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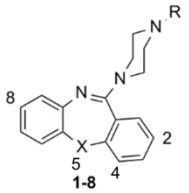

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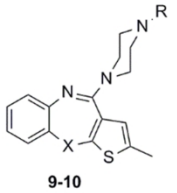

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1. R = H	8-Cl	X = N	*N*-desmethylclozapine	**9**.	R = H	*N*-desmethylolanzapine
2. R = Me	8-Cl	X = N	Clozapine	**10**.	R = Me	Olanzapine
3. R = H	2-Cl	X = O	*N*-desmethylloxapine			
4. R = Me	2-Cl	X = O	*L*oxapine			
5. R = H	8-F	X = C	*N*-desmethylfluperlapine			
6. R = Me	8-F	X = C	Fluperlapine			
7. R = H	H	X = S	*NH*-piperazinyl quitiapine			
8. R = $(CH_2CH_2O)_2H$	H	X = S	Quitiapine			

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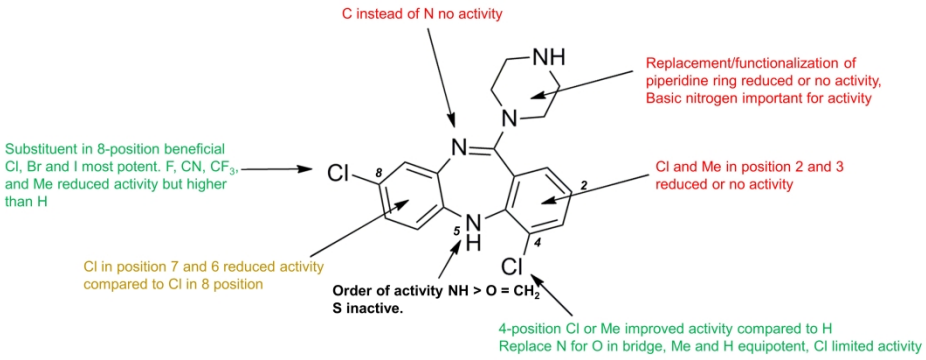

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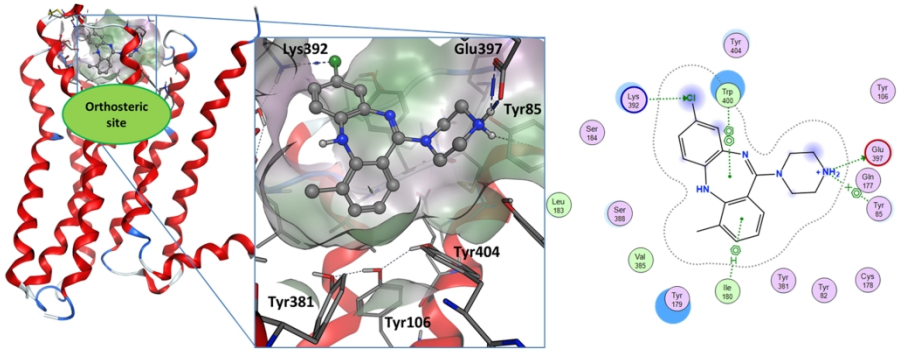

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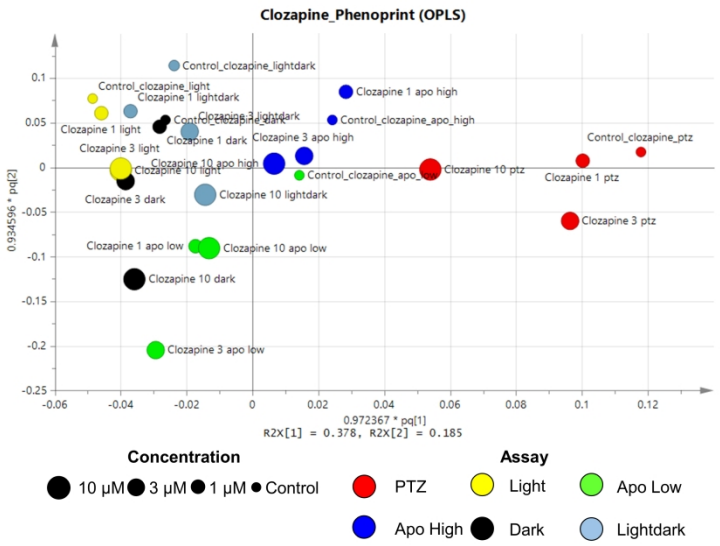

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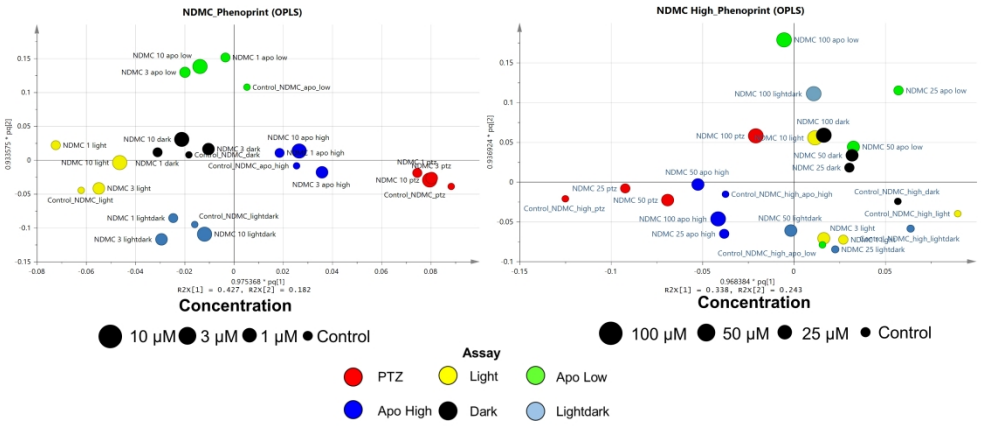

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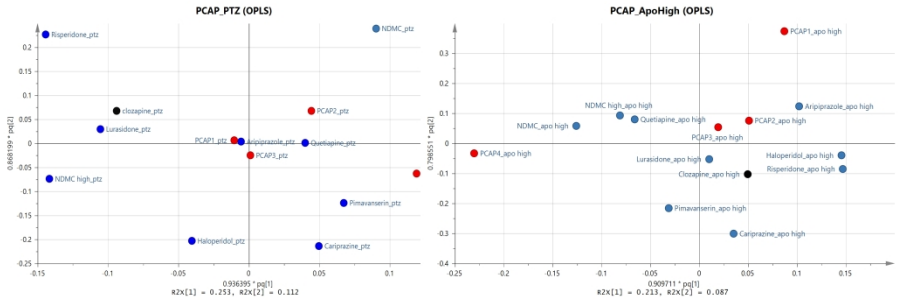

Figure 6. Phenotypical behavioural profiling of PCAPs and reference antipsychotic drugs in PTZ and ApoHigh assays using multivariate analysis (OPLS).

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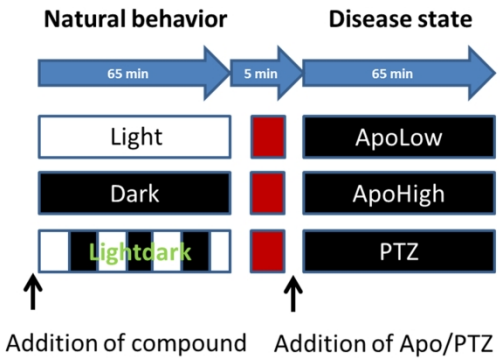

Figure 7. Illustration of the behavioural profiling experiments in 10 dpf zebrafish larvae.

