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Short communication

Decreased binding affinity of olanzapine and clozapine for human muscarinic receptors in intact clonal cells in physiological medium

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Abstract

The binding affinity of olanzapine, clozapine and atropine for muscarinic receptor subtypes in clonal Chinese hamster ovary (CHO) cell lines was compared in intact cells in physiological media to disrupted cells in hypotonic buffer. The affinity of olanzapine and clozapine, but not atropine, for muscarinic receptor subtypes (M_1–M_5) was significantly reduced in intact cells in physiological medium compared to disrupted cells in hypotonic buffer. The affinity of olanzapine for muscarinic M_1 receptors was most affected with a reduction of K_i value from 2.5 to 73 nM in intact cells. These data suggest that the affinity of olanzapine and clozapine for muscarinic receptors have been significantly overestimated.

Keywords: Muscarinic receptor; Olanzapine; Clozapine; Binding; Antipsychotic

1. Introduction

Olanzapine is an atypical antipsychotic agent that has relatively high and comparable affinity in radioligand binding studies for a number of neuronal receptors including dopamine D_1–D_5, 5-$HT_{2A,B,C}$, α_1-adrenoreceptor, histamine H_1 and muscarinic M_1–M_5 receptor subtypes (Bymaster et al., 1996; Schotte et al., 1996). However, the relatively high affinity of olanzapine and clozapine for muscarinic receptors in in vitro radioligand binding experiments does not correlate with results from in vitro functional studies, in vivo studies in animals and clinical results in humans. For example, olanzapine has high affinity for muscarinic receptor subtypes in in vitro binding studies (K_i values for M_1–M_5 range from 1.9 to 25 nM), but it was shown to be a relatively weak antagonist of muscarinic agonist-induced changes in second messenger levels with K_i values ranging from 70 to 622 nM (Bymaster et al., 1996, 1999). In contrast, olanzapine in in vitro functional studies antagonized 5-HT_2, α_1-adrenoreceptors and histamine H_1 receptors at nearly the same potency as in binding studies. In addition, olanzapine relative to its blockade of dopamine D_2 and 5-HT_{2A} receptors, weakly blocked ex vivo and in vivo binding of muscarinic receptor ligands in rats (Schotte et al., 1996; Zhang and Bymaster, 1999). Lethality in mice produced by the cholinesterase inhibitor physiostigmine was not readily reversed by olanzapine, suggesting modest antagonism of muscarinic receptors in vivo (Arnt and Skarsfeldt, 1998). Olanzapine did not significantly impair spatial memory in the Morris water maze up to relatively high doses, whereas the potent muscarinic antagonist scopolamine blocked acquisition of the spatial memory trace (Moore et al., 1997). In addition, olanzapine patients reported a relatively low rate of anticholinergic events in clinical trials (Beasley et al., 1996). The interaction of olanzapine with muscarinic receptors is further complicated by the finding that clozapine and olanzapine have apparent partial agonist activity in cell line based functional studies (Zorn et al., 1994; Zeng et al., 1997; Olianas et al., 1999). However, another study has shown only antagonist effects in cell lines with low receptor expression density (Bymaster et al., 1999). Thus, there seems to be a contradiction in the high affinity of olanzapine for muscarinic receptor in binding experiments vs. lesser effects in functional, in vivo and clinical studies.

We have investigated these apparent discrepancies by performing in vitro radioligand binding studies in intact

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Chinese hamster ovary (CHO) cells transfected with the respective human muscarinic receptor subtypes. These binding experiments were performed in physiological medium instead of low ionic strength buffer used in previous binding studies (Bymaster et al., 1996; Schotte et al., 1996). Binding in intact cells using physiological medium and with the receptor coupled to G protein and signal transduction apparatus may be a more appropriate biological milieu for compounds like olanzapine. We also compared the inhibition of muscarinic radioligand binding by olanzapine to clozapine and the classical muscarinic antagonist atropine.

2. Materials and methods

2.1. Cell culture

Cell culture of CHO-K1 cells was performed according to a previously described method (Bymaster et al., 1996). The CHO-K1 cells transfected with human muscarinic receptor subtypes (Dorje et al., 1991) were obtained from Dr. Mark Brann at the University of Vermont.

