- and D_2-dopamine receptors derived from 4-(3-(10-(2-trifluoromethyl) phenothiazinyl) propyl)-1-(2-aminoethyl)-piperazine hydrochloride are described and their interactions with D_1- and D_2-dopamine receptors of the bovine caudate nucleus have been characterized. The bromoacetylamido-, maleinimido-, and isothiocyanato-derivatives expressed low selectivity and moderate affinity for both categories of dopamine receptors. 4-(3-(10-(2-Trifluoromethyl) phenothiazinyl)propyl)-1-(2-(isothiocyanatobenzoyl)ethyl)-piperazine hydrochloride did not discriminate among the two subclasses of the dopamine receptors, but showed an extremely strong irreversible binding to the D_1-receptors and thus is promising as a highly specific affinity ligand for biochemical and pharmacological studies of the D_1-dopamine receptors.

Two categories of the dopamine (DA) receptors, termed D_1 and D_2 have been distinguished on the basis of biochemical and pharmacological criteria (reviewed by Seeman 1980; Stoof & Kebabian 1984; Seeman & Grigoriadis 1987). These receptor subtypes have been pharmacologically differentiated mainly on the basis of their different affinities for various ligands (Creese et al 1983). Activation and/or inactivation of the D_1- and D_2-receptors results in different biochemical responses thus demonstrating their different functions within the central nervous system (Stoof et al 1986). At present, little is known about structural features essential for these differences. An approach that yields a wealth of information in relation to the structure of minor membrane constituents such as hormone neurotransmitter receptors including DA receptors is the use of affinity and photoaffinity labelling techniques (Bayley 1983; Bayley & Staros 1984). During the last decade, several DA agonists (Neymeyer et al 1980; Hall et al 1987) and antagonists (Schuster et al 1982; Amlaiky & Caron 1985; Wregget 1987) which irreversibly bind to DA receptors have been synthesized and their interaction with these receptors studied. Among DA antagonists, much attention has been paid to phenothiazine-mustards, which have been shown to be non-selective irreversible ligands for both D_1- and D_2-receptors (Schuster et al 1982; Wregget 1987). In our search for suitable irreversible DA receptor labels, we have synthesized several phenothiazine derivatives by linking different reactive groups to the same phenothiazine pharmacophore. In this paper the syntheses and in-vitro evaluation of these phenothiazine compounds as potential DA receptor ligands are described.

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Materials and Methods

Chemicals

[^{3}H]Spiperone (spec. act. 70·5 Ci mmol^{-1}) and [^{3}H]SCH 23390 (spec. act. 80 Ci mmol^{-1}) used as radioactive labels for the D2- and D1-receptors, respectively, were purchased from Amersham Buchler GmbH, Braunschweig, Germany.

(+)-Butaclamol, SCH 23390 (*R*-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-3-benzazepine-7-ol) and SKF 38393(1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol) were obtained from Biochemical Research Inc, Natick, MA, USA; spiperone was from Janssen Pharmaceutica Beerse, Belgium; bromoacetyl bromide, *N*-ethoxycarbonyl maleimide, iron powder and *p*-nitrobenzoyl chloride were supplied by Aldrich Chemie, Steinheim, Germany: silica gel and thiophosgen were obtained from Merck, Darmstadt, Germany and Hepes from Serva, Heildelberg, Germany. All other chemicals were purchased from established commercial sources and were of analytical grade.

Membrane preparation

Nuclei caudata were dissected from bovine brains obtained from a local abattoir 2 h after death. Synaptosomal membranes were prepared as described by Šoškić et al (1985). Briefly, the tissue was cut, minced and homogenized in 8 vol of homogenizing solution (0·32 M sucrose, 2 mM EDTA, 5 mM Hepes, pH 7·4) in a motor-driven Teflon homogenizer (2 min, 1000 rev min^{-1}). The homogenate was centrifuged (900 *g*, 10 min) and the post-mitochondrial supernatant, collected and recentrifuged (10 000 *g*, 30 min, 4°C). The pellet was made hypotonic by the addition of 20 vol ice-cold distilled water and resuspended. The suspension was centrifuged (20 000 *g*, 30 min, 4°C), the resulting pellet was resuspended in two vol 100 mM NaCl, 2 mM EDTA, 20 mM Hepes, pH 7·4 and stored in liquid nitrogen until use.

