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ORIGINAL INVESTIGATION

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The role of M1 muscarinic receptor agonism of *N*-desmethylclozapine in the unique clinical effects of clozapine

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Abstract *Rationale:* Clozapine is a unique antipsychotic, with efficacy against positive symptoms in treatment-resistant schizophrenic patients, and the ability to improve cognition and treat the negative symptoms characteristic of this disease. Despite its unique clinical actions, no specific molecular mechanism responsible for these actions has yet been described. *Objectives and methods:* To comprehensively profile a large library of neuropsychiatric drugs, including most antipsychotics, at human monoamine receptors using R-SAT, an in vitro functional assay. *Results:* Profiling revealed that *N*-desmethylclozapine (NDMC), the principal metabolite of clozapine, but not clozapine itself, is a potent and efficacious muscarinic receptor agonist, a molecular property not shared by any other antipsychotic. To further explore the role of NDMC muscarinic receptor agonist properties in mediating the physiological actions of clozapine, systemically administered NDMC was found to stimulate the phosphorylation of mitogen-activated protein kinase (MAP kinase) in mouse CA1 hippocampal neurons, an effect that was blocked by scopolamine, confirming central M1 muscarinic receptor agonist activity in vivo. Lastly, an analysis of clozapine and NDMC serum levels in schizophrenic patients indicated that high NDMC/clozapine ratios better predicted improvement in cognitive functioning and quality of life than the levels of either compound alone. *Conclusions:* The muscarinic receptor agonist activities of NDMC are unique among antipsychotics, and provide a possible molecular basis for the superior clinical effects of clozapine pharmacotherapy.

Keywords Muscarinic acetylcholine receptors · Agonist · *N*-desmethylclozapine · Clozapine · *N*-desmethylclozapine/clozapine ratios · Schizophrenia · Antipsychotic · Cognition

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Introduction

Schizophrenia, which affects about 1% of the adult population, is characterized by disturbances in reality testing, cognition, affect, and behavior, which often cause persistent impairments in work and social function (Wong and Van Tol 2003). Treatment with typical antipsychotic drugs, e.g., haloperidol, controls positive symptoms (delusions, hallucinations and bizarre behavior) in about 70% of patients. However, the effects of typical antipsychotic drugs on negative symptoms (e.g., affective flattening, anhedonia, anergia or avolition) and cognitive impairment are much less than the effects observed on positive symptoms (Leucht et al. 2003). The efficacy of antipsychotic drugs to reduce positive symptoms is related to their ability to antagonize central dopaminergic neurotransmission mediated by dopamine D_2 receptors in the mesolimbic system, while antagonism of striatal D_2 receptors is responsible for their ability to produce a wide range of immediate and delayed extrapyramidal symptoms (EPS), which may secondarily cause or worsen negative symptoms (Carlsson 1978). In contrast to the clinical profile of typical antipsychotics, the dibenzodiazepine agent clozapine is unique, demonstrating significantly greater efficacy against positive and negative symptoms in treatment-resistant schizophrenic patients with a low incidence of EPS (Kane et al. 1988), efficacy

against suicidality in schizophrenia (Meltzer et al. 2003), efficacy and motoric tolerability in Parkinson's Disease patients with psychosis (The Parkinson Study Group 1999), and, importantly, the ability to improve cognitive function (Hagger et al. 1993). These characteristics have led to an intensive search for the molecular mechanism(s) of action of clozapine that differentiate it from the rest of this class of agents.

Antipsychotics possess a complex pharmacology across the biogenic amine receptor family, which is largely based on affinity constants derived from radioligand-binding techniques (http://pdsp.cwru.edu/). However, these techniques lack the ability to detect functional drug-receptor interactions and to discriminate between agonists and antagonists (which include partial agonists, neutral antagonists, and inverse agonists). Antipsychotic drug-receptor interactions have been assessed using functional assays; yet technical aspects of these studies often limit these analyses to only a small subset of drugs at a given receptor, and require the comparison of results from different assays (stimulation or inhibition of cAMP or GTPγS, or PI hydrolysis assays). We reasoned that systematic profiling, utilizing a single functional assay, of a large number of clinically useful therapeutic agents, including nearly all known antipsychotics and their metabolites, might reveal previously unappreciated aspects of their drug actions. The importance of inclusion of metabolites is illustrated by risperidone, whose active principle in 90% of patients is its major metabolite, 9-hydroxyrisperidone (Spina et al. 2001).

Methods

Molecular profiling of clinically relevant drugs was performed at all known monoaminergic receptor subtypes except the Dopamine D_4, Serotonin 5_A, and Histamine H_4 receptors using Receptor Selection and Amplification Technology (R-SAT) assays. R-SAT assays were performed as previously described (Weiner et al. 2001; Spalding et al. 2002; Wellendorph et al. 2002; Weissman et al. 2003). Briefly, HIH/3T3 cells plated at 70–80% confluency were transfected with various receptor cDNA (10–100 ng receptor and 20 ng β-Gal reporter/well of a 96 well plate) using the Polyfect Reagent (Qiagen, Inc.) as described in the manufacture's protocol. One day after transfection, ligands were added in Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 2% Cyto-SF3. After 4–6 days, the media was aspirated off, the cells were lysed, *O*-Nitrophenyl-beta-D-Galactopyranoside (ONPG) was added and the resulting absorbance was measured spectrophotometrically. Concentration response curves were performed as eight-point concentration response experiments run in duplicate, where the maximal antipsychotic concentrations varied from 10 to 25 μM and data were analyzed using Excel fit and Graph Pad Prism. Reported EC_{50} values represent the concentration of a ligand that produces a half-maximal response from a receptor in the absence of other ligands, and IC_{50} values represent the concentration of a ligand that inhibits half of the agonist-induced activity. Competitive antagonist IC_{50} data were adjusted for agonist occupancy using the equation $K_i = IC_{50}/\{1 + [\text{agonist}]/EC_{50}\ \text{agonist}\}$. Data are reported as negative log values (pEC50 and pK_i). Sources of the drugs utilized in this study were previously described (Weiner et al. 2001; Wellendorph et al. 2002) with the exception of *N*-desmethylclozapine (NDMC), which was acquired from Sigma, Inc., and *N*-desmethylolanzapine, which was synthesized by ACADIA Pharmaceuticals. A list of the compounds screened can be found as "Supplemental information".

