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Identification of 2-fluoro-8-methyl-11-(1-methyl-1,2,3,6-tetrahydropyridin-4-yl)-5*H*-dibenzo[b,e][1,4]diazepine with clozapine-like mixed activities at muscarinic acetylcholine, dopamine, and serotonin receptors

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Clozapine is the golden standard for treatment-resistant schizophrenia (TRS), and is more effective than olanzapine or quetiapine, which have a similar chemical structure.[1,2] These antipsychotics have polypharmacological action, *i.e.* having affinity for dopamine D_1/D_2, 5-HT_{2A}, *etc.*, which led Melzer to propose a "serotonin-dopamine antagonist theory".[3] Great efforts have been made to clarify the putative "magic receptor" responsible for the unique actions of clozapine.[4] Clozapine is metabolized *via* the hepatic microsomal enzymes (CYP 3A4 and 1A2), and the main metabolite is *N*-desmethylclozapine (NDMC).[5] In this regard, the agonistic activity of clozapine and NDMC at M_1 muscarinic acetylcholine receptors is of interest, and it is conceivable that the M_1 muscarinic acetylcholine receptor is the single molecular target responsible for the unique actions of clozapine (so-called M_1-hypothesis).[6] Xanomeline, a M_1/M_4 agonist, demonstrated efficacy in the treatment of schizophrenia, which also supported the M_1-hypothesis.[7] Weiner *et al.* reported that the M_1 agonistic activity of NDMC was stronger than that of clozapine, suggesting that NDMC plays a role in its therapeutic efficacy.[8] The effective plasma trough concentrations of clozapine and NMDC for TRS were reported to be 421 ng/ml (1.29 μM) and 262 ng/ml (0.83 μM), respectively.[9] We evaluated M_1 agonistic activity of clozapine and NDMC up to 10 μM. Consequently, the M_1 agonistic activity of clozapine was weak (EC_{50}; >10 μM, Emax; 26 ± 4%), whereas NDMC exhibited robust M_1 agonistic activity (EC_{50}; 0.048 μM, Emax; 75 ± 3%), consistent with a previous report.[8] We hypothesized that clozapine itself does not function as a M_1 agonist and behaves as an antagonist-like manner in the brain, thus we added clozapine and NDMC to M_1 receptor stably expressing CHO cells simultaneously at an appropriate concentration ratio and assessed their M_1 agonistic activity. As a result, activation of M_1 receptor by NDMC was attenuated by the concomitant presence of clozapine, and clear M_1 agonistic activity was observed when mixed at a 2.5:1 ratio (Emax; 60%, Fig. 1). As it is difficult to measure the clozapine/NDMC concentration in the human brain, M_1 agonism in the brain is complicated because it is like the co-administration of an M_1 agonist and antagonist. This may be a reason for the difficulty in understanding the M_1-hypothesis accurately. In the clinical use of clozapine for TRS, both responders and non-responders are present, but the plasma clozapine/NDMC ratio is consistent.[10] Comparing the structure of clozapine and NDMC, NDMC has an additional hydrogen bond donor, which should theoretically reduce blood–brain barrier penetration. Rodent animal pharmacokinetics (PK) revealed that the brain clozapine concentration exceeded the NDMC concentration by 3–4 fold.[11,12] Our rodent animal PK studies also confirmed this (clozapine/NDMC ratio in the brain was 3.2–6.2, data not shown). We hypothesized that brain penetration of NDMC differed from individual to individual, and highly permeable individuals may be responders, leading to M_1 agonism.

Agranulocytosis and clozapine-induced hypersalivation (CIH) are problematic side effects of clozapine. Agranulocytosis is life-threatening and it limits the wide use of clozapine. Reactive metabolite formation was proposed as a potential mechanism of agranulocytosis caused by clozapine.[13] CIH is a bothersome, socially stigmatizing side effect, which may affect nearly 30% of patients who take clozapine. Anti-cholinergic agents are useful to manage CIH and it is considered to be caused by systematic M_3 receptor stimulation.[14]

Compounds with a clozapine-like GPCR binding profile with strong M_1 agonism have not been reported so far. In order to demonstrate the clozapine M_1-hypothesis, we considered the following profile to be required: (i) robust M_1 agonism, (ii) clozapine-like binding affinity toward various GPCRs, (iii) diminish or reduce reactive metabolite formation, (iv) no or weak M_3 agonism, and (v) high brain permeability. We reported compound **1**, which has clozapine-like D_1/D_2 antagonistic activity with reduced reactive metabolite formation currently

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(Figure 2).[15]

As shown in Table 1, compounds **1**–**3** did not have clear M_1 agonistic activity up to 10 μM, whereas its analogue, compound **4,** was active (EC_{50}; 4.7 μM, Emax; 64 ± 4%). Comparing the structure of **2** and **4**, we thought the hydrogen atom should be placed as an R^3 substituent to yield M_1 agonistic activity. Thus, we explored the structure and activity relationship (SAR) study based on compound **4** to identify compounds with ideal profiles.

