Behavioural and pharmacological profiles of zebrafish administrated pyrrolidinyl benzodioxanes and prolinol aryl ethers with high affinity for heteromeric nicotinic acetylcholine receptors

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ABSTRACT

Rationale

Prolinol aryl ethers and their rigidified analogues pyrrolidinyl benzodioxanes have a high affinity for mammalian $\alpha4\beta2$ nicotinic acetylcholine receptors (nAChRs). Electrophysiological studies have shown that the former are full agonists and the latter partial agonists or antagonists of human $\alpha4\beta2$ receptors, but their *in vivo* effects are unkown.

Objectives and Methods

As $\alpha4\beta2$ nAChRs play an important role in cognition and the rewarding effects of nicotine we tested the effects of two full agonists and one antagonist on spatial learning, memory and attention in zebrafish using a T-maze task and virtual object recognition test (VORT).The effect of a partial agonist in reducing nicotine-induced conditioned place preference (CPP), was also investigated.

Results

In comparison with the vehicle alone, the full agonists MCL-11 and MCL-28 induced a significant cognitive enhancement as measured by the reduced running time in the T-maze and increased attention as measured by the increased discrimination index in the VORT. MCL-11 was 882 times more potent than nicotine. The two compounds were characterised by an inverted U-shaped dose–response curve, and their effects were blocked by the co-administration of the antagonist MCL-117, which alone had no effect.
The partial agonist MCL-54 induced CPP and had an inverted U-shaped dose–response curve similar to that of nicotine, but blocked the reinforcing effect of co-administered nicotine.
Binding studies showed that all of the compounds have a higher affinity for heteromeric [^{3}H]-epibatidine receptors than [^{125}I]-αBungarotoxin receptors. MCL-11 was the most selective of heteromeric receptors.

Conclusions.

These behavioural studies indicate that full-agonist prolinol aryl ethers, are very active in increasing spatial learning, memory and attention in zebrafish. The benzodioxane partial agonist MCL-54 reduced nicotine-induced CPP, and the benzodioxane antagonist MCL-117 blocked all agonist-induced activities.

Key words

Full agonist, partial agonist, antagonist, αBungarotoxin, epibatidine, nicotinic receptors, T-Maze, VORT , zebrafish.

INTRODUCTION

The cholinergic pathways in the central nervous systems of animals and humans have always been considered highly relevant to cognitive and behavioural functions. There is now a large body of data showing that nicotine can enhance information processing and cognitive function in experimental animals and human non-smokers (Terry and Callahan, 2019) by acting on neuronal nicotinic receptors (nAChRs). In addition to its pro-cognitive effects (Lendvai et al. 2013), nicotine has neuroprotective activity in multiple disease models (*in vitro* and *in vivo*), and this indicates that nicotine or nicotinic agonists might have both symptomatic and disease-modifying effects on neurodegenerative illnesses such as Alzheimer's disease (AD) and Parkinson's disease (Picciotto and Zoli, 2008; Geerts 2012) and other neurospsychiatric diseases (Radek et al. 2010; Wallace and Bertrand 2013).

nAChRs are a hetereogeneous family of ligand-gated receptors that regulate neuronal excitability and neurotransmitter release by modulating the flux of cations across cell membranes, and influence the physiological processes that affect neurobehavioural functions such as cognitive enhancement, neuroprotection and addiction (Zoli et al. 2015). A number of nAChR subtypes are present in mammalian brain, but the most widely expressed are the homopentameric α7 and heteropentameric $\alpha4\beta2$ receptors (Picciotto and Zoli, 2008). The activation of α4β2 or α7 nAChRs enhances synaptic plasticity *in vitro* (McKay et al. 2007) and improves memory performance in various animal cognition tests (Leiser et al. 2009; Levin 2012; Sabri et al. 2018). They are also the most frequently targeted receptor subtypes in drug discovery programmes, especially those aimed at cognitive disorders.

Nicotine is the main addictive chemical in tobacco and greatly stimulates the mesolimbic dopaminergic neurotransmitter system. Smoking leads to neuroadaptations in various brain areas that sustain the habit, whereas smoking cessation disrupts the equilibrium reached in the presence of nicotine and induces an unpleasant sensation. In rodents, the withdrawal syndrome is characterised by somatic signs and affective changes (including increased anxiety, anhedonia and irritability) that are similar to those observed in humans (reviewed in Paolini and De Biasi, 2011; Baldwin et al. 2011). Furthermore, it decreases the function of the reward system and dramatically increases reward thresholds in intracerebral self-stimulation experiments (Epping-Jordan et al. 1998) decreases dopamine release and leads to changes in the number of dopamine receptors (reviewed in Pistillo et al. 2015).