The protein concentration was measured by the method of Markwell et al (1978) using bovine serum albumin as a standard.

Radioligand binding assays

[^{3}H]Spiperone binding was determined in solution containing 100 mM NaCl, 5 mM EDTA, 20 mM Hepes, pH 7·4 (buffer A) at a membrane protein concentration of 0·3 mg mL^{-1} of incubation mixture (incubation time 60 min, 23°C), as described by Šoškić et al (1985). Free ligand was separated by rapid filtration through Whatman GF/C filters, followed by three washes with 5 mL of ice-cold buffer A. The 5-HT component of [^{3}H]spiperone binding was corrected for by the addition of 20 mM ketanserin to all incubation mixtures (Hamblin et al 1984). Non-specific binding was determined in the presence of 1 μM (+)-butaclamol. In competition binding experiments, varying concentrations of phenothiazine ligands and 0·2 nM [^{3}H]spiperone were used, and in equilibrium binding studies 0·01–2·0 nM [^{3}H]spiperone was applied.

[^{3}H]SCH 23390 binding to synaptosomal membranes of the bovine caudate nuclei was examined by the same rapid filtration method employed for [^{3}H]spiperone binding determination. The incubation mixture (total volume 1·0 mL) contained protein 0·3 mg mL^{-1}, buffer A, 0·25 nM [^{3}H]SCH 23390 and different concentrations of phenothiazine derivatives tested in competition binding experiments or varying concentrations of this radioligand (0·01–2·0 nM) in equilibrium binding analyses. Non-specific binding was determined as described above.

Experiments with phenothiazine derivatives

Synaptosomal membranes were resuspended in buffer A to a protein concentration of 0·2–0·3 mg mL^{-1} and appropriate concentrations of phenothiazines were added. Suspensions were incubated at three different temperatures (23°, 30° and 37°C) in the presence or in the absence of the competing ligands (10 μM apomorphine, SKF 38393, spiperone or fluphenazine). After 60 min of incubation, the membranes were washed by rapid centrifugation with 30 mL of buffer A at 23°C (10 min, 10 000 rev min^{-1}). In between the washings, membrane suspensions were incubated (30 min, 23°C) to allow dissociation of the ligands from the receptor molecules. After the sixth washing, membranes were collected by centrifugation, resuspended in the same buffer and used in binding experiments with 2 nM [^{3}H]spiperone (D_2-receptors) or [^{3}H]SCH 23390 (D_1-receptors).

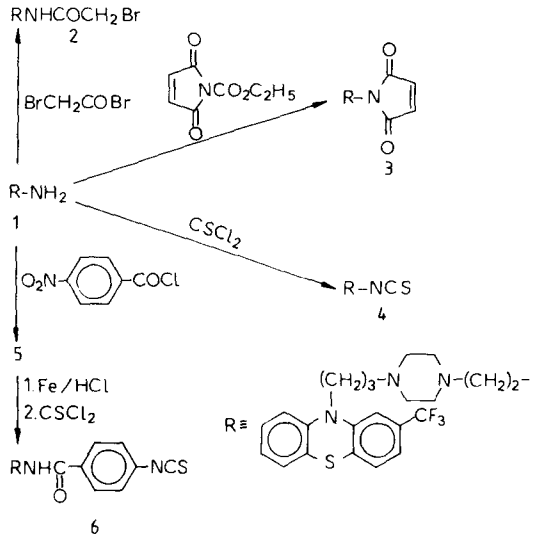
Radioactivity retained on the filters was measured by introducing dried filters into 10 mL of toluene-based scintillation liquid and counting in a 1219 Rackbeta, LKB Wallac scintillation counter at an efficiency of 51–55% for tritium.

Data analysis

Both saturation and competition curves were analysed using the iterative non-linear least-squares curve-fitting program LIGAND (Munson & Rodbard 1980), where the data are fitted to a hyperbola.