PI hydrolysis assays were performed on Chinese Hamster Ovary cells stably transfected with the human M1 muscarinic receptor cDNA as previously described (Spalding et al. 2002), and the data are derived from six or eight-point concentration response experiments performed in duplicate.

MAP Kinase assays utilized C57BL6 mice treated subcutaneously with either vehicle, clozapine, or NDMC with or without scopolamine, sacrificed 2 h later, and phospho-MAPK immunoreactivity was assayed as previously described (Berkeley et al. 2001). Briefly, after treatments which were administered s.c. at 60 min., mice were perfused with 100 ml of 4% paraformaldehyde followed with 100 ml of 10% sucrose. Brains were removed and cryoprotected in 30% sucrose overnight at 4°C. The next day, 50 μm slices were cut on a sliding microtome. Slices were rinsed, treated with 3% H_2O_2 for 10 min at room temperature and rinsed again. Slices were blocked in PBS containing 10 μg/ml avidin (Vector Laboratories Burlingame, CA), 0.1% triton-X and 4% normal goat serum (NGS) for 1 h. Slices were rinsed and incubated in PBS containing 50 μg/ml biotin (Vector Laboratories Burlingame, CA), 2% NGS, and phospho-ERK1/2 antibody (Cell signal Technologies, Beverly, MA) at a concentration of 1:250 and allowed to incubate overnight at 4°C. The next day, slices were rinsed and placed in PBS containing 2% NGS and biotinylated goat anti-rabbit (Vector Laboratories Burlingame, CA) at a concentration of 1:100 for 1 h at 4°C. Slices were rinsed and placed in horseradish peroxidase-conjugated avidin–biotin complex (Vector Laboratories Burlingame, CA) for 1 h at 4°C. Slices were rinsed and incubated in TSA Fluorescein tyramide for 10 min at room temperature. Slices were treated with 10 mM $CuSO_4$ for 30 min, mounted onto glass slides with Vectashield mounting media (Vector Laboratories Burlingame, CA). Slides were visualized via a fluorescence microscope and digital images were analyzed with Scion image analysis software (Scion Corp. Frederick, MD).

Stepwise multiple-regression analysis, including the dependent measure, dose, age, and gender was utilized to assess the contribution of NDMC to treatment response in schizophrenic subjects (Hasegawa et al. 1993; Lee et al. 1999). The analysis was adjusted for baseline level of symptom severity, age, and dose, since dose was not fixed. The plasma samples chosen for the analyses were obtained

at 6 weeks and 6 months after initiation of therapy, were related to the clinical measures obtained at those times, and were drawn 12 h after the last clozapine dose. Only subjects who had received at least 100 mg of clozapine per day were included in the analysis, and some data were unavailable for these subjects at some time points. Regarding co-treatment with anticholinergic agents, only two subjects in this sample were treated with benztropine. The results did not differ when data from these two subjects were omitted (data not shown). Lastly, ten of the patients in this study were treated with benzodiazepines at the time the levels of clozapine and NDMC were measured. Benzodiazepines have not been reported to affect the metabolism of clozapine.

Results and discussion

We profiled a library of 462 clinically relevant drugs for functional activity at 33 of the 36 known human monoaminergic *G*-protein coupled receptors using the mammalian cell-based functional assay R-SAT. Table 1 illustrates data on representative antipsychotic agents for receptors at which the most potent activities were observed. Competitive antagonism of D_2 receptors, and inverse agonism of 5-HT_{2A} receptors was nearly uniform throughout this class, with typical agents demonstrating low $5HT_{2A}/D_2$ ratios, and atypical agents demonstrating high ratios (Meltzer et al. 1989; Weiner et al. 2001). Inverse agonism of H_1 receptors was commonly observed, where clozapine and olanzapine displayed particularly high potency (Weiner et al. 2001). Many compounds showed antagonist activity at $alpha_1$-adrenergic receptors, fewer agents exhibited potent 5-HT_6 activity, while many, particularly risperidone, displayed potent inverse agonist activity at 5-HT_7 receptors. Clozapine, olanzapine, and a number of typical agents (e.g., thioridazine, data not shown), were found to possess potent muscarinic receptor antagonist properties. Importantly, no single antagonist activity differentiated clozapine from all other agents.

In contrast to the widespread antagonist activity of these compounds, very few agents possessed agonist activity. Figure 1 reports the results of the functional agonist screen of this compound library at the human M1 muscarinic acetylcholine receptor. Only four compounds, the known muscarinic receptor agonists arecoline and carbachol, moperone and NDMC, the major metabolite of clozapine (Gauch and Michaelis 1971), were identified. Moperone displayed only a very low potency (EC_{50}>1 μM) interaction. In contrast, NDMC displayed an EC_{50} of 100 nM with 80% efficacy (relative to carbachol) in this study. We then sought to confirm this result in a second functional assay, PI hydrolysis. As depicted in Fig. 1, clozapine displays limited agonist efficacy in this assay, precluding accurate potency determinations, whereas NDMC displayed high potency (93±22 nM, *n*=3) and greater agonist efficacy (56±8%, *n*=3) relative to carbachol. In fact, when assayed against carbachol for competitive antagonist activity, clozapine behaved as an antagonist, while NDMC only partially reversed carbachol-induced PI

Table 1 Pharmacological activities of antipsychotics at human monoamine receptors. Potency data for five representative antipsychotics and the clozapine metabolite *N*-desmethylclozapine (NDMC) at 13 human monoamine receptor subtypes are shown. Potency data are reported as pK_i values for the competitive antagonist studies, while inverse agonist data are reported as pEC_{50} values, both derived from three to eight separate determinations ± standard error. *Asterisks* indicate the presence of agonist activity where the muscarinic receptor agonist potencies are reported in Table 2. Ziprasidone displays limited but detectable agonist efficacy at human 5-HT_{1A} receptors (<30% relative to 8-OH-DPAT), and a K_i>1 μM when assayed as a competitive antagonist. NDMC *N*-desmethylclozapine, 5-HT serotonin, H histamine, M muscarinic, D dopamine, and Alpha-alpha adrenergic, and nr no response defined as no significant antagonist or inverse agonist activity at concentrations up to 1 μM