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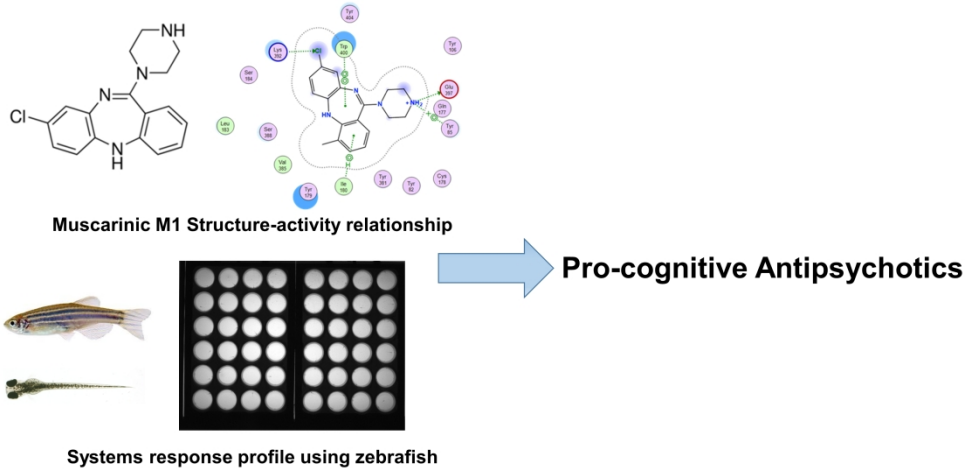

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Abstract

Current antipsychotic drugs are notably ineffective at addressing the cognitive deficits associated with schizophrenia. *N*-desmethylclozapine (NDMC), the major metabolite of clozapine, displays muscarinic M_1 receptor (M_1) agonism, an activity associated with improvement in cognitive functioning. Preclinical and clinical data support that M_1 agonism may be a desired activity in antipsychotic drugs. However, NDMC failed clinical phase II studies in acute psychotic patients. NDMC analogues were synthesised to establish a structure–activity relationship (SAR) at the M_1 receptor as an indication of potential pro-cognitive properties. In vitro evaluation revealed a narrow SAR in which M_1 agonist activity was established by functionalisation in the 4- and 8-positions in the tricyclic core. In vivo behavioural response profiles were used to evaluate antipsychotic efficacy and exposure in zebrafish larvae and peripheral side effect related M_1 activity in adult zebrafish. The NDMC analogue **13f** demonstrated antipsychotic activity similar to clozapine including M_1 agonist activity. Co-treatment with trospium chloride, an M1 peripheral acting antagonist, counteracted peripheral side effects. Thus, the NDMC analogue **13f**, in combination with a peripherally acting anticholinergic compound, could be suitable for further development as an antipsychotic compound with potential pro-cognitive activity.

Keywords Zebrafish, Antipsychotics, Muscarinic agonist, Structure-activity relationship, N-desmethylclozapine, Behavioural profiling

Introduction

Schizophrenia is a chronic disabling disease that affects about 0.4% of the adult population.[1] The disease is associated with positive symptoms (e.g., hallucinations, delusions and extreme emotions), negative symptoms (e.g., social withdrawal, poverty of speech and apathy) and disturbances in cognitive function.[2-3] Current antipsychotic drug therapies for schizophrenia have limited efficacy and are notably ineffective at addressing the cognitive deficits associated with this disorder.[4] Clinically, muscarinic anticholinergic drugs adversely affect cognitive performance, especially on measures of learning and memory, in both elderly persons and patients with schizophrenia and are linked to impaired concentration, confusion, delirium and attention deficit.[5-7] In fact, some of the approved antipsychotic drugs, such as chlorpromazine, olanzapine, quetiapine, fluperlapine and clozapine, display anticholinergic effects. Clozapine is the gold standard for the treatment of resistant schizophrenia and, despite its anticholinergic effects, shows cognitive benefits in some patients. However, in 2003–2004, Merck & Co. and ACADIA Pharmaceuticals independently suggested that *N*-desmethylclozapine (NDMC), the major metabolite of clozapine—which, in contrast to clozapine, displays M_1 receptor agonism—is behind the unique efficacy of clozapine in improving cognition and negative symptoms in the treatment of patients with schizophrenia.[8-9] The metabolite NDMC was detected in concentrations 20%–150% of that observed for clozapine in humans.[10]

NDMC (100 mg and 200 mg twice daily) was tested in a phase II study in acutely psychotic subjects with schizophrenia. Disappointingly, the study did not meet its primary endpoint of antipsychotic efficacy, and dose-escalating studies were prohibited mainly because of dose-dependent adverse events (e.g., salivation, tachycardia and dyspepsia), which may be related to peripheral M_1 activity.[11-12] Clozapine is given in the range of 300–600 mg daily, a comparable dose to what was used in the NDMC study.[13] This leads to speculations that NDMC does not reach sufficient brain concentrations, that the polypharmacological profile is not efficient enough for antipsychotic activity, or both. Rodent models displaying hyperactivity (MK-801 and amphetamine) showed that a higher dose of NDMC was needed compared with clozapine: sevenfold in MK-801 and fourfold in the amphetamine assay.[14] Furthermore, in earlier ex vivo studies comparing blood-brain barrier penetration and unbound fraction in the brain, clozapine was found to be about 2–3 times more brain penetrating, depending on kinetics and concentrations, and displayed about 2–3 times higher unbound brain fraction compared with NDMC.[15] Taken together, these results may partially explain the lack of antipsychotic activity of NDMC. To explore the polypharmacological activity of potential pro-cognitive

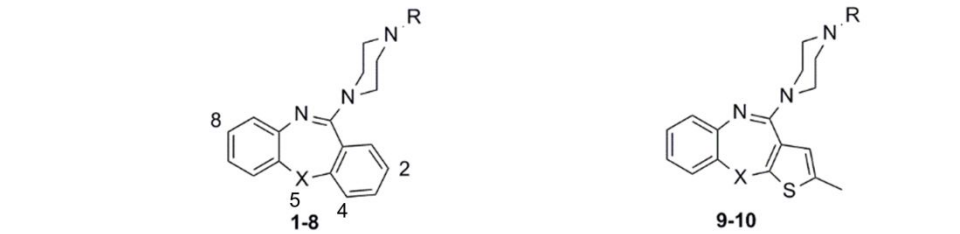
antipsychotics (PCAPs), we turned to phenotypic profiling in zebrafish larvae. In an earlier proof of concepts study to explore the translational value of behavioural profiling, we demonstrated that zebrafish and rats show similar phenotypes when treated with dopaminergic compounds.[16]

Previously, we have shown that NDMC analogues with novel receptor activity profiles comprising M_1 receptor agonism displayed pro-cognitive activity in the novel object recognition assay in rats.[17] M_1 receptor agonism is associated with improvements in cognitive functioning, and preclinical and clinical data support that M1 receptor agonism may be a desired pharmacological activity to incorporate into the profile of an antipsychotic drug.[9, 17] To facilitate the development of antipsychotic drugs that have pro-cognitive effect, knowledge of the structure–activity relationship (SAR) at the M_1 receptor is important. However, in addition to M_1 receptor activity, potential drugs need to have an antipsychotic effect, as the lack of the latter might be the reason for the failure of NDMC in the clinic. Given that there are limitations in in vitro profiling—for instance, testing so many targets are possible, and redundancy and adaptations occur in vivo but not in vitro—knowledge on how in vitro activities transfer to effects in vivo is limited. Therefore, in combination with receptor activity at M_1, in vivo systems response profiling in zebrafish larvae derived from six different behaviour assays (three based on spontaneous behaviour in different light settings and three from chemically induced disease states) was used in this study. They were analysed using multivariate analysis to identify compounds which combine M_1 agonism with antipsychotic effects.

Results and discussion

Summary of in vitro evaluation of M_1 agonist activity using R-SAT

Establishing a SAR at the M_1 of some known 11-piperazinyl-tricyclic-based antipsychotics and their *NH*-piperazinyl counterparts (**1**-**10**) as the initial step confirmed the uniqueness of NDMC (**1**), as none of the other antipsychotics or their corresponding *NH*-piperazinyl analog showed M_1 receptor agonism using the receptor selection and amplification (R-SAT) assay.