Partial nAChR agonists of the α4β2 subtype have been intensively studied because they help people to stop smoking by maintaining moderate levels of dopamine that counteract withdrawal symptoms and attenuate the rewarding effects of smoking by

preventing nicotine from accessing the α4β2 nAChRs (Cahill et al. 2016; Kathuria et al. 2018).

Varenicline is the most widely known partial agonist of $\alpha4\beta2$ receptors (Rollema et al. 2009). It activates $\alpha4\beta2$ receptors with a maximal effect that is ~50% than that of nicotine, reduces the desire to smoke and decreases attentional deficit during nicotine withdrawal (Prochaska and Benowitz, 2016). However, it has many side effects in smokers, including nausea, headache, insomnia and rare neuropsychiatric events (Anthenelli et al. 2016). In the search for new drugs that selectively act on dopamine release, particular attention has been given to cytisine, a nicotinic agonist that has been used as a smoking cessation drug in eastern and central European countries since the 1960s (Etter and Stapleton, 2006). At the level of the CNS, cytisine has the same activity as nicotine but only at higher doses, probably because of its poor penetration of the blood brain barrier.

The highly conserved nature of the reward pathway in the brain and the ability of drugs of abuse to stimulate the nervous system allow drug–associated reward to be modelled in non-mammalian species such as zebrafish, thus making them a promising animal model for screening psychoactive substances. Eight zebrafish nAChR subunit cDNAs (α2, α3, α4, α6, α7, β2, β3, and β4) have been cloned (Zirger et al. 2003; Ackerman et al. 2009; Ackermann and Boyd, 2016) and those that have been expressed in XENOPUS oocytes share pharmacological properties with mouse, rat and human nAChRs. Zebrafish have previously been used to study the mechanisms of nicotine reward and the role of nicotine and nAChRs in other complex behaviours .

Our research groups have long been working on the design, synthesis and characterisation of nicotinic ligands of unichiral 2-substituted 1,4-benzodioxanes, in particular 2-(2-pyrrolidinyl)-1,4-benzodioxanes (Pallavicini et al. 2006, 2009; Bolchi et al. 2007, 2011, 2014, 2017), and unichiral prolinol aryl ethers (Bolchi et al. 2015, 2016). The pharmacological characterisation of these compounds has shown that some have very high and selective affinity for the $\alpha4\beta2$ subtype and, depending on the flexibility of their structure and the substitution pattern at the aromatic ring, they can be $\alpha4\beta2$ full agonists, partial agonists or antagonists. Full agonism is shown by the flexible prolinol aryl ethers, whereas rigid pyrrolidinyl benzodioxanes behave as partial agonists or (when deprived of any decoration at the benzodioxane nucleus) antagonists.

With the aim of developing new nAChR ligands for use in a variety of diseases, we tested a selection of these compounds (Figure 1) for their effects on zebrafish behaviour. As it has been shown that prolinol aryl ethers (compounds MCL-28 and MCL-11), are $\alpha4\beta2$ receptor full agonists *in vitro* tests, we hypothesised that they may reinforce learning and memory. We therefore tested MCL-28 and MCL-11 and the antagonist pyrrolidinyl benzodioxane MCL-117 using the T-maze and a visual object recognition test (VORT) which respectively evaluate spatial memory and attention. We also tested

the pyrrolidinyl benzodioxane MCL-54 partial agonist, using conditioned place preference (CPP) in order to investigate its ability to block the rewarding effects of nicotine.

MATERIALS AND METHODS

Animals and housing

Five hundred and seventy adult male and female (approximately 50%-50%) wild type (short fin) zebrafish (*Danio rerio*) were obtained from the Department of Life and Enviromental Science of the Università Politecnica delle Marche (Ancona, Italy). The zebrafish were 6–12 months old, 3–4 cm long, that is typical of young age (Itou et al. 2012) and were housed in mixed age groups of 30 in 96 L tanks under standard conditions (28± 2 °C, 14–10 h day/night cycle, with lights on at 7:00 a.m.) for at least two weeks before the experiments.The tank water contained sea salts (Instant Ocean, Aquarium System, Sarrebourg, France) at a concentration of 0.6 g/10L, dissolved in water obtained by means of a reverse osmosis filter system. Water quality was kept at optimal levels, checked for pH (6.5-7.5) every day and for nitrates (<0.02 ppm) every three days. The fish were fed twice a day (two hours before each test in the morning and late in the evening) with commercial flakes (tropical fish food, Consorzio G5, Italy) supplemented with live brine shrimp. All of the fish were drug naive**.** All of the experiments followed the ARRIVE guidelines and were approved by the National Ethics Committee for the care and use of laboratory animals and the National Ministry of Health (Italian Government Decree N° 513/2018PR). In addition, the number of animals used and their suffering was minimized in all experiments.