Receptor	Haloperidol	Risperidone	Ziprasidone	Olanzapine	Clozapine	NDMC
Competitive antagonist (pK_i)						
D_2	10.0±0.1	9.3±0.1	8.3±0.3	8.4±0.2	7.7±0.1	7.2±0.1
5-HT_{2A}	7.3±0.1	9.7±0.1	8.6±0.1	8.6±0.1	8.3±0.2	8.3±0.2
5-HT_{1A}	nr	nr	nr*	nr	nr	nr
5-HT_{2C}	nr	7.2±0.3	7.4±0.2	7.4±0.1	7.4±0.2	7.8±0.2
H_1	nr	7.0±0.2	nr	8.4±0.1	9.5±0.2	8.2±0.2
M_1	nr	nr	nr	7.2±0.2	7.8±0.2	nr*
M_2	nr	nr	nr	6.9±0.1	nr*	nr*
M_3	nr	nr	nr	6.7±0.5	8.2±0.2	6.8±0.7*
M_4	nr	nr	nr	7.4±0.3	nr*	nr*
M_5	nr	nr	nr	7.2±0.2	7.5±0.3	nr*
D_3	9.7±0.1	7.9±0.4	7.5±0.3	7.6±0.4	6.3±0.1	nr
Alpha $_{1A}$	7.4±0.1	8.5±0.1	7.4±0.2	7.4±0.2	8.1±0.1	7.3±0.1
Alpha $_{2A}$	nr	7.7±0.1	nr	nr	nr	nr
Inverse agonist (pEC50)						
5-HT_{2A}	6.8±0.1	9.0±0.3	8.8±0.3	7.8±0.1	8.0±0.3	8.0±0.3
5-HT_{6A}	nr	nr	nr	7.4±0.2	7.0±0.2	6.9±0.1
5-HT_{7A}	nr	9.1±0.2	7.3±0.1	nr	7.4±0.1	7.3±0.1

hydrolysis (Fig. 1), consistent with the lack of an antagonistic response observed when NDMC was tested as a competitive antagonist at M1 receptors in R-SAT (Table 1). Finally, the agonist activity of NDMC was blocked by both atropine and clozapine (Fig. 1). These results confirm that NDMC is a potent, efficacious, M1 receptor agonist, distinguishing it from the M1 receptor antagonist properties of clozapine.

Having demonstrated the agonist activity of NDMC at human M1 receptors in multiple in vitro functional assays, we then profiled carbachol, clozapine, NDMC, olanzapine, the major olanzapine metabolite *N*-desmethylolanzapine, and the muscarinic agonist xanomeline (Shannon et al. 1994), at all five human muscarinic receptor subtypes using R-SAT (Table 2). Clozapine was found to be a very weak partial agonist at M1 receptors, a more efficacious agonist at M2 and M4 receptors, and to lack agonist activity at M3 and M5 receptors. NDMC also displayed high potency interactions with all five human muscarinic receptors, but with increased agonist efficacy at M1, M4, and M5 receptors when compared to clozapine (Table 2). In contrast, olanzapine and *N*-desmethylolanzapine, both structurally related to clozapine and NDMC, lacked agonist activity at human muscarinic receptors. Interestingly, xanomeline displayed a muscarinic receptor profile that is similar to that observed for NDMC, with the notable exception of higher agonist efficacy at M3 receptors. The agonist activities of clozapine, NDMC,