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8. R = $(CH_2CH_2O)_2H$	H	X = S	Quitiapine			

Figure 1. Structures of the 11-piperazinyl-tricyclic-based antipsychotics evaluated using the receptor selection and amplification (R-SAT) assay for M_1 receptor agonism.

To further explore the SAR at M_1, additional analogues (**11-15**) were evaluated (structure details can be found in Table S1, S2 and S3, Supporting information and in vitro M_1 receptor agonism activity in patent Ek et al).[14] A summary of in vitro M_1 receptor agonism activity extracted from patent Ek et al is outlined in Figure 2.[14] In general, few structural changes maintained M_1 agonist activity. A substituent at the 8-position is beneficial for M_1 receptor agonism; the best activity was shown for analogues with Br (**11d**) and I (**11e**), which were equipotent with NDMC. Furthermore, functionalisation in the 4-position (-Me, (**11p**) and –Cl (**11q**)) for diazepine analogues and only –Me for oxazepine (**13f**), in combination with Cl in 8-position, was beneficial for the M_1 agonism potency, efficacy, or both. Analogues with substituents in other positions in the tricyclic system demonstrated reduced or no M_1-agonist activity. Replacing the bridge nitrogen in position 5 with a methylene group (**13a**) or oxygen atom (**13b**) somewhat reduced the M_1 potency and efficacy compared with **1**. Surprisingly, the analogue **13c** containing a sulphur atom showed no muscarinic activity. We have previously demonstrated that the bridge atom (position 5) in the central azepine ring has a critical effect on the dihedral angle between the two planar aromatic moieties, which probably influences affinity to the M_1 receptor (Figure S1).[18] With this follows that the N to O exchange has an impact on the substituents in the 4-position; thus, 4-Me was still active, but 4-Cl showed no

activity. All structural modifications to the piperidine moiety and the amidine functionality in the central ring reduced the M_1 agonist activity.

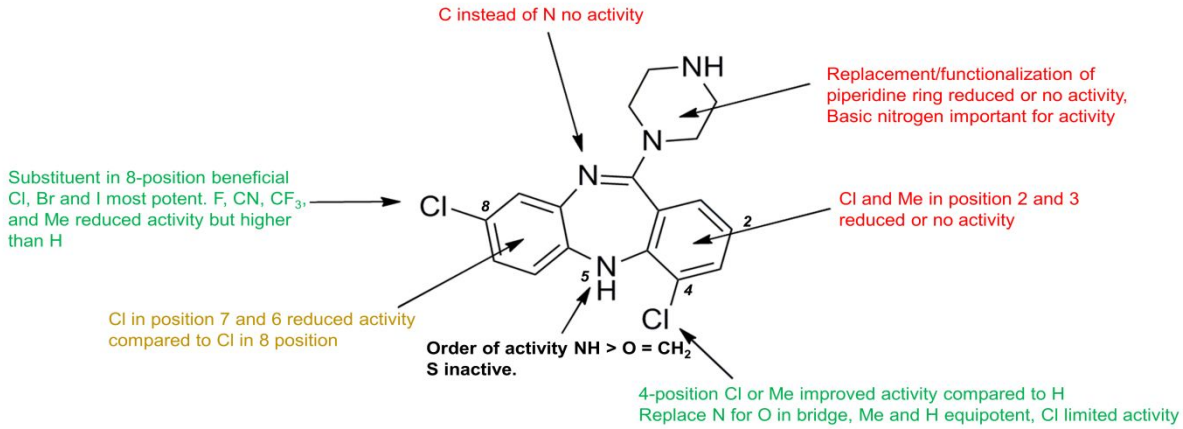

Figure 2. Summary of the M_1 agonist in vitro activity SAR for NDMC analogues.

Modelling of M_1 receptor and docking with NDMC analogues

To get an understanding of the narrow SAR established from the in vitro data, we turned to molecular modelling. However, the NDMC is known to be an allosteric agonist and positive allosteric modulator (PAM) of the human M_1 receptor, but the binding site and mechanism of action are unknown.[8] A limited number of mutation studies have been performed on M_1 with NDMC, but it has been shown that a tyrosine residue (Tyr381$^{6.51}$) in M_1 is highly involved in NDMC-induced signalling.[19] This mutation reduces both the affinity and efficacy of NDMC. Together with two additional tyrosine residues (Tyr106$^{3.33}$ and 408$^{7.43}$), Tyr381 forms a hydrogen bonding network which shields the orthosteric binding site from the allosteric one (Figure 3). These three positions are conserved in the primary sequences of the muscarinic receptor family, but they also show pronounced similarities in 3D structure when comparing the solved X-ray structures. Gregory et al. made a detailed mutation study of the muscarinic M_2 receptor involving both the orthosteric and the suggested allosteric sites by using various ligands, including NDMC.[20] They found that in addition to Tyr177$^{6.51}$, Tyr104$^{3.33}$ is also involved in both binding and signalling. The other mutations done in the allosteric site are not conserved between M_2 and M_1, but the results indicate that NDMC binds towards the extracellular side relative to the orthosteric site. These results prompted us to develop a 3D

model of the M1 receptor in order to identify the allosteric site, perform molecular docking to this site and evaluate if this explained the narrow SAR.

Human muscarinic receptors are highly conserved in the orthosteric binding pocket (M_1 to M_5) (Figure S 2-4, Supporting information), which may be the reason for the difficulty to achieve truly subtype selective ligands.[21] However, the sequence homology in allosteric sites is generally less conserved, so such sites can be suitable targets in the search for selective muscarinic ligands.[21]

A crystal structure of a human M_2 receptor in an active conformation is available, and it has a PAM present together with a co-bound agonist (pdb code: 4MQT)[22] that was considered appropriate to use as a template for a homology model (described in the method section). The PAM (LY2119620)[23] in the crystal structure binds in the region where NDMC is proposed to bind according to mutation studies, i.e., just above the allosteric binding site in the helix bundle and in between extracellular loops 2 and 3 (EC2 and 3). During the preparation of the manuscript an active conformation of the M1 receptor structure was solved using cryo-electron microscopy (cryo-EM) (pdb code: 6OIJ).[24] This structure had an orthosteric agonist present but lacked the allosteric modulator. This fact made our homology model more appropriate to use in the present study. The two 3D-structures were however very similar (see Figure S5, supporting information) and the allosteric pocket is predefined in the cryo-EM structure.

As the ligands tested show a moderate affinity and the span between the most potent (pEC_{50} = 7.4) and inactives (pEC_{50} < 5) is narrow, differentiating actives from inactives can be difficult (pEC_{50} and efficacy data can be found in reference 14). However, the ligands have efficacies from full agonism to weak partial agonism (antagonists) to not active. As the ligands to be studied have similar scaffolds, we assume that the agonists adopt similar binding modes to achieve an agonistic effect. A selection of clozapine analogues was docked into the suggested allosteric site of the M_1 receptor, including four agonists with high efficacy (**11p**, **11q**, NDMC and **13f**), seven analogues with medium/low efficacy (**11a**, **11i**, **11j**, **11m**, **12a**, **13a** and **13b**), one antagonist (clozapine) and five inactives (**11n**, **11o**, **13c**, **13d** and **13e**) (definition high/medium/low efficacy and pEC_{50} can be found in table 1 legend). The top-scored docking solutions for each ligand indicate a common binding mode for the compounds (Figure 3 and Table 1).