The synthetic routes for compounds **4**–**14** are shown in scheme 1. The chloride intermediate (2, 8-disubstituted 11-chloro-5*H*-dibenzo[b, e][1,4]diazepine) was prepared according to the previously reported method.[16] The substituted 2-fluoronitrobenzene was reacted with substituted anthranilic acids by heating in DMF in the presence of Cs_2CO_3 (A1-A7), the iron-catalyzed hydrogenation of the nitro group (B1-B7), followed by reaction with WSC to produce a tricyclic intermediate. The intermediate was treated with phosphorous oxychloride under reflux in toluene in the presence of *N,N*-dimethylaniline to form a chloride intermediate (C1-C7). Suzuki-Miyaura coupling of the chloride intermediate and 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1,2,3,6-tetrahydropyridine gave compound **4**, and **7**–**12**. The coupling of the chloride intermediate (C1) and 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridine, alkylation of nitrogen atom at pyridyl group by ethyl iodide or isopropyl iodide, then reduction by sodium borohydride gave compounds **5** and **6**, respectively. In the case of compound **13**, coupling of the C1 with BOC-protected boronate and then the de-BOC group by trifluoroacetic acid yielded target compound **13**. Compound **14** was prepared according to a similar method to **13**.

The SAR of the test compounds on M_1 agonism is summarized in Table 2. Replacement of the methyl group at R^4 in **4** with ethyl (**5**) and *i*-propyl (**6**) resulted in the loss of M_1 agonistic activity. The results of compound **2**, **5**, and **6** suggested that a sterically large substituent around a basic nitrogen atom is unacceptable. Compound **7**, in which the fluorine atom at R^2 in **4** was replaced with hydrogen, demonstrated > 10-fold stronger M_1 agonistic activity, but unfavorable M_3 agonistic activity accompanied. Its potency was the same as that of NDMC. M_3 receptors are expressed on salivary glands and their agonist causes salivation. Such hypersalivation was noted in a phase II clinical study of NDMC.[17] Thus, compound **7** can be considered to have a hypersalivation risk. Next, we replaced the chlorine atom at R^1 in **4** with a methyl group; the molecular size of a methyl group is similar to that of a chlorine atom, but the electronic behavior is opposite. The EC_{50} of compound **8** was 0.31 μM, over > 10-fold stronger than compound **4**. The hypersalivation risk of **8** was considered to be lower than that of clozapine because **8** did not exhibit M_3 agonism. Based on **8,** we replaced the R^2 substituent, and the EC_{50} of methyl (**9**), ethyl (**10**), and methoxy (**11**) groups was over 10 μM, whereas the Emax was reduced according to the substituent size, suggesting a sterically small substituent to be preferable as R^2. Swapping the R^1 and R^2 substituents of **4** diminished the activity (**12**). Compounds **13** and **14** were desmethylated analogues of compounds **4** and **8,** respectively. Of note, their M_1 agonistic activity was diminished (EC_{50}; >10 μM), differing from clozapine *versus* NDMC. Thus, a hydrogen atom as R^4 was not acceptable in the present series of compounds. These results suggested that the SAR on M_1 agonism is narrow, and a methyl group as R^1 and R^4, and a fluorine atom as R^2 were revealed to be a favorable combination for strong and selective M_1 agonistic activity. Furthermore, compound **8** exhibited binding affinity toward D_1 and D_2 receptors, and its *Ki* values were similar to those of clozapine and NDMC. In CHO cells stably expressing human D_1 or D_2 receptors, compound **8** antagonized dopamine-stimulated Ca^{2+} accumulation to a similar degree as clozapine.

We previously reported that compounds **1**–**3** were superior to clozapine in terms of reactive metabolite formation.[15] Dansylated glutathione (dGSH) was used as the trapping agent for the quantitative estimation of reactive metabolites. Test compounds were incubated with dGSH and human hepatocyte microsome fraction, and the amount of test compound-dGSH conjugate was measured by fluorescence detector in

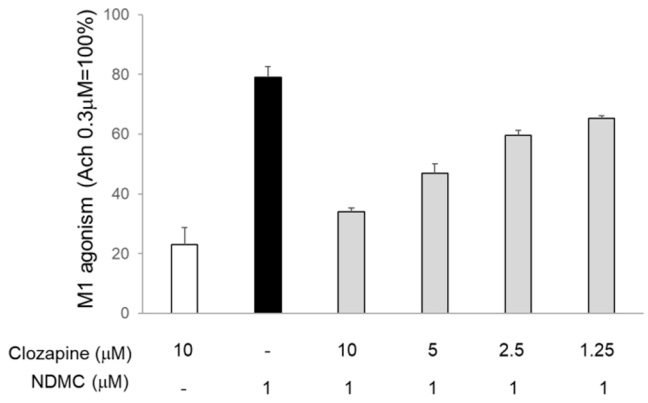


Fig. 1. Antagonist-like behavior of clozapine on NDMC-induced muscarinic M_1 agonism.