2.2. Binding determination

After growing to about 70–80% confluency, the cells were harvested with 0.25% trypsin/1 mM EDTA solution, centrifuged and resuspended in Earle's balanced salt solutions (EBSS) containing 120 mM NaCl, 26 mM sodium bicarbonate, 5 mM KCl, 1 mM NaH_2PO_4, and 2 mM $MgCl_2$, pH 7.4. About 0.4 million CHO cells transfected with the respective muscarinic receptor subtypes were added to 1 ml total volume of EBSS medium containing 0.24 nM [^{3}H]*N*-methylscopolamine (84 Ci/mmol, New England Nuclear) and various concentrations of drugs. After incubation for 30 min at 37°C, the cells were filtered through glass fiber filters (Whatman, GF/c) with vacuum. The filters were rinsed three times with 1 ml cold buffer, and placed in scintillation vials containing 10 ml of scintillation fluid (Ready Protein$^+$, Beckman, Fullerton, CA). Filters were presoaked in 0.1% polyethylenimine for several hours. Radioactivity trapped on the filters was determined by liquid scintillation spectrometry at approximately 40–50% efficiency. Nonspecific binding was determined in the presence of 1 μM atropine.

2.3. Data analysis

The mean IC_{50} values were obtained from at least three separate experiments performed in triplicate with at least 6–11 concentrations of drugs. Hill coefficients and IC_{50} values determined using the Allfit software program (De Lean et al., 1978) and inhibition constants (K_i values) were calculated utilizing the Cheng–Prusoff equation (Cheng and Prusoff, 1973). The K_d values for [^{3}H]*N*-methylscopolamine binding to M_1, M_2, M_3, M_4 and M_5 receptors were 0.212, 0.235, 0.174, 0.077, and 0.45 nM, respectively.

2.4. Drugs

Olanzapine was synthesized in the Lilly Research Laboratories, clozapine was purchased from RBI (Natick, MA) and atropine from Sigma (St. Louis, MO). Cell culture reagents were purchased from GIBCO (Gaithersburg, MD) and all other chemicals used were reagent grade and were purchased from Sigma.

3. Results

The radioligand [^{3}H]*N*-methylscopolamine bound readily to intact CHO cells with muscarinic M_1–M_5 receptors in physiological medium. Cells transfected with muscarinic M_1 receptors had representative nonspecific binding of 199 ± 23 dpm in intact cells in physiological medium and nonspecific binding of 136 ± 8 dpm in broken cells in 50 mM sodium phosphate medium, suggesting little if any uptake of [^{3}H]*N*-methylscopolamine into the intact cells. Olanzapine and atropine inhibited binding of [^{3}H]*N*-methylscopolamine to intact CHO cells transfected with the muscarinic M_1 receptor in a concentration dependent fashion (Fig. 1). However, atropine and particularly olanzapine less potently inhibited binding to muscarinic M_1 receptors in intact cells in physiological medium than to broken cells in hypotonic medium. In physiological medium and intact cells, the K_i values of olanzapine, clozapine and atropine

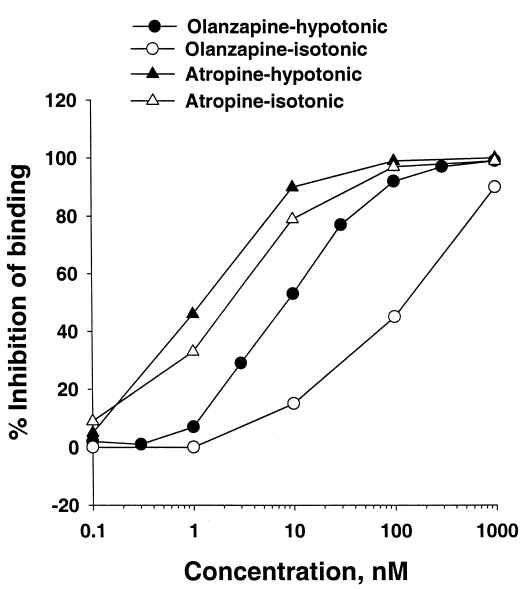

Fig. 1. Concentration-dependent inhibition by olanzapine and atropine of [^{3}H]*N*-methylscopolamine binding to muscarinic M_1 receptors in intact CHO cells in physiological medium (isotonic, open symbols) and disrupted membranes in 50 mM Na phosphate and 2 mM $MgCl_2$ buffer (hypotonic, filled symbols).

Table 1
Inhibition constants of olanzapine, clozapine and atropine for [^{3}H]*N*-methylscopolamine binding to clonal human muscarinic receptors subtypes in intact CHO cell suspensions in physiological medium, in membranes in 50 mM Na phosphate buffer and blockade of agonist-induced functional effects in clonal cells