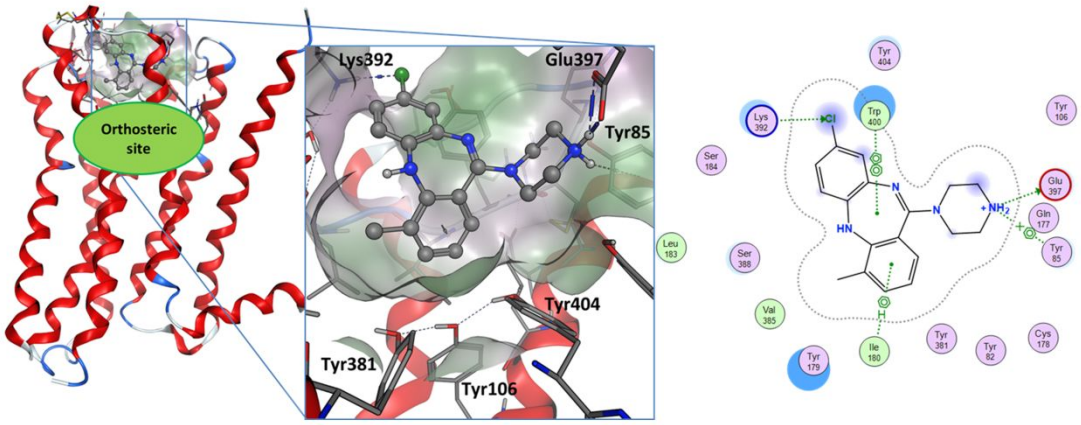

Figure 3. *Left*: The 3D model of the M_1 receptor with a close-up view of **11p** docked into the allosteric binding site. The ligand adopts the proposed important interactions with Lys392 and Glu397. The π-system of Tyr85 shields the basic nitrogen in the ligand, and the tyrosine residues (Tyr106, 381 and 404) that separate the orthosteric and allosteric sites interact with each other. *Right*: A schematic view of **11p** with the surrounding residues where purple represent polar residues, green greasy, and blue/purple shades receptor/ligand solvent accessibility.

Table 1. Ranked docking results for a selection of clozapine analogues docked into the homology model of the M_1 receptor. Efficacy was categorised into high (>75%), medium (40%–75%) and low (<40%). pEC_{50} was categorised into high (>7.0), medium (6.0–7.0) and low (< 6.0).

Analogue	Efficacy	pEC_{50}	ΔE (kcal·mol^{-1})[a]	Cl – Lys392[b]	Docking score
11p	High	High	3.7	X	−9.5
11q	High	High	4.1	X	−9.4
11j	Medium	Low	4.7		−9.4
NDMC (1)	High	High	4.0	X	−9.0
13e			3.7	X	−7.9
13f	High	Medium	3.1	X	−7.8
13b	Medium	Medium	3.4		−7.0
11i	Medium	Medium	4.7		−6.1
Clozapine[25] (antagonist)	Low	High			No hit
11a	Low	Medium			No hit
13a	Medium	Medium			No hit
11m	Low	Medium			No hit
11n					No hit
11o					No hit
12a	Medium	Medium			No hit
13c					No hit
13d					No hit

a] The conformational energy cutoff of the docked ligands is 5 kcal mol^{-1}. [b] Interaction between the basic nitrogen in Lys392 and the negative belt of the chlorine in the ligands.

The scores from the docking trails of the ligands were in good agreement with the pEC_{50} value of the compounds, indicating that the suggested binding mode is plausible. We found two amino acids in the allosteric binding pocket which seem to be important for binding. One is the positively charged lysine residue (Lys392) located in EC3, which interact with an ionic hydrogen bond directed towards the negative belt of the chlorine atom of the 8-chloro moiety,[26] and the other is the acidic glutamic acid ($Glu397^{7.32}$) which interacts with all agonists via ionic interactions. The other hydrogen in the basic amino function interacts via a π-cation interaction with Tyr85. This may be a major contributor to the lower efficacy of clozapine, which holds an *N*-methyl moiety. Abdul-Ridha et al. have made mutations in the allosteric site of the M_1 receptor together with modelling studies, and they suggest that $Trp400^{7.35}$ was most sensitive for mutations.[27] This residue was shown to be involved in PAM binding compared with the crystal structures of activated M_2 receptors.[22] In the M_1 receptor model, Trp400 and Tyr179 in EC2 form a hydrophobic sandwich with the aromatic rings in the NDMC analogues. The docking pose of the most potent analogue (**11p**) has a methyl substituent in the 4-position which fits into a hydrophobic pocket. The shape of the pocket indicates that substituents in the 2- and 3-positions would be clearly unfavourable (Figure 3).

All docked analogues with high efficacy fitted nicely into the binding pocket, interacted with Lys392 and were top scored. Three out of six medium efficacy analogues adopted the suggested agonist binding mode but did not interact with Lys392. One analogue (**13e**), which had a limited in vitro activity on muscarinic receptors, also turned out to adopt the suggested agonist binding mode. However, this ligand was shown to increase gastrointestinal (GI) tract motility when evaluated in adult zebrafish, which is associated with muscarinic agonism (see discussion below Table 3). The remaining inactive analogues docked were discriminated in the docking trail.

In vivo evaluation in zebrafish larvae

With the aim to identify a compound with similar proven efficiency as clozapine but with the added pro-cognitive effects of M_1 agonism, we selected **11q** (**PCAP2**) and **13f** (**PCAP3**) for further evaluation in vivo. Similar to clozapine, these compounds are antagonists at D_2 and 5-HT_{2A}, but they display M_1 agonism instead of antagonism. The second aim was to identify compounds with a similar in vitro profile as NDMC but with improved brain exposure for enhanced in vivo efficiency. The structural analogues **11p** (**PCAP1**) and **12a** (**PCAP4**) showed a similar in vitro profile as NDMC, which was emphasised by agonism/partial agonism on the D_2 receptor in combination with agonism on M_1 and antagonism on 5-HT_{2A}. These compounds were evaluated in zebrafish larvae and compared with eight marked antipsychotic drugs with

diverse in vitro receptor profiles by using the in-house-developed platform for behavioural phenotyping (Table 2). This behavioural platform consists of six different models, three of which are based on natural behaviour under different lighting conditions, (dark, light and alternating dark–light cycles). In these three assays, the optimal results would be that the drug induces a phenotype similar to the drug naïve state (control group). The three other assays are chemically induced disease models performed in dark conditions, two of which are based on different concentrations of apomorphine, low (ApoLow) and high (ApoHigh) for the induction of hypoactivity and hyperactivity, respectively. A third assay used a stable sub-convulsive concentration of pentylenetetrazole (PTZ) for the induction of bi-polar/psychotic behaviour.[28] These chemically induced models give a robust reproducible phenotype in zebrafish larvae 10 days post-fertilisation (dpf). This age was selected because zebrafish larvae are reported to have a fully developed blood–brain barrier at 10 dpf. Of note is that for several years, we have tried to establish the use of MK801 as a model in zebrafish larvae, which is a model frequently used in rodents. However, we have not able to detect any stable locomotor alterations suitable for establishing a model nor for reproducing any of the effects reported in the literature, except for effects on cognition.[29] These six assays are evaluated using multivariate analysis (Orthogonal partial least squares, OPLS), which is similar to a recently reported methodology using rodents;[30] 51 variables (the specification and definition can be found in the Supporting information) were analysed during 13 five-minute periods. The variables are based on positions in the well and natural swimming patterns, both singular actions and their combinations into more complex patterns (i.e. c-turns, o-turns and j-turns).[31-32] These were identified specifically in zebrafish larvae 10 dpf by using an unsupervised approach.[16] With this approach, we hope to analyse similar compounds structurally and pharmacologically, as well as differentiate and examine activity on a high detailed level in order to drive a multidimensional SAR.

Table 2. In vitro receptor profile for compounds evaluated in vivo on M_1, D_2 and 5-HT_{2A} receptors in 10 day-old zebrafish larvae or adult zebrafish (NA = no activity up to 10 μM, ANT = antagonist, AG = agonist, PA = partial agonist, and IA = inverse agonist). Values in table refer to pK_i unless otherwise noted.

