Biochemical Pharmacology, Vol. 34, No. 10, pp. 1701–1704, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ELLIPTICINE DERIVATIVES INTERACT WITH MUSCARINIC RECEPTORS

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(*Received* 6 *August* 1984; *accepted* 12 *November* 1984)

Abstract—Ellipticine derivatives or analogues, tetracyclic alkaloids used in human cancer treatment, have been evaluated with regard to their interaction with several neurotransmitter receptors, in order to explain or to predict the side effects which occur in man. These drugs were recently found to be reversible non-competitive inhibitors of cholinesterases. In this study, we have shown that ellipticines are also potent muscarinic antagonists, only 100-fold less active than atropine in inhibiting 50% of the specific binding of (^{3}H) quinuclidinyl benzilate on rat brain preparation of muscarinic receptors. That the interaction with muscarinic receptors is quite unique has been demonstrated by the lack of interaction with three other neurotransmitter receptors. Tertiary amines show relatively less blockade of muscarinic receptors, while substituted ammonium ions are better inhibitors of the QNB binding. The possible mechanisms of *in vivo* action of these alkaloids is discussed.

Ellipticines are tetracyclic alkaloids with significant antineoplastic activity. Although the mechanism of action remains unclear, it has been demonstrated that these drugs are DNA intercalating agents *in vitro*. Among the numerous synthetic derivatives of ellipticine, some have been the subject of further preclinical and clinical study on the basis of their high therapeutic index and broad spectrum activity [1]. The most active members of this series are: elliptinium‖ (2-methyl 9-hydroxy ellipticinium, NSC 264137), with established activity in human breast and kidney cancer; 9-methoxy-ellipticine with activity in human acute myeloblastic leukemia; and an ellipticine isomer, 9-hydroxy olivacinium. Their structural formulas are shown in Fig. 1.

These drugs possess a lipophilic skeleton with hydrophilic substitutions. The most important substitution is that occurring on the nitrogen of the isoquinoline ring: 9-methoxy-ellipticine does not possess any such substitution and acts as a teritary amine in contrast to the two other compounds, which have substituted ammonium ions.

In a previous study, we have shown that ellipticine and derivatives are reversible non-competitive inhibitors of true and pseudo-cholinesterases [2]. The quaternary ammonium derivatives produced significant inhibition of these enzymes, whereas tertiary amines are only poor inhibitors. These results are in good agreement with previous observations, indicating that positively charged drugs may interact with cholinesterases. Several cholinesterase inhibitors which possess a charged pole are also described to be muscarinic ligands [3].

The purpose of the present study, therefore, was to determine the *in vitro* interactions between key ellipticine derivatives and neurotransmitter receptors. Since ellipticine side effects seem to involve an interaction with acetylcholine, the study of muscarinic receptors and nicotine acceptors sites were included in this work. The study of two other types of receptors (β-adrenergic and benzodiazepine, central type) was performed in order to determine the specificity of the interaction described.

MATERIALS AND METHODS

Drugs. Radioactive ligands: (^{3}H) quinuclidinyl benzilate (QNB), (^{3}H) *dl*-nicotine, (^{125}Iodo) cyanopindolol (CYP) and (^{3}H) diazepam, were obtained from New England Nuclear Co. (Boston, MA). Ellipticine derivatives were provided by Institut Pasteur Production (Paris, France) and by Dr. N. Dat-Xuong (Gif-sur-Yvette, France). Atropine, propranolol, diazepam were obtained from Sigma Chemical Co. (Saint-Louis, MO). Nicotine was a generous gift of Pr. M. M. Plat. The source of (^{14}C) elliptinium acetate (sp. act. 55 mCi/mmole) was SANOFI Recherche (Paris, France). Bio-Rad protein assay kits were obtained from Bio-Rad Lab. (München, F.R.G.). All other compounds were of the best grade available and were obtained from commercial sources.