Behavioural testing

Behavioural testing took place between 09:00 and 14:00 h during the light phase. Animals from different tanks were chosen using a simple randomisation method based on a specific statistical random table**.** For each test, the tank was positioned in front of a webcam in order to ensure optimal video recording for later video-aided analysis. Each video was evaluated by three trained observers blinded to treatment. To reduce handling stress during the T-maze and VORT tasks, groups of 16 fish first underwent two habituation trials of 1 h each every day for three days during which, they were allowed to freely explore the entire apparatus. To minimise acute social isolation stress, the number of fish was gradually reduced: starting with 16 on day 1, to eight fish on day 2, and four fish on day 3 with individual fish testing starting on day 4 (Sison and Gerlai (2010). After each habituation trial, the fish were returned to their home tank until the next habituation. Each fish was used only for one test. Ten fish per group were used for each drug concentration based on our (Braida et al. 2014a, 2014b; Ponzoni et al., 2014, 2016a, 2016b) and on other studies (Karnik et al. 2012; Kundap et al. 2017) and each fish only received a single dose of one compound.

Treatment

Zebrafish body weight was measured according to Braida et al. (2007). Each fish was removed from its tank using a net and placed in a container of tank water positioned on a digital balance, and its weight was determined as the weight of the container plus the fish minus the weight of the container before the fish was added. The mean of three measurements was recorded. After weighing, each fish was put into an anesthetic solution of tricaine methanesulfonate (Sigma Aldrich, St. Louis, MO, USA) (150 mg/L) and upon loss of response to touch, was placed in a supine position. The drugs were injected into the abdominal cavity using a Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) at a volume of 2 µL/g. No more than the tip of the needle was inserted into the abdomen of each fish in order to prevent damage to its internal organs. After injection, each fish was immediately transferred back to its tank water for recovery.

T-maze

Zebrafish are capable of associative learning (i.e. they can acquire a memory of their environment after being allowed to explore it (Gomez-LaPlaza and Gerlai, 2010; Sison and Gerlai, 2010), and prefer to swim in areas with vegetation where they are protected from predators (Engeszer et al. 2007; Spence et al. 2006). We used a transparent Plexiglas T-maze (filled with tank water to a level of 10 cm) according to Braida et al. (2014a). The apparatus includes a start zone (30 x10 cm) separated from the rest of the maze by a transparent removable door. Behind this partition, there is a long (50 x10 cm) arm, at the end of which there are two short (20 x10 cm) arms perpendicularly to the right and the left and lead to the removable deep water chambers (30 x 30 cm). One of these chamber only contained water, the other known as reservoir, contains plants and a substrate of shells, stones and coloured marbles, thus making it possible to evaluate how well fish has learned and remembered the location of this desirable enriched environment. The location of the reservoir was the same for each subject during two training trials of exposure in the T-maze but, in each experimental group, 50% of the fish had the reservoir on the left, and the other 50 % on the right. To prevent viewing of the two chambers, two removable opaque partitions (4.5 cm×30 cm) were put, in a staggered way, at the beginning of each short arm. Each subject received two training trials of exposure in the T-maze, at an interval of 24 h. During each trial, each fish was placed in the start box for 5 min with its door closed. Then, the start box door was raised and then lowered after the fish had exited. Ten minutes was allowed to reach the reservoir or the other chamber. The running time taken to reach the reservoir and stay for at least 20 s was recorded by an experimenter blinded to the pharmacological treatments. After 20 s, each fish was returned to a home tank and housed in groups of two (of different sex). The fish were then given a second session 24 h later. The difference between the running time taken to reach the reservoir and stay for at least 20 s, during the first and the second trial was calculated as a measure of memory of the spatial location of reward.

Virtual Object Recognition test (VORT)

VORT was carried out according to Braida et al. (2014b) using a rectangular transparent plexiglas tank (70 cm long x30 cm high x10 cm wide) filled with tank water at a level of 10 cm. A central area of 20 cm is obtained by inserting two opaque barriers to visually isolate the two stimuli areas where two identical white geometrical shapes, on a black background, are shown on two iPod 3.5-in. widescreen displays, located externally to the opposite 10 cm wide walls. In order to minimize procedural novelty stress, zebrafish were habituated to the apparatus as described in the behavioural testing. Then, they were restricted for 5 min in a 20 cm central area delimited by two opaque barriers after which, each animal was submitted to a familiarisation trial (acquisition phase during T_1) followed by a shape recognition trial (test phase during T_2). Five min intervals separated the acquisition phase from the test phase. T_1 consisted of a 10-min session, during which two identical white geometrical shapes on a black background were shown on two 3.5-inch widescreen displays (iPod screens). Shapes were simple geometrical shapes (square, triangle, circle, cross, etc.) with equal surface (2.5 cm^2). Each shape was shown on a 3rd generation iPod Touch (Apple) through iTunes for the duration of the experiment (320 pixels horizontal axis and 480 pixels vertical axis). The luminosity of the screens was constant across the two screens and testing sessions. After T_1 the fish returned to the home tank. One of the two identical familiar shapes was replaced with a novel one and after 5 min, during T_2, each fish was placed again in the central area. Attention was paid to counterbalance the choice of the shapes within each treatment group that is that all the pairing of shapes were randomly chosen. Shape recognition was manually scored with a stopwatch, by an experimenter blind to the treatment, in terms of exploration time whenever the zebrafish approached to the iPod area (10 cm) and directed its head toward the shape.