Compound	**M_1**	**D_2**	**5HT_{2A}**
Aripiprazole[33]	NA	8.9 (PA)	7.8 (ANT)
Cariprazine[34]	NA	9.1 (PA)	7.8 (IA)
Haloperidol[35-36]	NA	9.3 (ANT)	8.5 (ANT)
Clozapine (**2**)[#]	8.0 (ANT)	7.0 (ANT)	8.7 (ANT)
NDMC (**1**)[*]	7.4 (AG)[A]	7.8 (PA)	7.8 (ANT)
Quetiapine (**8**)[37]	6.2 (ANT)	6.8(ANT)	7.0 (ANT)
Lurasidone[38]	NA	8.9 (ANT)	8.8 (ANT)
Risperidone[34]	5,4 (ANT)	8.8(ANT)	10.4 (ANT)

Pimavanserin[39]	NA	NA	9.3 (IA)
(11p) PCAP1[#]	7.2 (AG)[A]	6.0 (ANT)	7.9 (ANT)
(11q) PCAP2[#]	7.2 (AG)[A]	6.3 (ANT)	8.2 (ANT)
(13f) PCAP3[*]	6.7 (AG)[A]	6.5 (ANT)	7.9 (IA)[B]
(12a) PCAP4[*]	7.0 (AG)[A]	7.9 (PA)	7.5 (IA)[B]

* Data from reference 14, # data from reference 17 [A] pEC_{50}, [B] pIC_{50}

Phenoprofiling using multivariate analysis of behavioural data

As a starting point, we made phenoprints of clozapine and NDMC at comparable doses in the six assays. The initial water concentrations of the compounds were 1 μM, 3 μM and 10 μM, and 12 larvae were used for each dose. In addition, although the use of NDMC in concentrations higher than that of clozapine will be difficult in humans because of peripheral side effects, NDMC was also tested at about 10 times higher concentrations (25 μM, 50 μM, 100 μM) to evaluate if the uptake could be a problem and, if it is circumvented, to determine if NDMC would show antipsychotic activity.

Compounds with potential antipsychotic effect should show a dose-dependent deviation from the control groups in the the PTZ, ApoLow and ApoHigh models—larvae symbolising the disease—towards the controls in the dark assay that are representing the naïve healthy fish. As seen in Figure 4, clozapine exerts a dose-dependent effect in the three disease model assays, a clear dose-dependent response in PTZ and ApoHigh and a U-shaped response in ApoLow. What can also be noted in the assays with natural behaviour, dark, light and dark–light conditions is dose dependence towards a lower activity and sedation (parameters not shown for clarity). However, at comparable doses, NDMC-treated fish show no activity in any of the models; all doses are clustered around their respective controls. Thus, the NDMC phenoprint basically shows the overall diversity of the six models. The high dose regime of NDMC displays distinct activity patterns. For the highest dose of 100 μM, similar to clozapine at the highest concentration (10 μM), the NDMC phenotype clearly moves towards a sedative state, with one exception, the ApoHigh assay. Thus, the apomorphine counters the sedation, which also indicates that NDMC shows little or no antagonistic effect at the dopamine receptors, corroborating earlier results in vitro.[40] The up to 50 μM doses of NDMC seem to show reasonable activity, with the exception of the ApoHigh assay, which has about five times the dose of clozapine. Thus, pharmacologically, a major difference exists between clozapine and NDMC at the dopamine receptors, which have been the driver for the discovery of antipsychotic drugs for a while, the dopamine hypothesis that relates to the hyperactivity of dopamine signal transduction.

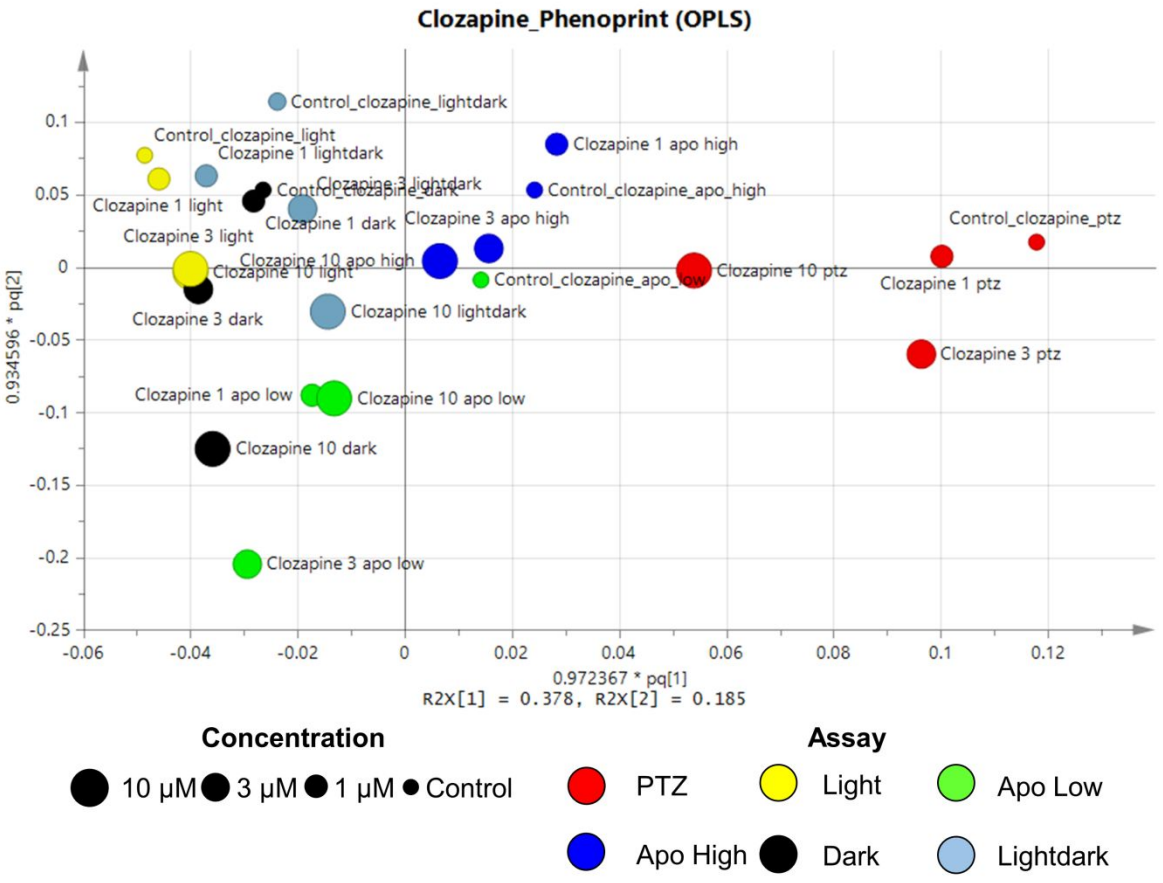

Figure 4. Phenotypical behavioural profiling of clozapine in six assays using multivariate analysis (OPLS).

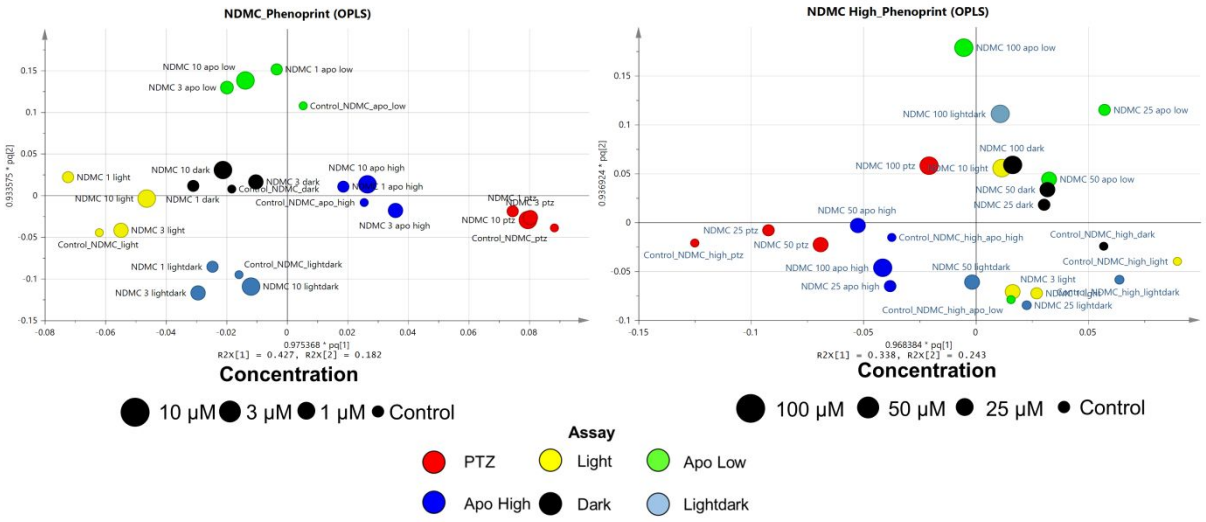

Figure 5. Phenotypical behavioural profiling of NDMC at normal (left) and high (right) dose regimes in six assays using multivariate analysis (OPLS).