Synthesis of phenothiazine derivatives

Synthesis of 4-(3-(10-(2-trifluoromethyl)phenothiazinyl)-propyl-1-(2-aminoethyl)-piperazine hydrochloride, the basic phenothiazine building block (compound 1, scheme 1) will be described elsewhere. In a typical synthesis of phenothiazine ligands, an aqueous solution of compound 1 (1·02 g in 30 mL H_2O, 2 mmol) was stirred with 60 mL of CH_2Cl_2 and 1·0 g of



sodium bicarbonate. After 5 min, 2·2 mmol of the corresponding reagent was added (bromoacetyl bromide, *N*-ethoxycarbonylmaleinimide, thiophosgene or 4-nitrobenzoyl chloride, for compounds 2, 3, 4, and 5, respectively, as the final products). After 60 min, the organic layer was separated, successively washed with dilute ammonium hydroxide solution (2 × 50 mL) and water (2 × 50 mL), dried over anhydrous sodium sulphate and purified by flash chromatography on silica gel columns (Still et al 1978) using CH_2Cl_2/MeOH as an eluant. The eluant was evaporated under reduced pressure and the oily residue dissolved in an ether–CH_2Cl_2 mixture (9:1). The hydrochloride salt was obtained as a precipitate after addition of an equivalent amount of HCl dissolved in ether. Precipitates were washed with ether, then with hexane and dried under vacuum. Compound 5 was reduced to the amine by iron powder and HCl in 60% boiling ethanol. The resulting amine was converted to the isothiocyanate derivative (compound 6) using thiophosgene, as described above.

The structure and purity of the synthesized phenothiazine derivatives was assayed by ^{1}H- NMR (Varian FT-80 A), IR spectroscopy (Perkin Elmer 457 grating infrared spectrophotometer) and chemical microanalyses.

Compounds 2,3,4,5 and 6 were isolated as their dihydrochloride salts with melting points of 137–140°, 177–180°, 160–163°, 151–153° and 133–135°C, respectively.

Results

Ligand properties of the phenothiazine derivatives were examined by competition binding assays using specific radiolabelled antagonists [^{3}H]SCH 23390 and [^{3}H]spiperone for the D_1- and D_2-dopamine receptors, respectively. Under the conditions of prevented [^{3}H]spiperone binding to 5-HT S_2 receptors (Hamblin et al 1984) a single class of the D_2-binding sites of high affinity ($K_D = 0{\cdot}23 \pm 0{\cdot}03$ nM) and saturability ($B_{max} = 207 \pm 15$ fmol mg^{-1} protein) was determined for the synaptosomal membrane preparation employed. [^{3}H]SCH 23390 as a radioligand, also labelled a single class of the D_1-binding sites of high affinity

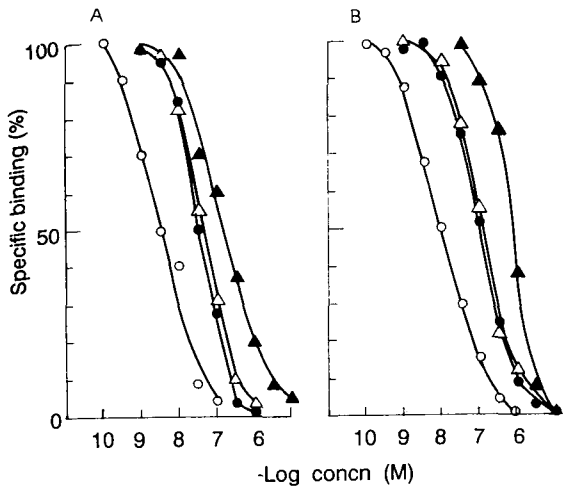


FIG. 1. Inhibition of [^{3}H]SCH 23390 and [^{3}H]spiperone binding to synaptosomal membranes of the bovine caudate nucleus by phenothiazine derivatives. Samples of the membrane suspensions (100 μL, 2–3 mg mL^{-1} protein) were incubated with varying concentrations of the following phenothiazines: 2 (○), 3 (●), 4 (▲) and 6 (△) used as the competitors of [^{3}H]SCH 23390 (0·25 nM; A) and [^{3}H]spiperone (0·2 nM; B) binding. After 60 min at 23°C, the incubation mixtures were rapidly filtered through Whatman GF/C filters which were washed three times with ice-cold buffer A. Data are from one representative experiment and are the means of triplicate determinations.