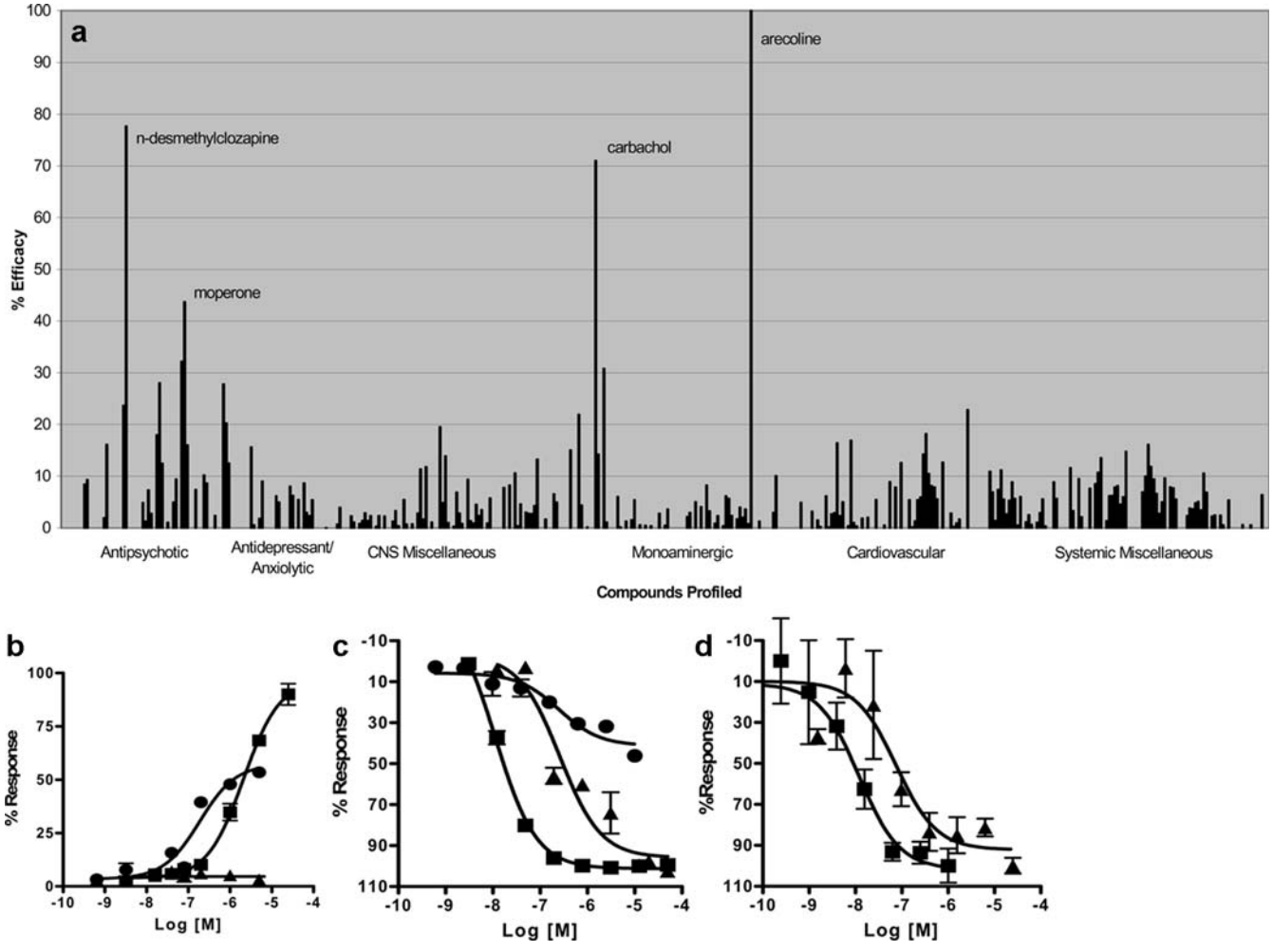

Fig. 1 Identification of *N*-desmethylclozapine as an M1 muscarinic receptor agonist. *Panel A* reports the muscarinic M1 receptor agonist activity of a library of 462 compounds as determined by R-SAT assays. M1 receptor efficacy data shown are derived from the 1 μM concentration of compound, and are reported as percentage efficacy relative to the maximal response observed for a saturating 40 μM concentration of carbachol (100%). *Panels B–D* report PI hydrolysis data utilizing Chinese Hamster Ovary cells stably transfected with the human M1 receptor gene. *Panel B* depicts agonist responses reported as the percentage response observed for carbachol. Drugs depicted are carbachol (*squares*), clozapine (*triangles*), and *N*-desmethylclozapine (*circles*), with observed potencies (pEC_{50}) of carbachol (5.7), *N*-desmethylclozapine (6.7), and clozapine (no response). *Panel C* depicts competitive antagonist responses obtained in the presence of a 3 μM concentration of carbachol, and are reported as the percentage response observed for atropine (100%). Drugs depicted are atropine (*squares*), clozapine (*triangles*), and *N*-desmethylclozapine (*circles*), with observed potencies (pK_i) of atropine (8.5), *N*-desmethylclozapine (no response), and clozapine (7.1). *Panel D* depicts competitive antagonist responses obtained in the presence of a 0.15 μM concentration of *N*-desmethylclozapine, and are reported as the percentage response observed for atropine (100%). Drugs depicted are atropine (*squares*), and clozapine (*triangles*), with observed potencies (pK_i) of atropine (8.4), and clozapine (7.6)

Table 2 Muscarinic acetylcholine receptor agonist activity of antipsychotics. Muscarinic receptor (M1–M5) agonist activity of clozapine, *N*-desmethylclozapine, olanzapine, *N*-desmethylolanzapine, xanomeline, and carbachol was determined using R-SAT as previously described (Spalding et al. 2002). Average efficacy (percentage relative to carbachol) and potency (pEC_{50}) ± standard error are reported for three or more replicate determinations. No response denotes the lack of agonist activity at concentrations up to 10 μM

Compound	M1		M2		M3		M4		M5	
	Efficacy	pEC50	Efficacy	pEC50	Efficacy	pEC50	Efficacy	pEC50	Efficacy	pEC50
Clozapine	24±3	7.63±0.37	65±8	6.23±0.14	No response		57±5	7.35±0.10	No response	
N-desmethylclozapine	72±5	7.26±0.07	106±19	6.47±0.21	27±4	6.49±0.18	87±8	6.87±0.17	48±6	7.63±0.25
Olanzapine	No response		No response		No response		No response		No response	
N-desmethylolanzapine	No response		No response		No response		No response		No response	
Xanomeline	121±6	7.20±0.08	106±9	6.30±0.23	66±6	6.63±0.21	116±9	7.46±0.14	86±12	6.59±0.22
Carbachol	101±2	6.11±0.03	101±5	6.23±0.09	102±3	6.53±0.04	96±3	6.53±0.05	105±3	6.76±0.12

and xanomeline at human muscarinic receptor subtypes are unique among all neuropsychiatric agents tested (Fig. 1, and Tables 1, 2).

The discovery of the efficacious muscarinic receptor agonist activity of NDMC prompted us to explore the possibility that muscarinic receptor agonism, and M1 receptor agonism in particular, may be achieved in vivo during pharmacotherapy with clozapine. We tested clozapine and NDMC for their ability to increase the phosphorylation of mitogen-activated protein kinase (MAP kinase) in the CA1 region of mouse hippocampus, a response that has been shown to reflect M1 receptor activation (Berkeley et al. 2001). As depicted in Fig. 2, subcutaneous administration of vehicle (Panel A), clozapine (Panel B), or scopolamine alone (data not shown) fails to stimulate phosphorylation of hippocampal MAP kinase. In contrast, NDMC induced phosphorylation of MAP kinase in hippocampal neurons in a dose dependent