The next step was to analyse PCAPs **1-4**. To put them in the context of marketed drugs and NDMC (the two dose regimens used in the phenoprint [NDMC and NDMC high]), we selected the following eight marketed drugs: aripiprazole, cariprazine, clozapine, haloperidol, lurasidone, quetiapine, pimavanserin and risperidone. Based on phenotype, we selected the PTZ model and ApoHigh for analysis. In these analyses, we couple dose and time period. The administration of drugs to zebrafish larvae in water is different from the administration of compounds, in general, to animals. The larvae are swimming in a given start concentration and are continuously absorbing the drug throughout the experiment to an equilibrium.

From the phenoprint, we know that NDMC showed no activity in this model, whereas clozapine showed a dose response towards healthy fish. Every dot in the plot is a dose response vector showing the results for three concentrations for a total of 36 individual zebrafish larvae. The four PCAPs (red dots) showed activity towards clozapine (black dot, Figure 6 and S6-S7, Supporting information). In this assay, **PCAP1-3** showed a similar profile as quetiapine and aripiprazole, whereas **PCAP4** showed quite a different profile which is closer to that of pimavanserin, a selective $5HT_{2A}$ inverse agonist with some activity at $5HT_{2C}$, as well (phenoprints PCAP3 and PCAP4 in Figure S7, Supporting information). In the ApoHigh assay, which predominantly displayed dopamine phenotypes, it can be seen that the two dose regimes of NDMC showed very little activity, and the potent dopamine compounds, risperidone, aripiprazole and haloperidol, are shown far to the right in the plot. This might be interpreted as increases in dopamine antagonistic activity from left to right. Pimavanserin, which does not have any activity at the dopamine receptors, are at the same level as **NDMC**-treated fish at the x-axis, as expected. However, cariprazine, a potent D_2 receptor partial agonist, shows about the same activity as clozapine, whereas aripiprazole, a compound with a similar profile, is far out to the right. An unexplained toxic drug–drug interaction was seen between apomorphine and the 10 µM dose of lurasidone in the ApoHigh and ApoLow assays. This effect was not seen in any of the four other assays of lurasidone or at lower doses of lurasidone. Therefore, the high 10 µM dose was removed in the ApoHigh model (Figure S9-S11, Supporting information). The ApoHigh model shows that **PCAP2** and **PCAP3** have similar phenotypes at the dopamine receptors as clozapine, whereas **PCAP1** indicates a strong dopamine receptor activity, along with thigmotaxis, indicating anxiety. Interestingly, **PCAP4** is on the left side of NDMC, indicating no to little activation of the dopamine receptors, which agrees with the in vitro activity at D_2. From the PTZ and ApoHigh assays, **PCAP3** and **PCAP4** were found to have interesting profiles—**PCAP3** for displaying a similar profile as clozapine but with M_1 agonist

activity and **PCAP4** for having a different dopamine receptor profile, which may be of interest for other diseases in need of cognitive enhancement.

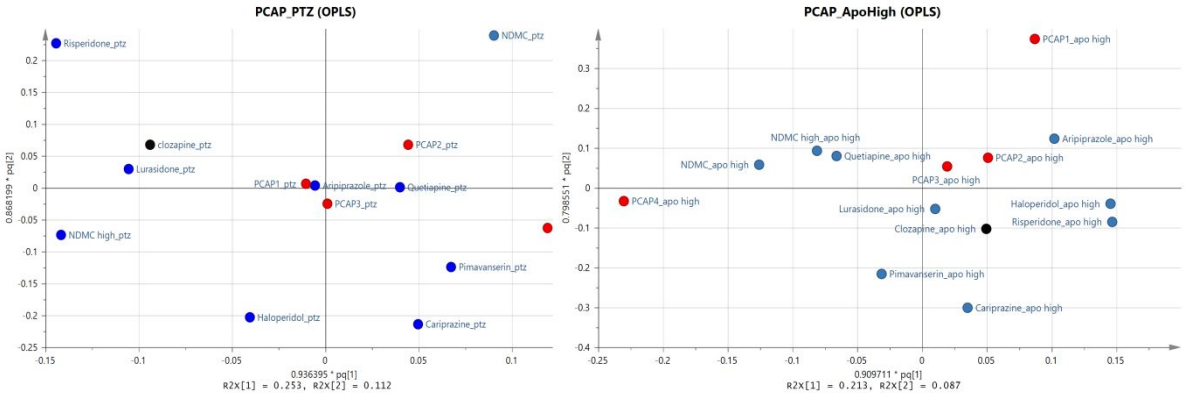

Figure 6. Phenotypical behavioural profiling of PCAPs and reference antipsychotic drugs in PTZ and ApoHigh assays using multivariate analysis (OPLS).

Behavioural analysis in adult zebrafish

Ideally, separating peripheral and central effects would be optimal, as unwanted drug-induced side effects from the peripheral activation of muscarinic receptors have so far limited the development of this class of compounds as therapeutics. Behavioural changes in zebrafish because of central effects have been well explored and can be manifested by a lack of body control, erratic movements/seizure, increased surface dwelling, hyper/hypoactivity and stigmotaxis.[41] In humans, M1 activation evokes a number of peripheral effects, including increased GI tract motility, e.g., diarrhoea.[42] This effect have also been seen in dogs, rodents and zebrafish.[43-45] To verify if we could detect signs of central effects, we first evaluated two antipsychotic drugs (clozapine and haloperidol), both with classical D_2 and $5HT_{2A}$ activity but with and without anticholinergic activity. Clozapine 10 µM added to the fish medium with adult zebrafish (n = 10) induced a fast transition from normal swimming at the bottom of the tank to surface dwelling, indicative of a drug-induced central effect (Table 3). This was accompanied by a minor amount of faeces (indication on GI motility, potentially via the metabolite NDMC) at the bottom. Haloperidol 10 µM also induced a transition to the surface, although it was slower compared with that with clozapine and was not accompanied by defecation. We continued to evaluate NDMC analogues with in vitro muscarinic activity. Compound **PCAP3** (10 µM) increased surface swimming, although not as fast as clozapine did. However, we noted substantial defecation in comparison with both the clozapine and control groups (Figure S12, Supporting information). **PCAP1**, **PCAP2** and **PCAP4** also induced defecation at 10 µM, although not to the same extent and rate as **PCAP3** did. The NDMC analogues have the highest in vitro activity on the M_1 receptor in comparison to M_2–

M_5 but are still not regarded as M_1 selective. Therefore, to further prove that it is the M_1 activity that increases the defecation, a selective M_1 agonist **AC260594** was evaluated.[46] This compound produced a rapid onset of defecation and surface swimming. Furthermore, the M_1/M_4 agonist xanomeline, a compound used in clinics for pro-cognitive effects in psychiatric disorders and degenerative diseases, induced surface swimming and defecation but to a lesser extent than **AC260594** did. The anticholinergic reference compound scopolamine did not induce increased defecation compared with the control group. Further evaluation of NDMC analogues **13d** and **13e** without M_1 in vitro activity produced no or minor defecation at 10 μM, although surface swimming indicated efficient uptake. Analogue **PCAP3** was also tested in combination with two muscarinic antagonists, scopolamine and trospium chloride (Figure S13, Supporting information); the latter is a peripheral active compound that does not enter the brain.[47] Pre-treatment (1 h) with both antagonists (20 μM) reduced defecation without any effect on the centrally mediated surface swimming.