Membrane preparations. Male Wistar rats were decapitated and their brain and lungs were removed rapidly. Cerebral cortices and striata were separated from other cerebral structures and placed in 0.1 M phosphate buffer, pH 7.4 (Buffer A), for the study of muscarinic receptors, in 20 mM Hepes buffer, pH 7.5 (Buffer B) for the study of nicotine acceptors sites and in 50 mM Tris–HCl buffer, pH 7.4 (Buffer C) for benzodiazepine receptors. The three preparations were then homogenized by Polytron action at 4°, followed by slow centrifugation (150 *g*, 10 min,

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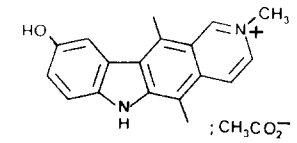
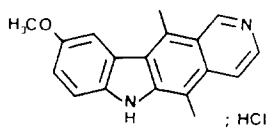
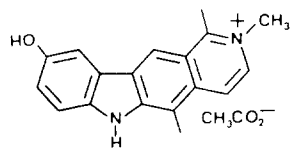
‖ Celiptium™ (Institut Pasteur Production).



ELLIPTINIUM



METOXY-ELLIPTICINE



9-HYDROXY OLIVACINIUM

Fig. 1. Structure of ellipticine derivatives included in the study: methoxy-ellipticine, elliptinium and 9-hydroxy-olivacinium.

4°). The supernatants were removed and centrifuged once (25,000 *g*, 30 min, 4°). The resulting pellets were resuspended in their respective ice-cold buffers and adjusted for a final protein concentration ranging from 0.5–1 mg per ml. Rat lungs were placed in 50 mM Tris–HCl buffer, pH 7.5 (Buffer D), and after two washes, were subjected to the same steps as above in order to obtain a lung membrane preparation for the study of β-adrenergic receptors.

Binding assays. All binding assays were performed in triplicate in 3–6 separate experiments.

The binding to muscarinic receptors [4] was determined by (^{3}H) QNB binding, 0.5 nM, in a final volume of 0.5 ml (Buffer A). Incubation was carried out for 45 min at 37° and terminated by rapid filtration through Whatman GF/C glass fibre filters. The filters were washed with ice-cold buffer until dryness and counted in scintillation fluid, on a Beckman counter. Each drug included in this study was tested in concentrations ranging from 10^{-9} to 10^{-3} M, and compared with atropine. Non-specific binding was determined with atropine, 10^{-6} M.

The binding to nicotine acceptors sites was determined with (^{3}H) nicotine, 40 nM in a final volume of 0.14 ml (Buffer B). The incubation time was 45 min, at 20° [5]. Non-specific binding was determined with the reference drug, *dl*-nicotine, 10^{-5} M. The methodology was the same as described above.

Benzodiazepine receptor binding was performed using (^{3}H) diazepam, 2.0 nM in Buffer C with incubation for 30 min, at 20° [6]. Non-specific binding was determined with the reference drug, diazepam, 10^{-5} M.

The binding of (^{125}I) cyanopindolol, 0.1 nM to β-adrenergic receptors on rat lung membranes was conducted at 20° for 30 min [7], using propranolol as the reference drug (non-specific binding: propranolol 10^{-6} M).

The binding of (^{14}C) elliptinium to rat brain was determined using the same conditions as described above for (^{3}H) QNB. When this binding was performed in the presence of (^{3}H) QNB, the fixation of each ligand was separately counted.

Receptor data analysis. The 50% inhibitory concentration (IC_{50}) defined as that concentration of drug which reduced specific binding by 50%, was corrected for radio-ligand occupancy shift according to the equation: $K_i = IC_{50}\,(1 + C/K_d)$, where K_i, K_d and C represent the inhibition constant, the dissociation constant and the concentration of the radio-ligand. The binding parameters were determined from the experimental data by non-linear least-squares regression analysis.

RESULTS

Muscarinic receptors

Inhibition plots of (^{3}H) QNB binding to muscarinic receptors are shown in Fig. 2. The K_i value for atropine was $7.8 \pm 2.4\ 10^{-10}$ M, whereas methoxy-ellipticine, elliptinium, and 9-hydroxy olivacinium inhibited 50% of the binding at $1.5 \pm 0.3\ 10^{-5}$ M, $4.9 \pm 2.2\ 10^{-8}$ M and $1.6 \pm 0.6\ 10^{-7}$ M respectively. Thus, elliptinium and 9-hydroxy olivacinium demonstrated considerable affinity for muscarinic receptors, only 100-fold less than that of the standard, atropine. Furthermore, in the case of elliptinium, the K_i value represents a concentration of the same order as that achievable in man following a standard-dose elliptinium infusion [8]. Hill plots of these data indicated that the binding of ellipticines to muscarinic