Compound	Preparation	K_i, nM (Hill coefficient)				
		M_1	M_2	M_3	M_4	M_5
Olanzapine	whole cells	73 ± 14 (0.80 ± 0.15)	96 ± 24 (1.13 ± 0.02)	132 ± 30 (1.10 ± 0.06)	32 ± 6 (1.15 ± 0.10)	48 ± 10 (1.50 ± 0.09)
	membranes[a]	2.5 ± 0.3	18 ± 5	13 ± 0.8	10 ± 0.6	6 ± 0.8
	functional[b]	70 ± 30	622 ± 179	126 ± 61	350 ± 171	82 ± 25
Clozapine	whole cells	31 ± 9 (1.01 ± 0.18)	204 ± 33 (0.96 ± 0.09)	109 ± 22 (1.06 ± 0.02)	27 ± 3 (0.92 ± 0.07)	26 ± 18 (1.19 + 0.18)
	membranes[a]	1.4 ± 0.3	10 ± 1	7 ± 1	6 ± 0.5	5 ± 1.2
Atropine	whole cells	1.1 ± 0.3 (0.91 ± 0.05)	0.9 ± 0.1 (0.88 ± 0.10)	0.8 ± 0.1 (0.98 ± 0.03)	0.3 ± 0.02 (0.89 ± 0.04)	0.5 ± 0.2 (0.85 ± 0.03)
	membranes[a]	0.2 ± 0.02	1.5 ± 0.7	0.2 ± 0.03	0.1 ± 0.03	0.6 ± 0.1
	functional[b]	0.7 ± 0.3	1 ± 0.4	0.1 ± 0.01	1 ± 0.1	0.4 ± 0.1

[a] Data from Bymaster et al., 1996.
[b] Functional data from Bymaster et al., 1999 using antagonism of oxotremorine-M-induced release of arachidonic acid for muscarinic M_1, M_3 and M_5 receptor subtypes and antagonism of oxotremorine-M effects on cAMP for muscarinic M_2 and M_4 receptor subtypes.

for muscarinic M_1 receptors were 73 ± 14, 31 ± 9, and 1.1 ± 0.3 nM; whereas, the K_i values in broken membranes and 50 mM Na phosphate buffer were 2.5 ± 0.3, 1.4 ± 0.3 and 0.2 ± 0.02 nM, respectively (Table 1; Bymaster et al., 1996). This represents a reduction of 29-, 22-, and 5-fold in affinity, respectively (Table 1). Similarly, olanzapine had lower affinity for muscarinic M_2, M_3, M_4 and M_5 receptors of intact cells in physiological medium with K_i values of 96 ± 24, 132 ± 30, 32 ± 6 and 48 ± 10 nM and the affinity was reduced 5-, 10-, 3.2- and 8-fold, respectively. The affinity of clozapine for muscarinic M_2–M_5 receptors was also significantly reduced in physiological medium, but the affinity of atropine was not appreciably altered. Hill coefficients for the binding in intact cells did not appreciably differ from unity except for the values of olanzapine in M_5 cells (Table 1). The K_i values obtained in functional studies measuring antagonism of muscarinic agonist-induced changes in second messenger levels (Bymaster et al., 1999) was in better agreement with binding in intact cells than membranes (Table 1).

4. Discussion

Olanzapine has been shown to be a relatively weak muscarinic antagonist in in vitro in functional studies, animal studies and in humans (Beasley et al., 1996; Schotte et al., 1996; Bymaster et al., 1999). However, radioligand binding studies conducted in low ionic strength buffer with muscarinic receptors from animal tissue, receptors from human tissue and with human muscarinic receptors transfected into cell lines suggest that olanzapine has high affinity for muscarinic receptor subtypes and might be expected to produce more potent muscarinic antagonism in vivo (Bymaster et al., 1996; Schotte et al., 1996). In this binding study using physiological medium and intact cells, olanzapine had reduced affinity for muscarinic receptor subtypes ranging from 32 to 132 nM. In particular, the affinity of olanzapine for the muscarinic M_1 receptor subtype was reduced 29-fold from 2.5 nM in broken cells in hypotonic medium to 73 nM in intact cells in physiological medium. Similarly, clozapine had reduced affinity for muscarinic receptors in this paradigm. On the other hand, the affinity of the potent muscarinic antagonist, atropine, was only appreciably shifted in the M_1 cells with a 5.5-fold decrease in affinity and less for the other receptor subtypes. Thus, the affinity of olanzapine and clozapine, but not atropine, was significantly overestimated in binding studies utilizing broken cell membranes in low ionic strength buffer. This overestimation may account, at least in part, for the contradiction between high radioligand binding affinity and the relatively weak muscarinic antagonism observed with olanzapine in animal studies and in humans.