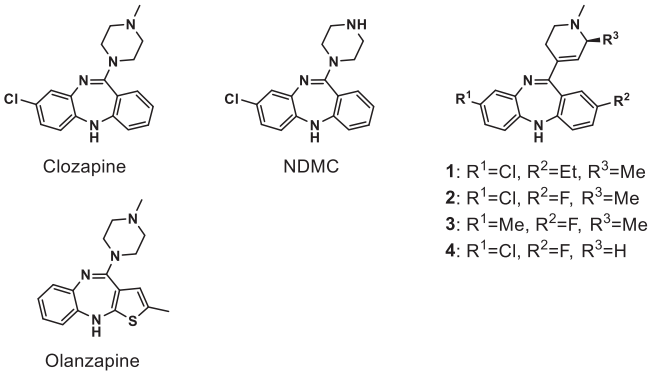


Fig. 2. Structures of antipsychotic drugs and compounds **1**–**4.**

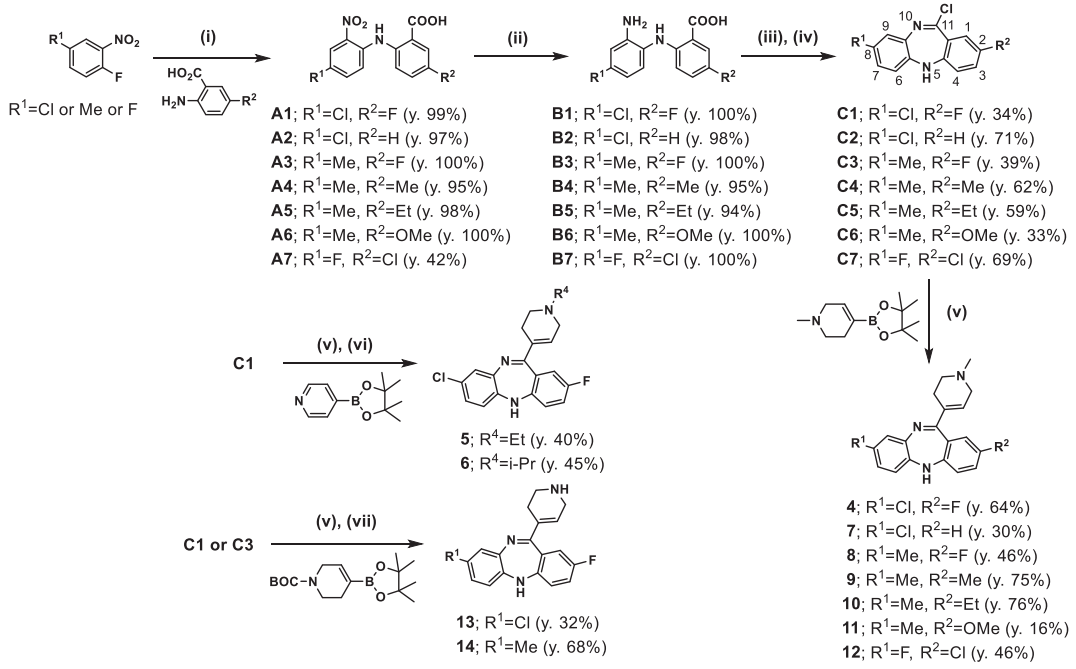
Table 1
M_1 agonistic activity of reference drugs and compounds **1**–**4.**

Compound	M_1 agonism	
	EC_{50} (μM)	Emax
Olanzapine	>100	<5%
Quetiapine	>10	<5%
Clozapine	>10	26 ± 4%
NDMC	0.048	75 ± 3%
1	>10	13%
2	>10	42%
3	>10	28%
4	4.7	64 ± 4%

Emax values are shown as the mean ± SE of two or more experiments

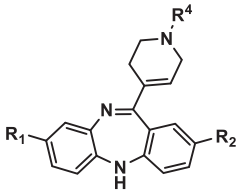
HPLC analysis. The amounts of conjugate in compounds **4**–**14** were 25-fold lower than that in clozapine (Table 2). The reactive metabolite formation in clozapine was initiated by the activation of a nitrenium ion (nitrogen atom at the 11th position of clozapine), but the nitrogen atom was replaced with carbon in the present series of compounds; therefore, we hypothesized that reactive metabolite formation was suppressed.[18] This suggested that our compounds have a low risk in terms of agranulocytosis due to reactive metabolites. Considering all results, we selected compound **8** for further evaluation.