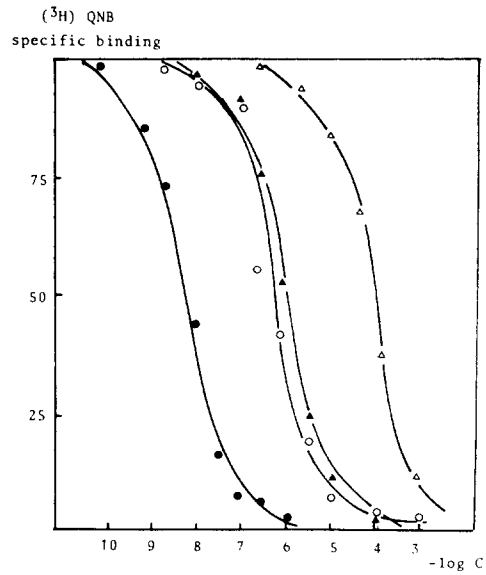


Fig. 2. Inhibition of (^{3}H) QNB binding by ellipticine derivatives: comparison with atropine. The reference drug atropine (●) has a K_i value of 0.78 nM, while elliptinium (○) and 9-hydroxy-olivacinium (▲) have comparable values (K_i = 49 and 160 nM, respectively). Methoxy-ellipticine (△), a tertiary amine compound, is only a poor inhibitor of the binding (K_i = 15 μM).

receptors was not consistent with a two-site binding equation. The Hill coefficient (slope of the Hill plot) for atropine was 0.94 ± 0.08, a value commonly obtained for muscarinic antagonists [9]. Hill coefficients for the ellipticine derivatives were as follows: 9-methoxy ellipticine, 1.06 ± 0.14; elliptinium, 0.89 ± 0.12; and 9-hydroxy olivacinium, 0.95 ± 0.05. These data could suggest that the compounds behave as muscarinic antagonists. Further experiments on isolated organs are in progress in order to confirm these results.

Nicotine sites

dl-Nicotine exhibited a K_i value of $3.5\ 10^{-8}$ M, a value in good agreement with previous observations. Ellipticine derivatives failed to inhibit (^{3}H) nicotine binding at concentrations below 10^{-4} M. Only 10% of the total binding is inhibited at this concentration. Furthermore, these compounds were unable to interact with α-bungarotoxin binding sites (data not shown). However, nicotine sites are not directly reliable with nicotinic receptors, nicotinic compounds always inhibit the binding of (^{3}H) nicotine with its sites [5]. The negative result obtained with ellipticine derivatives could indicate that these compounds are not nicotinic ligands.

Benzodiazepine receptors

The reference drug, diazepam, was determined to have a K_i value of $1.25\ 10^{-8}$ M. No displacement was observed when the binding was conducted with the ellipticine derivatives.

β-adrenergic receptors

However, (^{125}I) CYP does not discriminate between the two subtypes, i.e. β_1 and β_2, adrenoceptors, the binding of this ligand indicated predominantly the presence of β_2 receptors, according to the fact that the ratio β_2/β_1 in rat lungs is about 3. Propranolol inhibited 50% of (^{125}I) CYP binding at $4.0\ 10^{-9}$ M. The K_is of the ellipticine derivatives were as follows: 9-methoxy ellipticine, $1.25\ 10^{-4}$ M; elliptinium, $8.2\ 10^{-5}$ M; 9-hydroxy olivacinium, $9.2\ 10^{-5}$ M. Thus, only poor affinity of ellipticines for adrenergic receptors could be demonstrated.

Figure 3 indicates the binding inhibition obtained with elliptinium, 10^{-6} M (therapeutic levels), as compared to the inhibition induced by the respective reference drugs (atropine, nicotine, diazepam, propanolol), all at a concentration of 10^{-8} M. The specificity of the interaction of ellipticine derivatives with muscarinic receptors is significant. No notable interaction was observed with the three other receptors.

Thus, it seems evident that the cholinergic system is a target for ellipticines, since interaction with two components of this system (cholinesterases and muscarinic receptors) has now been clearly demonstrated. It is possible that ellipticine derivatives react with the anionic site of the muscarinic receptor via the quaternary nitrogen group [10]. Furthermore, the lipophilic nucleus of the ellipticines may be easily attracted to the hydrophobic zones of the muscarinic receptor, and by this means, may block such receptors.