The reason for the significantly reduced binding affinity of olanzapine and clozapine for muscarinic receptors in physiological medium and intact cells is not entirely clear. Since clozapine and olanzapine are apparent partial agonists at muscarinic receptors (Zorn et al., 1994; Zeng et al., 1997; Olianas et al., 1999) and high concentration of salts particularly reduce binding of muscarinic agonists (Birdsall et al., 1979), this may play a role in the reduction of the affinity of the compounds in isotonic medium. Moreover, the affinity of clozapine for dopamine D_2 and D_4 receptors is reduced in medium with sodium salts (Seeman and Van Tol, 1993), suggesting that receptor interactions of this type of molecule are particularly sensitive to changes in ionic strength of the buffer. In addition, the intact cells containing transfected muscarinic receptors have a dynamic equilibrium between receptors, G proteins and the entire signal transduction apparatus that may alter affinity of olanzapine and clozapine for the receptor. Attempts at determining affinity constants for ligand receptor interactions in broken cells and particularly in whole cells are complicated by the heterogeneity of receptor equilibrium

(high and low affinity states, desensitization, down regulation, etc.) G protein stoichiometry and effector availability. This underscores the importance of apparent in vivo potency in estimating in vivo drug responses. Further studies are needed to understand the role of ionic strength and binding in intact cells on the affinity of olanzapine and clozapine for muscarinic receptors and other neuronal receptors.

References

Arnt, J., Skarsfeldt, T., 1998. Do novel antipsychotics have similar pharmacological characteristics? A review of the evidence. Neuropsychopharmacology 18, 63–101.

The Olanzapine HGAD Study Group, Beasley, C.M., Tollefson, G.D., Tran, P., Satterlee, W., Sanger, T., Holman, S., 1996. Olanzapine versus placebo and haloperidol: acute phase results of the North American double blind olanzapine trial. Neuropsychopharmacology 14, 111–123.

Birdsall, N.J.M., Burgen, A.S.V., Hulme, E.C., Wells, J.W., 1979. The effect of ions on the binding of agonists and antagonists to muscarinic receptors. Br. J. Pharmacol. 67, 371–377.

Bymaster, F.P., Calligaro, D.O., Falcone, J.F., Marsh, R.D., Moore, N.A., Tye, N.C., Seeman, P., Wong, D.T., 1996. Radioreceptor binding profile of the atypical antipsychotic olanzapine. Neuropsychopharmacology 14, 87–96.

Bymaster, F.P., Nelson, D.L., DeLapp, N.W., Falcone, J.F., Eckols, K., Truex, L.L., Foreman, M.M., Lucaites, V.L., Calligaro, D.O., 1999. Antagonism by olanzapine of dopamine D1, serotonin$_2$, muscarinic, histamine H_1 and α_1-adrenergic receptors in vitro. Schizophr. Res. 37, 107–122.

Cheng, Y.C., Prusoff, W.H., 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. Biochem. Pharmacol. 22, 3090–3108.

De Lean, A., Munson, P., Rodbard, D., 1978. Simultaneous analysis of families of sigmoidal cures: application to bioassay, radioligand assay and physiological dose–response curves. Am. J. Physiol. 235, E97–E102.

Dorje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E., Brann, M.R., 1991. Antagonist binding profiles of five cloned muscarinic receptor subtypes. J. Pharmacol. Exp. Ther. 256, 727–733.

Moore, N.A., Leander, J.D., Benvenga, M.J., Gleason, S.D., Shannon, H., 1997. Behavioral pharmacology of olanzapine: a novel antipsychotic drug. J. Clin. Psychiatry 58 (suppl 10), 37–44.

Olianas, M.C., Maulla, C., Onali, P., 1999. Mixed agonist–antagonist properties of clozapine at different human cloned muscarinic receptor subtypes expressed in Chinese hamster ovary cells. Neuropsychopharmacology 20, 263–270.

Schotte, A., Janssen, P.F.M., Gommeren, W., Luyten, W.H.M.L., Van Gompel, P., Lesage, A.S., De Loore, K., Leysen, J.E., 1996. Risperidone compared with new and reference antipsychotic drugs: in vitro and in vivo receptor binding. Psychopharmacology 124, 57–73.

Seeman, P., Van Tol, H.H.M., 1993. Dopamine receptor pharmacology. Curr. Opin. Neurol. Neurosurg. 6, 602–608.

Zeng, X.P., Le, F., Richelson, E., 1997. Muscarinic m4 receptor activation by some atypical antipsychotic drugs. Eur. J. Pharmacol. 321, 349–354.

Zhang, W., Bymaster, F.P., 1999. The in vivo effects of olanzapine and other antipsychotic agents on receptor occupancy and antagonism of dopamine D_1, D_2, D_3, $5HT_{2A}$ and muscarinic receptors. Psychopharmacology 141, 267–278.

Zorn, S.H., Jones, S.B., Ward, K.M., Liston, D.R., 1994. Clozapine is a potent and selective muscarinic M4 receptor agonist. Eur. J. Pharmacol., Mol. Pharmacol. Sect. 269, R1–R2.