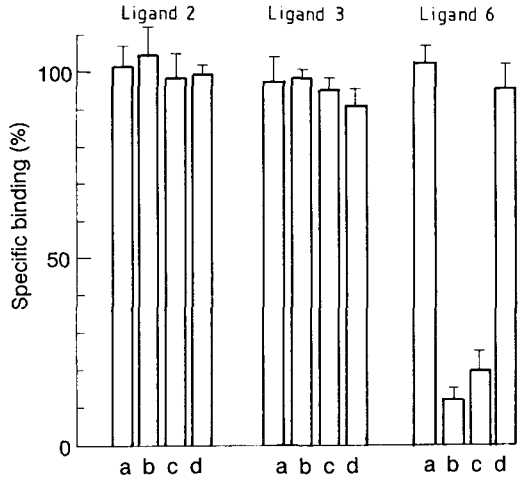


FIG. 2. Inactivation of the D_1-receptors by phenothiazines. Data are from one representative experiment performed by incubation of synaptosomal membrane preparation with phenothiazines at 37°C for 60 min. Membrane suspensions were washed six times. After each washing the incubation was repeated (30 min, 23°C). Upon the sixth washing, the membranes were collected by centrifugation and used in competition binding experiments. The extent of labelling was estimated by measuring the amount of specifically bound [^{3}H]SCH 23390 and expressed as relative concentrations of the radioligands in the absence of the tested phenothiazines (a), in the presence of the respective phenothiazine ligand (b), in the presence of both the respective phenothiazine ligand and 10 μM SCH 23390 (c) and when only 10 μM SCH 23390 was added (d). The results are means ± s.e.m.

($K_D = 0{\cdot}27 \pm 0{\cdot}03$ nM) in a concentration of 397 ± 20 fmol (mg protein)$^{-1}$ (B_{max}). These K_D value means for both D_1- and D_2-binding sites were employed in the calculation of the inhibition constants (K_i) for the phenothiazine compounds examined.

In Fig. 1, representative examples of competition binding experiments are shown and the corresponding K_i values for the synthesized phenothiazine derivatives are listed in Table 1.

All phenothiazine compounds tested expressed a limited selectivity for the D_1- and D_2-receptors. Compound 4 had a weak affinity for both categories of receptors and was excluded from further studies.

The reversibility of interaction of ligands 2, 3 and 6 with both D_1- and D_2-receptors was examined by incubating synaptosomal membrane preparations at three different temperatures (23°, 30° and 37°C) with 10-fold concentrations of the corresponding K_i values. No significant incubation temperature-related differences, over the short time of incubation, in the ligand-receptor interaction were observed. After thorough washing of the membranes, the number of receptor binding sites was measured using saturating concentrations of [^{3}H]SCH 23390 and [^{3}H]spiperone for the D_1- and D_2-receptors, respectively.

Under the experimental conditions applied, only ligand 6 showed a significant irreversible interaction with the D_1-

Table 1. Inhibition by phenothiazine derivatives of [^{3}H]SCH 23390 and [^{3}H]spiperone binding to synaptosomal membranes of the bovine caudate nuclei.

	K_i (nM)	
Phenothiazine	D_1-receptors	D_2-receptors
2	7·6 ± 0·5	2·5 ± 0·4
3	56·0 ± 3·0	32·0 ± 5·0
4	376·0 ± 42·0	216·0 ± 18·0
6	61·0 ± 8·0	35·0 ± 4·0

K_i values were calculated on the basis of K_D values obtained in saturation binding experiments with [^{3}H]SCH 23390 and [^{3}H]spiperone as specific radioligands for the D_1- and D_2- receptors, respectively. All other details are given in caption to Fig. 1. Each value represents the mean ± s.e.m. from at least three independent experiments done in triplicate.