The study of elliptinium binding to the rat brain preparation was performed using (^{14}C) elliptinium as tracer (1%), resulting in a total elliptinium concentration ranging from 10^{-7} to 10^{-5} M. Figure 4 shows the fixation of elliptinium compared to the fixation of (^{3}H) QNB, as a function of the total elliptinium concentration. There was a certain non-specificity of the fixation, because of the interaction with phospholipids. (^{3}H) QNB binding was blocked by elliptinium, but, in addition, for each molecule of QNB which was displaced, approximately 300 molecules of elliptinium were bound. This result is in good agreement with the observation that ellipticine derivatives interact in a very important way with membranes [11]. This study shows that, although the interaction of ellipticines with neurotransmitter receptors is quite specific for the cholinergic system, the fixation of these drugs to membranes is even more important.

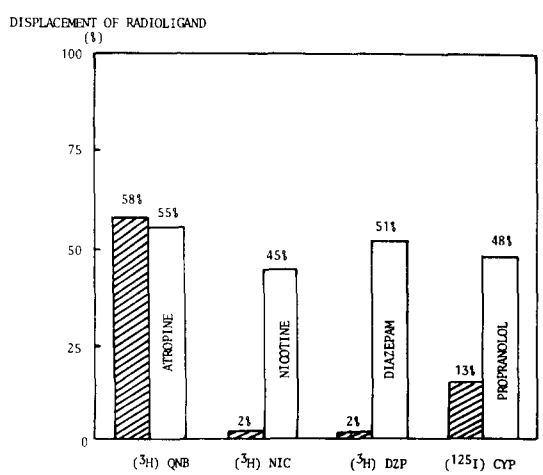


Fig. 3. Binding inhibition obtained with elliptinium 10^{-6} M (hatched bars) in comparison to the inhibition induced by the respective reference drug 10^{-8} M (open bars). This figure indicates that ellipticine derivatives interact only with muscarine receptors.

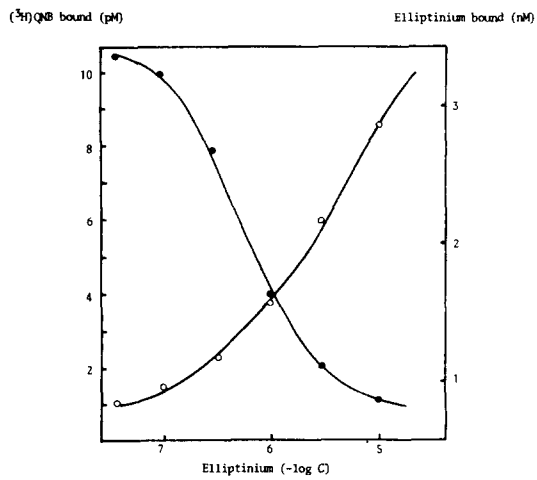


Fig. 4. Fixation of elliptinium vs (^{3}H) QNB to rat brain, as a function of the total elliptinium concentration (10^{-5} to 10^{-7} M), using (^{3}H) QNB 0.5 nM and (^{14}C) elliptinium as tracer (1%). The fixation of elliptinium (○) is approximately 300-fold greater than that of QNB (●).

DISCUSSION

The results of the present study allow for some important clinical correlations with regard to ellipticine, the member of this series with the greatest clinical use and least toxicity. Two observed side effects of ellipticine may involve the cholinergic system: asialia, which occurs in approximately 40% of the patients, and diarrhoea, occurring in as many as 80–100% of patients [12]. Asialia is a common side effect resulting from the administration of parasympatholytic agents. In addition to the present demonstration of significant affinity of ellipticines for muscarinic receptors, ellipticine is known to concentrate significantly in salivary glands [1], although no resultant histologic abnormalities occur.

Generally, the diarrhoea associated with cytotoxic chemotherapy is related to a non-specific action of such drugs upon intestinal muscosal cells. However, in the case of ellipticine it is possible that this side effect is also related to an interaction with the cholinergic system. During chronic toxicity studies of rats receiving ellipticine, considerable diarrhoea was consistently observed in association with intestinal dilatation.

Studies are in progress to determine the *in vivo* action of ellipticine derivatives upon markers of the cholinergic system.

Acknowledgements—This study was supported in part by Grant 82D10 of Institut Gustave-Roussy and by SANOFI Recherche. We thank J. Guery for typewriting this manuscript.

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