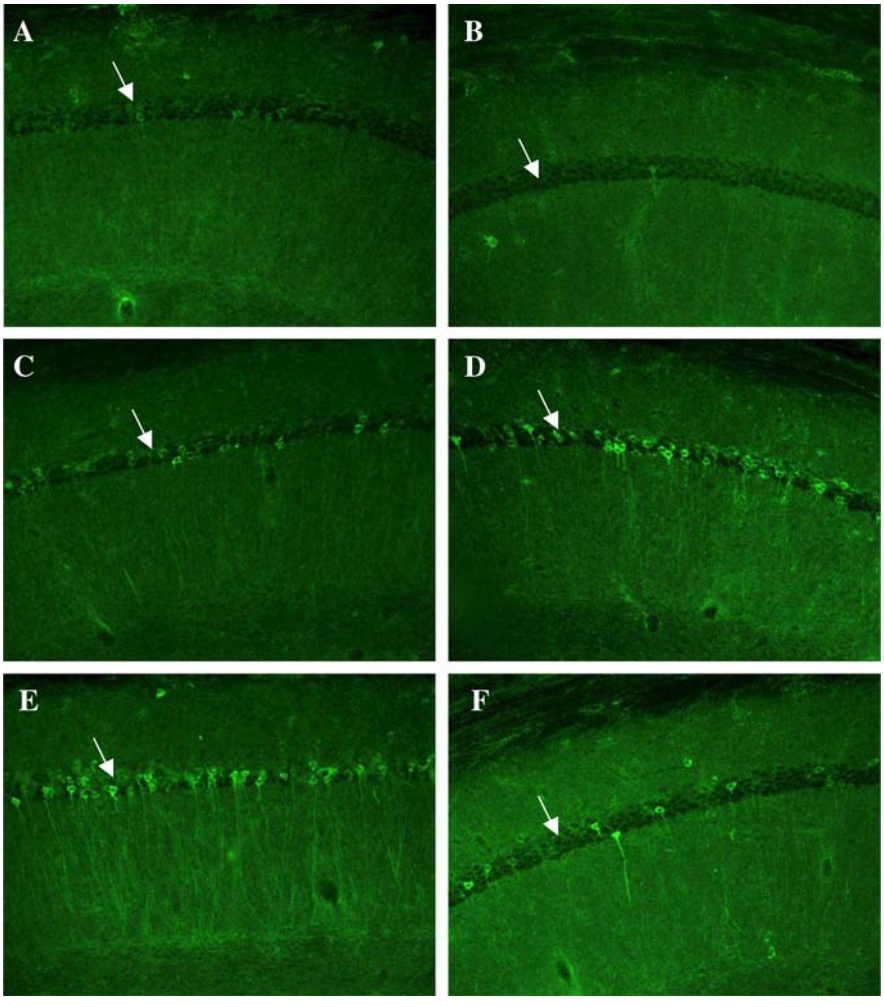

Fig. 2 M1 muscarinic receptor agonist activity of *N*-desmethylclozapine in mouse hippocampus. Phospho-MAPK immunoreactivity in the cell bodies and proximal dendrites of CA1 pyramidal cells (highlighted by *arrows*) is shown following the administration of vehicle **a**, clozapine at 30 mg/kg **b**, *N*-desmethylclozapine at 10 **c**, 30 **d**, 100 **e**, or *N*-desmethylclozapine (30 mg/kg) and **f** scopolamine (0.3 mg/kg, i.p.)

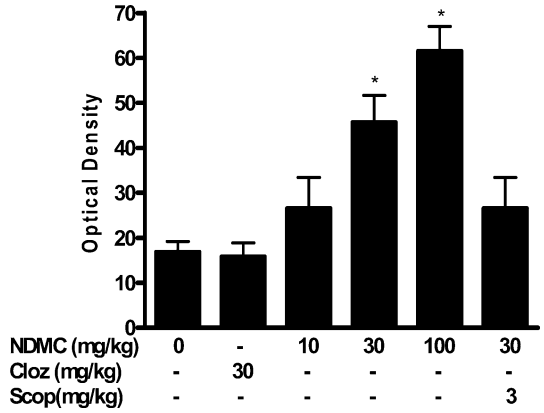

Fig. 3 Quantification of M1 muscarinic receptor agonist activity of *N*-desmethylclozapine in mouse hippocampus. Quantification of phospho-MAPK immunoreactivity was performed via computer calculated optical density measurements of the CA1 region of the hippocampus from four mice, where (*asterisk*) indicates a significant difference to vehicle treatment using a one factor ANOVA post hoc Dunnett's test ($F_{(5,23)}$=10.88, P<0.0001)

manner (Panels C, D, and E), an effect that was blocked by pretreatment with scopolamine (Panel F). Quantification of this effect demonstrates statistically significant M1 receptor activation at NDMC doses of 30 mg/kg and greater (Fig. 3). Clozapine fails to behave as an agonist under these experimental conditions, which likely reflects either insufficient metabolism to NDMC after acute administration in mouse, or direct antagonist effects at the M1 receptor as demonstrated in the in vitro studies. These data confirm that NDMC passes the blood brain barrier and activates hippocampal M1 receptors in vivo.

It has long been appreciated that antagonism of central muscarinic receptors can attenuate the EPS induced by antipsychotics (Miller and Hiley 1974). Initial investigations of the anti-muscarinic properties of antipsychotics defined the high potency of clozapine for these receptors in rodent brain, and elucidated the inverse correlation between muscarinic receptor antagonism and propensity to induce EPS (Snyder et al. 1974). Following the elucidation of five muscarinic acetylcholine receptor subtypes (Bonner et al. 1987), clozapine was described as a potent competitive antagonist (Bolden et al. 1991). Functional studies in various cell lines subsequently documented that clozapine has significant agonist activity at M2 and M4 receptors, and low agonist efficacy at M1 receptors (Zorn et al. 1994; Olianas et al. 1999), consistent with the results reported herein. In humans, clozapine has two major metabolites, NDMC and clozapine-*N*-oxide (Gauch and Michaelis 1971). After steady state dosing, NDMC represents a large proportion of total detectable moieties, with concentrations ranging from 20 to 150% of that observed for clozapine, with mean values of 60–80% (Bondesson and Lindstrom 1988; Perry et al. 1991). That NDMC is an active metabolite is supported by the present data, as well as by prior reports documenting D_1, D_2, and 5-HT_{2C} receptor competitive antagonist activity (Kuoppamaki et al. 1993), and a recent report of M1 receptor agonist activity (Sur et al. 2003). In contrast, the other major clozapine metabolite, clozapine-*N*-oxide, displays only very low potency (pK_i's<6.0) functional activity at human monoaminergic receptors (data not shown). While varying degrees of brain penetration of NDMC have been reported in rodents (Baldessarini et al. 1993; Weigmann et al. 1999), the present results, the observation that systemically administered NDMC activates cFOS expression in rodent brain (Young et al. 1998), and the detection of NDMC in human cerebrospinal fluid following parenteral administration of clozapine (Nordin et al. 1995), demonstrate that NDMC is brain penetrant and centrally active.