In addition, we could also detect signs of inferior systemic exposure for some of the evaluated compounds in the assay based on in vitro potency/efficacy and the expected in vivo effect. NMDC (10 μM) induced less defecation despite a higher M_1 receptor in vitro activity compared with **PCAP3**, and required higher concentrations to induce surface swimming and increased defecation. This supports the finding that NDMC has a lower systemic uptake and brain exposure in zebrafish, which is in line with previous ex vivo and in vivo results.[15] The compounds **PCAP4**, **PCAP1** and **PCAP2** showed a slower onset and degree of surface swimming compared with clozapine and **PCAP3**, which could also be related to central and peripheral exposure.

Thus, M_1 activation is most likely responsible for the increased defecation in adult zebrafish and confirms the in vivo activity on this receptor, supporting the findings in other species and further demonstrate the translational value of zebrafish in drug discovery.[43, 48] More importantly, we can separate drug-induced central and peripheral effects, as well as identify compounds with uptake liability.

Table 3. Results from the drug evaluation of the induction of surface swimming and defecation in adult zebrafish (N = 10 per experiment) over 60 min. The results were manually scored from no effect (−) to maximal effect (+++); the time point for surface swimming was logged.

Compound (10 μM)	Fecal release	Surface swimming at 1 h	Time point Surface swimming
Control	-	-	-
Clozapine	+	+++	10 min
Haloperidol	-	+++	30 min
PCAP1	++	+	1 h
PCAP2	++	++	1 h
PCAP3	+++	+++	15 min
PCAP4	++	+	1 h*
13d	-	++	1 h
13e	+	++	1 h
NDMC	+	-	-
NDMC 50 μM	+	+++	30 min
NDMC 100 μM	++	+++	<5 min
Scopolamine	-	-	-
AC260584	+++	++	10 min
Xanomeline	++	++	60 min
PCAP3 + Trospium Chloride	+	+++	15 min
PCAP3 + Scopolamine	+	+++	15 min

***PCAP4** dose escalation up to 80 μM before surface swimming similar to clozapine.

Trospium chloride and scopolamine (20 μM) were added 1 h before the addition of **PCAP3** (10 μM).

Conclusion

Several of currently approved antipsychotic drugs are hampered by anticholinergic effects, which have been linked to delirium and dementia, as well as peripheral effects, such as constipation and tachycardia, which increase the risk of mortality. This is particularly true for older adults who are more sensitive to these side effects because of physiological and pathophysiological changes that often accompany the aging process. Therefore, adding M_1 agonist activity to an antipsychotic drug profile might be beneficial for drugs aimed at this group of patients. In this study, we have identified an NDMC analogue **13f** (**PCAP3**) with a

combined potential pro-cognitive and antipsychotic profile by using a combination of in vitro M_1 activity data, computer-aided receptor modelling and docking with in vivo systems behavioural profiling in larval and adult zebrafish. We have also showed that **13f** (**PCAP3**) in combination with a peripherally acting anticholinergic compound (trospium chloride) could led to a pro-cognitive antipsychotic treatment without peripheral M1 related side effects.

Method

Animal ethics

This study was conducted in accordance with the national legislation of Sweden and with European Community guidelines for animal studies. All procedures were approved by the ethical committee in Malmö-Lund (permit M23-15, Zebrafish larvae and 5.8.18-05993/2018 adult zebrafish).

Fish maintenance

The zebrafish larvae used in this study were from intercrosses of the wild-type AB strain. Embryos were collected and raised in a 14:10-hour light–dark cycle at 28.5°C on Petri dishes containing E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2 and 0.33 mM MgSO4) in an incubator up to 5 days post-fertilisation (dpf). At the age of 5 dpf, the larvae were transferred into 0.8 L aquaria and placed in an Aquaneering, Inc. (San Diego, CA) recirculating system held at 26±1.5°C, where feeding was initiated. Larvae were fed with a commercial larval diet, ZM000 (ZM Fish Food & Equipment, Winchester, UK), three times daily until the age of 10 dpf. Behavioural experiments were conducted at 10 dpf. The age of the zebrafish larvae was chosen to ensure a developed blood–brain barrier, which is an important feature when evaluating potential pharmaceuticals for central nervous system diseases.

Compounds

Aripiprazole (SML0935, Sigma-Aldrich), cariprazine (HY-14763, MedChem Express,), haloperidol (Haldol®, Janssen-Cilag AB), clozapine (C6305, Sigma-Aldrich), N-Desmethylclozapine (NDMC, D5676, Sigma-Aldrich), xanomeline (X2754, Sigma Aldrich), pimavanserin (S8183, Selleckchem), AC260584 (HY-100336, MedChemExpress), lurasidone (HY-B0032, MedChemExpress), scopolamine hydrobromide (S1875, Sigma-Aldrich), quetiapine (Q3638, Sigma-Aldrich, and risperidone (R3030, Sigma-Aldrich) were commercially available.

Compounds **11-15** were synthesised according to previously described procedures.[14]

Pharmacology

The compounds were dissolved in 100% dimethyl sulfoxide to generate stock solutions (10 mM). These solutions were then diluted in E3 medium to 10 μM, 3 μM, and 1 μM. NDMC (D5676, Sigma-Aldrich, St. Louis, MO) was dissolved in 100% dimethyl sulfoxide to generate a stock solution (50 mM). The stock solution was then diluted in E3 medium to 100 μM, 50 μM, and 25 μM.

Behavioural profiling in larval zebrafish

Each experiment was performed using 48 zebrafish larvae in two 24-well microtiter plates (Cat. No. 303002; Porvair Sciences, Leatherhead, UK) milled to a depth of 9 mm to reduce shadow artefacts. The microtiter plates had white walls to increase the contrast between the larvae and the background and to prevent larvae in adjacent wells from acting as visual stimuli. The behavioural setup consisted of a 300 fps digital camera (Genie HM640, Teledyne DALSA, Waterloo, Canada) connected to a computer with video-recording software (CamExpert v7.00.00.0912, Teledyne DALSA, Waterloo, Canada; LabVIEW™ 2011 v11.0, National Instruments, Austin, TX). To maintain the environment in the wells at 28°C, the microtiter plates were placed parallel to each other in a water bath containing a temperature control unit (Neoheater 25 W thermostat, AQUAEL, Warsaw, Poland). The microtiter plates were placed on a light box containing LED strips (SMD5050 infrared 850 nm LEDs and SMD5050 white light LEDs) for camera backlight and illumination of the light box. For a schematic image of the experimental setup, see Figure S4 in the study of Anderson et al.[29]

At 10 dpf, the larvae were transferred to the microtiter plates containing 1 ml of the E3 medium. All individuals were observed for abnormal swimming behaviour and body deformities prior to the experiments. Damaged individuals were removed and replaced. The behavioural effects were evaluated in six conditions for 65 min each, pairwise arranged according to Figure 7. Light; constant visual light (2,000 lux), dark; constant dark (only IR backlight) and light–dark: three cycles of light for 10 min, followed by dark for 10 min and, finally, 5 min of light. Compounds were added immediately before the start of the first session without any previous habituation of the larvae. All disease states were performed in the dark condition with the addition of apomorphine (low = 0.5 μM, high = 50 μM) and PTZ (2.5 mM) immediately before the start of the second recording session. There was a 5 min break in between the recording sessions.