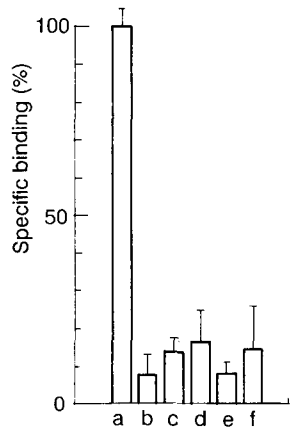


FIG. 3. Inhibition of [^{3}H]SCH 23390 binding to the D_1-receptors in the synaptosomal membranes of the bovine caudate nucleus by ligand 6. Data are from one representative experiment performed by incubation at 37°C for 60 min. Synaptosomal membranes were incubated, washed and collected as described in caption to Fig. 2. The amount of labelling was assessed by determining the concentration of specifically bound [^{3}H]SCH 23390. The data are expressed as relative radioligand concentrations in the absence of ligand 6 (a), in the presence of ligand 6 (b) and in the presence of both ligand 6 and the respective competing ligand (10 μM): SCH 23390 (c), fluphenazine (d), apomorphine (e) and SKF 38393 (f). The results are means ± s.e.m.

receptor (Fig. 2). Prolonged incubation of the synaptosomal membrane suspension supplied with this ligand (24 h, 23°C) and three additional washings with buffer A during this time interval did not alter the observed inhibitory effect on [^{3}H]SCH 23390 binding to the D_1-binding sites. The results (Fig. 3) demonstrate that incubation with D_1-receptor agonists (apomorphine and SKF 38393) or antagonists (SCH 23390 and fluphenazine) at a concentration of 10 μM in the incubation of the synaptosomal membrane suspension with compound 6 did not prevent the inhibition of the D_1-receptor interaction with the specific radioligand.

Discussion

Fluphenazine and α-fluphenthixol mustards are useful tools frequently applied in biochemical and pharmacological studies of the dopamine receptors (Schuster et al 1982; Amlaiky & Caron 1985; Wregget 1987). However, the application of these two ligands is limited by their low affinity and selectivity for the D_1- and D_2-receptors. The aim of our work was to design irreversible dopaminergic antagonists which would not suffer from these disadvantages. All ligands tested in this work showed a high affinity for both D_1- and D_2-binding sites with the exception of ligand 4. Irreversibility of interaction of ligands 2, 3 and 6 with the DA receptors was checked by incubation with synaptosomal membrane preparations at different temperatures and for different times. After washing out the excess ligand, specific binding of [^{3}H]SCH 23390 and [^{3}H]spiperone was measured under saturating concentrations of the radiolabels. In this type of experiment, no irreversible interaction between the ligands tested and the D_2-receptors was detected. The same was found for the interaction of ligands 2 and 3 with the D_1-receptor. However, ligand 6 preincubated with synaptosomal membrane suspension greatly reduced specific binding of [^{3}H]SCH 23390 to the D_1-binding sites. This effect was impossible to reverse with long-lasting incubations combined with excessive washings, indicating a slow dissociation of the ligand from the D_1-receptor. Simultaneous addition of the D_1-receptor agonists, apomorphine and SKF 38393, or antagonists, fluphenazine and SCH 23390 to the incubation mixture did not attenuate ligand 6-related loss of the D_1-binding site. At the moment, we have no rational explanation for such an effect. On the basis of the results obtained it can be concluded that although ligand 6 did not discriminate between the two subclasses of the DA receptors, it showed an irreversible binding only to the D_1 class of these receptors and thus, seemed promising as an irreversible, highly specific ligand for biochemical and pharmacological studies of the D_1-receptors. This could mean that radiolabelled compound 6 might provide a suitable probe for irreversible labelling of the D_1-receptors within the central nervous system. The addition of a radioactive atom may even enhance the binding affinity, as observed recently by Murphy et al (1990) who synthesized iodobenzamide analogues as potential D_2-receptor imaging agents and observed a great increase of binding affinity upon the introduction of an isotopic iodine atom into the molecule.

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