Our results suggest that clozapine, acting through its predominant metabolite NDMC, may function as a direct acting muscarinic receptor agonist in vivo. During pharmacotherapy with clozapine, the agonist actions of NDMC would be attenuated by the antagonistic actions of the parent compound. Thus, high NDMC levels, and particularly high NDMC/clozapine ratios, would increase agonist efficacy at muscarinic receptors, as predicted by mass action and by agonist/antagonist mixing studies (Brauner-Osborne et al. 1996). Clinical data support this notion. Not only does clozapine therapy usually lack the traditional anti-cholinergic side effects of dry mouth, blurred vision, and urinary retention common to classical muscarinic antagonists, it is unique in its ability to frequently produce sialorrhea (Baldessarini and Frankenburg 1991) an effect that can be blocked by the muscarinic antagonist pirenzepine (Fritze and Elliger 1995). Thus, the muscarinic receptor agonist activity of NDMC likely mediates this peripheral effect, while the muscarinic receptor subtype responsible is still unknown, receptor subtypes in addition to the M3 have been implicated (Bymaster et al. 2003).

We hypothesized that the muscarinic agonist properties of NDMC reported herein may underlie some of the unique central effects of treatment with clozapine. This hypothesis is attractive, as multiple lines of evidence support a pro-cognitive effect of potentiating central cholinergic neurotransmission, including the clinical effects of acetylcholinesterase inhibitors and direct acting muscarinic receptor agonists (Davis et al. 1993). High dose clozapine therapy in treatment refractory schizophrenics may actually serve to raise brain levels of NDMC to achieve central muscarinic receptor agonist activity, particularly M1 receptor stimulation, rather than recruiting additional lower potency receptor interactions that clozapine and NDMC possess (Table 1). If this hypothesis is correct, NDMC/clozapine ratios should be a better predictor of therapeutic response to clozapine, particularly for cognition, than absolute clozapine levels.

To test this hypothesis, we have re-analyzed data on clozapine and NDMC plasma levels and clinical response that were prospectively gathered as part of two clinical trials which included 59 neuroleptic resistant patients (Hasegawa et al. 1993), and 33 neuroleptic responsive patients (Lee et al. 1999) with schizophrenia. Patients were classified as treatment-resistant or not by standard criteria (Kane et al. 1988), and clinical ratings and neuropsycho-

logical test scores were obtained by trained raters who were blinded to plasma drug levels. The mean daily dosages of clozapine, as well as clozapine and NDMC serum levels, and NDMC/Clozapine ratios after 6 weeks and 6 months of treatment are reported in Table 3. Both time points were analyzed because improvement in psychopathology and cognition with clozapine may take 6 months or longer (Hagger et al. 1993). Thirteen of the 92 patients (14.1%) had NDMC/clozapine ratios ≥1. Of these thirteen patients, the highest ratio was 1.77 and the median was 1.05. The Spearman rank order correlation between clozapine and NDMC levels was 0.82 and 0.89 at 6 weeks and 6 months, respectively (P=0.0001). The correlation between NDMC/clozapine ratios at 6 weeks and 6 months was 0.92 (P=0.0001), indicating remarkable stability of NDMC/clozapine ratios within subjects. Importantly, dose and NDMC/clozapine ratios were not significantly correlated at either time point ($\rho<0.10$) in neither the neuroleptic-resistant nor neuroleptic-responsive patients.

We utilized stepwise multiple-regression to determine the best predictors of outcome from each of these measures, including baseline levels of the dependent measure, dose, age, and gender, since all have been shown to significantly predict response to clozapine (Table 3). In all the models tested, baseline levels of the dependent measure predicted the largest share of the variance in the model. As predicted, the NDMC/clozapine ratio was the next most frequent predictor of response; the ratio significantly predicted response in 8/24 (33.3%) of the models, all in the expected direction: the higher the ratio, the better the outcome. This result contrasts with the lack of predictive power of clozapine levels alone, NDMC levels alone, or their sum. The exception was that higher NDMC levels alone predicted greater improvement in two subscales of the Quality of Life scale (Heinrichs et al. 1984) (data not shown). As shown in Table 3, higher NDMC/clozapine ratios predicted improvement in multiple measures of cognition, as well as the Scale for the Assessment of Negative Symptoms—Attention subscale, which has been suggested to be more related to cognition than negative symptoms. The ratio also predicted improvement in quality of life-total score, including the instrumental role function factor, which has been shown to be dependent upon cognitive status (Green 1996), and negative symptoms, which have been found to correlate with cognition. The ratio also predicted improvement in delusions, but not hallucinations, with clozapine treatment. Dose did not contribute to the prediction of any of the models in Table 3. Dose is significantly correlated with plasma levels of clozapine and NDMC (P=0.01–0.001) but not, as noted above, with the NDMC/clozapine ratio. This provides further evidence that the absolute levels of clozapine and NDMC, while important in identifying responders and non-responders (Fabrazzo et al. 2002) are

Table 3 Serum *N*-desmethylclozapine levels and clinical response in schizophrenia. Statistical analysis of the correlation between clinical outcome and serum levels of clozapine and *N*-desmethylclozapine (NDMC) for a cohort of 92 clozapine treated schizophrenics are reported. Group one reports the clozapine dose, clozapine level, NDMC levels, and NDMC/clozapine ratios for all treatment-resistant (TR) subjects, responders, non-responders, and all subjects at 6 weeks and 6 months. *P** reports statistically significant differences between responders and non-responders. Group two reports the major relationships of interest for the prediction of the contribution of NDMC to response to clozapine treatment, including quality of life, negative symptoms, and cognition, analyzed by multiple linear regression. R^2** refers to the model applied. NS not significant, BPRS Brief Psychiatric Rating Scale, SANS Scale for the Assessment of Negative Symptoms, SAPS Scale for the Assessment of Positive Symptoms, WISC Wisconsin Card Sorting test