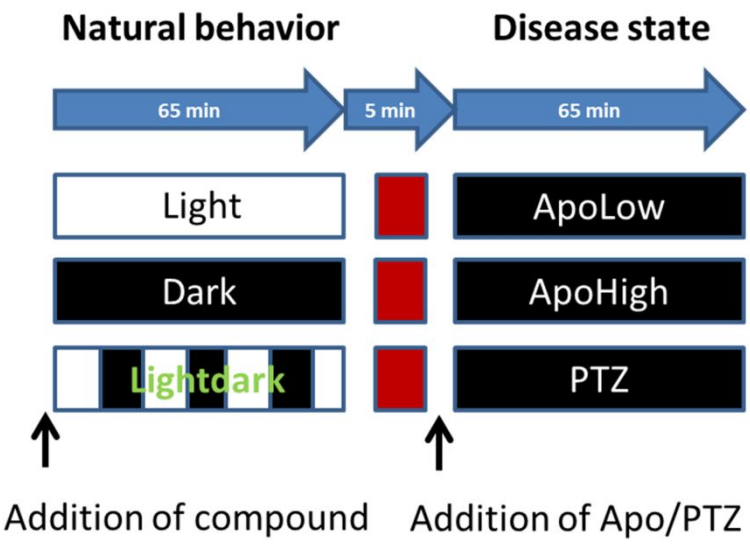

Figure 7. Illustration of the behavioural profiling experiments in 10 dpf zebrafish larvae.

Zebrafish larvae were analysed in three treatment groups (1 µM, 3 µM and 10 µM) and a control group in all six conditions (n = 12 larvae/dose). Haloperidol was also analysed at a lower dose range (0.1 µM, 0.3 µM and 1.0 µM) and NDMC at a higher dose range (25 µM, 50 µM and 100 µM).

The dataset was generated by automated imaging analysis of the video recordings of the zebrafish larvae (10 dpf). Imaging analysis of the video files was performed after the recording by using a previously described in-house-developed tracking program.[16] The position and angular information from the tracking were then used to calculate 51 parameters (the list and definitions can be found in the Supporting information).

Statistical Analysis. Zebrafish. Multivariate data analyses were performed using SIMCA-P+, v.13 (Umetrics AB), principal component analysis (PCA), projections to latent structures discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA). To identify outliers, we conducted PCA analysis of the different treatment groups. We identified that <8% larvae were outside of the 99% confidence interval; these were evenly spread amongst the treatment groups, and no specific localisation of the well in the plate was observed. These larvae were removed from the analysis. In addition, we filtered from the data set extreme values that resulted from errors in tracking technology.

Behavioural analysis in adult zebrafish

Adult zebrafish (n = 10) were transferred to a beaker with a 500 mL fish medium maintained at 23°C. Compounds (for concentrations, see Table 3) were then added as DMSO stock solutions (10 mM). The behavioural changes were manually monitored (open study), and the

time point for the transition of the fishes from the bottom to the surface was registered. After 1 h, the relative defecation and surface swimming were graded from – (no faeces/no surface swimming) to +++ (the highest amount of faeces/intense surface swimming).

Modelling of M_1 receptor and docking with NDMC analogues

A crystal structure of a human M_2 receptor in an active conformation is available, and it has a PAM present together with a co-bound agonist (pdb code: 4MQT).[22] A receptor structure in an active conformation with only an orthosteric agonist bound was also reported. These two structures are shown to be structurally similar in allosteric binding site, except for the conformation of a tryptophan residue (Trp422$^{7.35}$). A side chain rotamer of Trp422 in the PAM-binding structure allows the PAM to bind deeper down in the pocket. This also means that the active M_2 receptor lacking the PAM has a pre-defined allosteric binding site. The hypothesis that the receptor opens at the extracellular side during receptor activation is strengthened by the superimposition of the two inactive and antagonist bound receptor structures, M_2-(3UON)[49] and M_1- (5CXV)[50], in which the allosteric pocket is considerably more narrow. The allosteric pocket is located just above the orthosteric binding site, between the second and third extracellular loops (EC2 and EC3); this result is in agreement with NDMC's binding site identified in the mutation studies. Therefore, the M_2 structure with a PAM present was considered suitable as a template for the construction of a homology model of M_1. The sequence homology between human M_1 and M_2 receptors is 65.2% (when excluding the third intracellular loop), which is sufficient for building homology models that can be used further for docking (see sequence identities and alignments in the Supporting information). To retain the active receptor conformation, the selective M_1 receptor PAM (benzoquinazolinone 12)[51] was present during the modelling procedure, together with acetylcholine in the orthosteric binding site. Acetylcholine was manually docked into its binding site, guided by the agonist present in the crystal structure (iperoxo), and benzoquinazolinone 12 was docked into the M_2 template structure. The ligands were present in their respective binding sites during the receptor modelling procedure in order to preserve an agonist-induced conformation. Ten models were constructed, and for each model, all side chain conformations were sampled three times by using the homology modelling tool implemented in the MOE software (*Molecular Operating Environment (MOE)*, version 2016.0802; Chemical Computing Group Inc.: Montreal, QC, 2016). From these 30 models, one was selected (see the Ramanchandran plot in the Supporting information) for a docking trail of the series of clozapine analogues. Before the docking trail, acetylcholine was removed from the 3D receptor model, followed by energy minimisation, as the vitro studies were performed without an orthosteric agonist present. The initial M_1 receptor

homology model was unable to discriminate the agonists from the inactive ligands when using extra precision docking (XP) in Glide together with the OPLS3 force field[52], as implemented in the Schrödinger suite. Induced fit docking, in which both the ligand and the receptor are flexible, neither distinguished actives from inactives.

Supporting information

The Supporting information is available free of charge; **1**. Structures of NDMC analogs Table S1-S3; **2**. Effect of the bridging atom on the α-angle Figure S1; **3**. Modelling of the M_1 receptor Figure S2-S5; **4**. Multivariate analysis of zebrafish larvae experiments Figure S5-S10; **5**. Illustration of increased defecation after exposure to muscarinic agonists Figure S11-S12; **6**. Definition parameters for behavioral analysis

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Author Contributions Study conception and design: F.E. J.O and R.O. Acquisition of zebrafish data: K.H, and F.E. Computer model: MM. Analysis and interpretation of data: F.E., and R.O. Drafting of manuscript: F.E., R.O., and J.O; Critical revision: K.H, J.O, M.M, R.O and F.E.

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Notes

The authors declare no competing financial interest.

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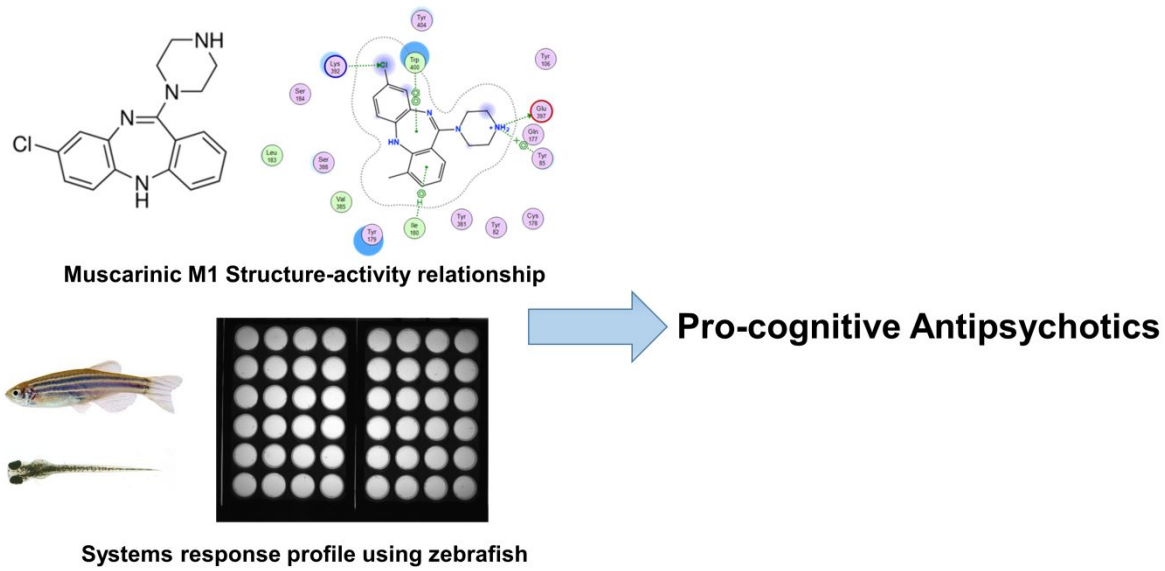

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