We evaluated the binding affinity of **8** toward various GPCRs. As shown in Table 3, the affinity of **8** was similar with those of clozapine and NDMC. As dopamine D_1/D_2 and $5HT_{2A}$ are important targets to exert antipsychotic activity, we consider compound **8** to be a "serotonin-dopamine antagonist". We performed a mouse PK study of **8** by oral administration, and the blood and brain sample were collected one hour after dosing. Compound **8** displayed good brain penetration



Scheme 1. Synthetic route for compounds **4**–**14**. Reagents and conditions: (i) 5-fluoroanthranilic acid, Cs_2CO_3, DMF, 120 °C. (ii) Fe, NH_4Cl, EtOH, reflux. (iii) HOBt, WSC, DMF, rt. (iv) $POCl_3$, *N,N*-dimethylaniline, toluene, reflux. (v) $Pd(PPh_3)_4$, Na_2CO_3 aq., THF, reflux, (vi) TFA, rt, (vii) EtI or ^{i}PrI, MeCN, 60 °C, then $NaBH_4$, MeOH, 0 °C-rt.

Table 2
SAR exploration of compounds **4**–**14**.



Compd	R^1	R^2	R^4	EC_{50}; μM (Emax)				(*Ki*; nM)		dGSH (μmol/L)
				M_1	M_2	M_3	M_4	D_1	D_2	
Clozapine				>10 (26 ± 4%)	>10	>10	>10	44	389	7.5
NDMC				0.048 (75 ± 3%)	>10	0.67 (52%)	>10	80	489	
4	Cl	F	Me	4.7 (64 ± 4%)	>10	>10	>10	35	410	0.3
5	Cl	F	Et	>10 (<5%)			>10		340	0.27
6	Cl	F	i-Pr	>10 (<5%)			>10			0.29
7	Cl	H	Me	0.35 (64%)	>10	0.64 (52%)	>10	380	1387	0.21
8	Me	F	Me	0.31 (75 ± 3%)	>10	>10	>10	95	447	0.12
9	Me	Me	Me	>10 (38%)			>10	57	694	0.22
10	Me	Et	Me	>10 (19%)			>10	90	274	0.08
11	Me	OMe	Me	>10 (<5%)			>10		329	0.07
12	F	Cl	Me	>10 (<5%)			>10		108	
13	Cl	F	H	>10 (15%)	>10	>10	>10			
14	Me	F	H	>10 (1%)	>10	>10	>10		848	0

Emax values are shown as the mean ± SE of two or more experiments

Table 3
Binding affinity of compounds **4** and **8** toward various GPCRs.

compound	M_1		*Ki* (nM)				
	EC_{50} (nM)	Emax	D_1	D_2	$5HT_{2A}$	$5HT_6$	H_1
Clozapine	>10,000	26 ± 4%	44	389	5.4	6.4	1.2
NDMC	48	75 ± 3%	80	549	4.0	8.76	3.11
4	4,700	64 ± 4%	35	410	7.2	6.3	11.4
8	310	75 ± 3%	95	447	1.5	11.4	4.0

Emax values are shown as the mean ± SE of three experiments

(concentration: 1843 nM for brain, 360 nM for plasma, brain/plasma ratio: 5.2), and **14**, a desmethylated metabolite of **8,** was observed in the brain (63 nM). However, the concentration ratio of **14**/**8** in brain was 0.03, being negligible. Different from clozapine and NDMC, **14** will not function as an M_1 antagonist in brain when **8** is dosed, therefore compound **8** can be used to confirm the muscarinic M_1-hypothesis. Further pharmacological evaluation of **8** is underway.

In conclusion, we investigated clozapine-like compounds for the treatment of TRS based on the muscarinic M_1-hypothesis. We optimized the substituents of **4**, a lead compound, and identified **8** as the best

compound. It had robust M_1 agonistic activity, good selectivity over M_3 receptors, and clozapine-like binding affinity toward various GPCRs, including dopamine D_1/D_2 and 5-HT_{2A} receptors. Thus, **8** is considered to be a unique muscarinic acetylcholine-serotonin-dopamine modulator from a pharmacological point of view. The reactive metabolite-derived agranulocytosis risk of compound **8** was considered to be lower than that of clozapine. The mouse PK study indicated that brain penetration of **8** was good and the amount of desmethylated metabolite **14** was negligible. These results suggested that compound **8** is a good candidate to confirm the M_1-hypothesis of clozapine.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127911.

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