Drug measure	All TR subjects (59)	Responders (26)	Non-responders (25)	*P**	All subjects at 6 weeks (86)	All subjects at 6 months (92)
Dose (mg/day)	468±190	485±205	433±178	NS	369±169	417±197
NDMC Level (ng/ml)	260±203	308±243	171±123	0.01	194±136	235±190
Clozapine level (ng/ml)	393±301	453±328	268±207	0.02	287±190	365±285
NDMC/clozapine	0.75±0.36	0.70±0.22	0.81±0.48	NS	0.83±1.08	0.71±0.30

Clinical measure	Beta	*F*	*P*	R^2**	df
Dependent variable: 6 weeks					
BPRS-withdrawal/retardation	−0.52	3.73	0.06	0.32	3.73
SANS attentional impairment	−0.28	5.65	0.02	0.26	33.65
SAPS global delusions	−1.00	3.87	0.05	0.60	3.55
Quality of life scale: total	17.50	5.20	0.03	0.50	2.40
Quality of life scale: objects and activities	2.91	7.10	0.01	0.43	2.40
Quality of life scale: instrumental role	13.80	14.84	0.01	0.54	2.39
WISC-R maze	2.27	4.10	0.05	0.75	4.33
Dependent variable: 6 months					
Petersen's consonant trigram test	7.45	6.75	0.01	0.47	4.47
WISC-categories formed	1.35	3.67	0.06	0.47	3.48

not as important as their ratio when baseline levels of the dependent measure are included in the model. Although additional analyses in larger cohorts are necessary, this analysis, as well as recent reports (Frazier et al. 2003; Mauri et al. 2003) all suggest that the NDMC/clozapine ratio is a better predictor of clinical response to clozapine than clozapine levels alone, and support the hypothesis that NDMC is a critical mediator of clozapine action.

The muscarinic receptor agonist properties of NDMC may also contribute to the efficacy of clozapine therapy against positive symptoms. Not only did high NDMC/clozapine ratios predict response to delusions as noted above, but additional support comes from the observation that there are several similarities between the central effects of muscarinic receptor agonists and dopamine D_2 receptor antagonists (Pfeiffer and Jenney 1957; Mirza et al. 2003). For example, behavioral pharmacological experiments with mice harboring targeted deletions of each of the five muscarinic receptor subtypes have shown that the M1 receptors plays a central role in DA-mediated behaviors (Gerber et al. 2001). In addition, xanomeline (which displays some selectivity for M1 and M4 receptors) inhibits amphetamine-induced locomotion (Shannon et al. 2000). Clinically, xanomeline was found to diminish hallucinosis and aggression in Alzheimer's Disease patients (Bodick et al. 1997), and has been shown to display activity against both positive and negative symptoms in a recent, small, Phase two study in schizophrenia (Schekhar et al. unpublished data).

The central dopaminergic and muscarinic cholinergic systems are well known to be functionally interrelated (Miller and Hiley 1974). The muscarinic antagonist properties of clozapine are thought to contribute to its low propensity to cause EPS, yet the anti-EPS effects of clozapine are more robust than those obtained by the adjunctive use of anticholinergics agents like trihexyphenidyl, and some EPS producing antipsychotics, e.g., thioridazine, also possess potent muscarinic receptor antagonist properties. These observations suggest that although antagonism of central muscarinic receptors can confer anti-EPS effects, cholinergic modulation of the motoric effects of D_2 receptor blockade are more complex than previously appreciated. Present data suggest that agonism, not antagonism, of certain muscarinic receptor subtypes expressed within critical basal ganglia structures (Weiner et al. 1990), may be a more efficacious mechanism to lessen these adverse motor effects. Further, the widespread use of adjunctive anticholinergics should be re-evaluated in light of the present data on the pro-cognitive benefits conferred by the central muscarinic receptor agonist properties of NDMC.

In summary, functional characterization of therapeutically useful neuropsychiatric drugs has revealed the potent, efficacious, muscarinic receptor agonist activity of NDMC. This activity was found to be unique among neuropsychiatric agents as a class. We demonstrate that NDMC can cross the blood brain barrier and function as an M1 receptor agonist in vivo. Consideration of the contribution of NDMC to improvement in cognition and quality of life in clozapine treated patients suggests that NDMC mediates clinically relevant aspects of treatment response that differentiate clozapine from other agents used to treat schizophrenia. These findings, when interpreted in the setting of the known clinical effects of clozapine and cholinergic agonists, provide additional evidence that muscarinic receptor agonism mediates the unique clinical properties of clozapine, and that M1 muscarinic receptor agonists (Spalding et al. 2002), including NDMC itself, may be efficacious atypical antipsychotic agents.

Supplemental information

Drugs screened, grouped according to clinical class, included: antipsychotics; amoxapine, amisulpiride, amperozide, bromperidol, butaclamol, chlorproethazine, chlorpromazine, chlorprothixene, *Cis*-flupentixol, clothiapine, clozapine, droperidol, fananserin, fluphenazine, fluspirilene, haloperidol, loxapine, mazapertine, M100907, melperone, mesoridazine, molindone, *N*-desmethylclozapine, *N*-desmethylolanzapine, ocaperidone, octoclothepin, olanzapine, perazine, perlapine, pimozide, pimpamperone, promazine, prothypendyl, quetiapine, remoxipride, risperidone, sertindole, spiperone, sulpride, sultopride, telfludazine, thioridazine, thiothixene, tiapride, moperone, tiospirone, *trans*-flupentixol, trifluoperazine, trifluoperidol, triflupromazine, and ziprasidone. Antidepressants/anxiolytics; acetyltryptophan, acetyltryptophanamide, alaprocate, alprazolam, amitriptyline, barbital, bromazepam, buproprion, buspirone, chloral hydrate, clobazam, clonazepam, clomipramine, clorgyline, chlordiazepoxide, chlormezanone, continine, compazine, desipramine, deprenyl, desmethyldiazepam, diazoxide, doxepin, flumazenil, flunitrazepam, fluoxetine, flurazepam, fluvoxamine, imipramine, indatraline, iproniazid, maprotiline, meprobamate, milnacipram, minaprine, mirtazepine, modafinil, nitrazepam, nomifensine, nortriptyline, oxazepam, pargyline, phenelzine, prazepam, protripytline, rolipram, tracazolate, tranylcypromine, trazadone, triazolam, trihexaphendyl, trimipramine, viloxazine, zimelidine, zolpidem, and zopiclone. CNS miscellaneous; 3PPP, 5-aminopentanoic acid, 5-hydroxy MDA, 5-methoxy DMT, 5-methoxytryptamine, acetaminophen, acetylsalicylic acid, alprenelol, amantadine, amiodarone, AMPA, apocodeine, apomorphine, atropine, baclofen, balperidone, benztropine, bicuculline, bradykinin, bretylium, BRL 37344, bromocriptine, cannabidiol, carbemazepine, carbidopa, cyproheptadine, cirazoline, D-amphetamine, (D-Ser2)-Leu Enkephalin-Thr, (Leu 5) enkephalin, D-phenylalanine, dibucaine, diclofenac, dihydroergotamine, DOI, domperidone, ebalzotan, edrophonium, ephedrine, etadolac, ethosuxamide, felbamate, fenbufen, GABA, gabaxadol, galanthamine, gamma-vinyl GABA, gabapentin, (−) GMC III, (+) GMC III, heroin, himbacine, I-4-AA, ICI 204448, indoprofen, isoguvacine, ketamine, ketaprofen, labetalol, lamotrigine, levallorphan, lidocaine, lisuride, L-745–870, melatonin, metoclopromide, memantine, mescaline, naftopidil, nalbuphine, *N*-

allyl SKF 38393, naloxone, naltrexone, naltrindole, neostigmine, nicotine, nipecotic acid, *N*-methyl ICI 118–551, *N*-methyldopamine, *N*, *N*-dimethyl MDA, norapomorphine, norcodeine, norfenfulramine, normetazocine, oxethazine, pemoline, pergolide, PCP, phaclofen, phenacetin, phenteramine, phenoxybenzamine, phenytoin, physostigmine, *P*-iodoclonidine, pirenzepine, prilocaine, primodone, procaine, prochlorperazine, propranolol, pseudoephedrine, quinpirole, raclopride, rauwolscine, reserpine, rimcazole, RO-05-3663, RS 100329, RX 821002, saclofen, salicylamide, SCH 12679, SCH 23390, scopolamine, SKF 81297, SKF 38393, SKF 82948, SKF 82957, SKF 83566, SR 141716A, SR 144528, succinylcholine, tenoxicam, terguride, tetracaine, tolazoline, tropicamide, UK 14304, valproate, vigabatrin, WIN 55212-2, xylazine, yohimbine, and zomepirac. Monoaminergic; 7-OH-DPAT, 8-0H-DPAT, alpha methyl serotonin, arecoline, astemizole, bethanacol, carbachol, CGS 12066A, cinanserin, chlorpheniramine, cimetidine, clobenpropit, CPP, dihydroergocristine, dimaprit, diphenhydramine, doxylamine, eltoprazine, famotidine, histamine, imetit, isomaltane, ketanserin, loperamide, L-tryptophan, LY 53857, mCPP, mesulergine, metergoline, methergine, methiothepin, methysergide, mexamine, mianserin, MK 212, mepyramine, pheniramine, phenylbiguanide, pimethixene, piperazine, pirenpirone, prazosin, promethazine, pyrilamine, quiapazine, ranitidine, ritanserin, SB 204741, SB 206553, serotonin, spiroxatrine, sumitriptan, thioperamide, tripellenamine, triprolidine, and WB 4101. Cardiovascular; acetazolamide, adenosine, albuterol, atenolol, amiloride, amrinone, bepridil, caffeine, catopril, CGS-15943, CGS-21680, CGP-12177A, chlorothiazide, clonidine, debrisoquin, digitoxin, digoxin, diltiazem, dipyridamole, disopyramide, dobutamine, doxazosin, DPCPX, epinephrine, enalapril, flunarizine, furosemide, guanabenz, guanethidine, hydralazine, hydrochlorothiazide, isoproterenol, isosorbide, lidocaine, linisopril, metaproterenol, methoxamine, metrifudil, metolazone, metoprolol, midodrine, minoxidil, *N*-acethylpocainamide, nicardipine, nifedipine, nimodipine, nitrendipine, norepinephrine, nylidrin, oxymetazoline, paraxanthine, pentoxifylline, phentolamine, pinacidil, pindolol, procainamide, propranalol, quinidine, spironolactone, theophylline, theoyphylline 1-3, timolol, triamterene, urapidil, verapamil, and warfarin.

Systemic miscellaneous; acyclovir, adephenine, allupurinol, amodiaquine, 6-bromo-APB, artemisinin, azathioprine, azithromycin, camphor, capsaicin, carbetapentane, carisoprodol, cefotaxime, cinchonidine, chloramphenicol, chloroquine, chlorpropamide, chlorzoxazone, clarithromycin, clofilium, clotrimazole, cyclobenzaprine, D-cycloserine, danazol, dantrolene, dextromethorphan, dimethadione, dropropizine, *E*-capsaicin, edoxudine, ethinimate, fipexide, fluconazole, foscarnet, gallamine, glibenclamide, glipizide, hypericin, ibuprofen, ifenprodil, indomethacin, isobutylmethylxanthine, kainic acid, ketoconazole, levorphanol, linopiridine, mazindol, meclizine, mefexamide, mefloquine, mephenesin, mesbeverine, methocarbamol, metoclopramide, metronidazole, MK 801, *N*-aminohexyl-5-chloronaphthalene-1-sulfonamide, *N*-methyl-D-aspartic acid, NCS 382, neophesperidin, nixoxetine, nocapine, octopamine, omeprazole, orphenadrine, oxyphenbutazone, papaverine, penicillamine, pentamidine, phenacemide, picrotoxin, pitrazepine, piracetam, piroxicam, primaquine, probenecid, pyrimethamine, quinine, ritodrine, saccharin, sulindac, suramin, SB 218795, thalidomide, tilorone, trimeprazine, tolazamide, tolbutamide, tolperisone, uridine, vidarabine, zaleplon, and zidovudine.

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