Swimming behavior

Each subject was placed in a transparent observation chamber (20 cm long×10 cm wide×15 cm deep) containing home tank water filled at a level of 12 cm. The floor of the chamber was divided into ten equal-sized 2×10-cm rectangles. Using a time sampling procedure, swimming activity was monitored by counting the number of lines crossed in a 30-s observation period every 5 min, for a total of six observation bins over 30 min (Swain et al. 2004). The mean of the six observation bins was calculated.

Conditioned Place Preference (manca half

The fish were tested in a two-chamber tank (10 cm × 20 cm × 15 cm) as previously described (Braida et al. 2007). The tank was divided into two halves (10 cm × 10 cm) containing distinct visual cues (three black polka dots in one halve, no dots in the other) with a perforated wall that allowed complete, albeit somewhat impeded, movement. On the first day, after a previous

introduction to the apparatus, the fish were tested for baseline preference by calculating the percent of time spent on a given side during a 15-min trial (pre-conditioning phase: PRE). Six hours later, the fish were given i.p. injections with nicotine (0.01 mg/kg) or one of different dose of MCL-54 or vehicle and then restricted to the least preferred side for 30 min. Twenty-four hours after, fish receiving vehicle or drug were confined in the opposite compartment for 30 min. Drug-texture pairings were always counterbalanced. On the third day (post-conditioning phase: POST), the fish were free to access to two sides for 15 min and the time spent in each compartment was recorded. The change (Δ) in preference, obtained by subtracting the baseline value from the final value in the drug-paired compartment, reflected rewarding or aversive properties.

Drugs

The drugs used were: nicotine bi-tartrate (0.001 and 0.02 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA), MCL-11 (0.0000001-0.001 mg/kg) (Bolchi et al. 2015), MCL-28 (0.0001-1 mg/kg) (Bolchi et al. 2015), MCL-117 (0.0001-1 mg/kg) (Pallavicini et al. 2006,2009) and MCL-54 (0.5-20 mg/kg) (Bolchi et al. 2011). The compounds MCL-28, MCL-54 and MCL-117 were tested as hydrochlorides, while the prolinol pyridyl ether MCL-11 as a free base. All drugs were dissolved in sterile saline (0.9%) + DMSO (2%) and prepared fresh daily. Treatment was done 20 min before each test. In the T-maze and VORT tasks the drugs or vehicle were administered i.p. 20 min before the first probe trial while in CPP test animals received the drugs or vehicle immediately before the conditioning phase. Vehicle group received sterile saline (0.9%) + DMSO (2%). When multiple treatments were needed, the drug solutions were put in the same syringe to avoid potential tissue trauma.

The doses of nicotine were chosen on the basis of their activity on T-maze and CPP tasks as previously described (Braida et al. 2014a; Ponzoni et al. 2014). The range of doses of nicotinic compounds was initially chosen on previous pilot studies performed in our laboratory. The dose of MCL-117, to antagonize nicotine, was chosen according to pilot studies in which doses from 0.01 to 1 mg/kg, previously tested for other nicotinic antagonists, were used. Only the rewarding and the maximally employed dose of each compound was tested in the swimming activity tank.

Binding assays and pharmacological experiments

Before the brain extraction, each fish was anesthetized in 0.2% Tricaine and then euthanized by incubation in ice water for 15 minutes according to (Gupta and Mullin, 2010). After dissection brains were immediately frozen in liquid nitrogen and stored at -80°C until later use. In each experiment, the tissues from 40-60 zebrafish were pooled and homogenized in 5 ml of 10 mM Na phosphate pH 7.4, 137 mM NaCl, 4 mM KCl and 2 mM phenylmethylsulfonylfluoride (PMSF) with a Potter homogenizer. The homogenates were then diluted and centrifuged for 1.5 h at 60,000g. The procedures of homogenization, dilution and centrifugation of the total membranes were performed twice, after which the pellets were collected, rapidly rinsed and then resuspended in the same

buffer containing a mixture of 10 µg/ml of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A and aprotinin. Binding studies were performed as previously described (Ponzoni et al. 2014) with minor modifications.

[^{3}H] epibatidine binding

(±)-[^{3}H] epibatidine with a specific activity of 56-60 Ci/mmol was purchased from Perkin Elmer (Boston MA) and the non-radioactive αBungarotoxin, nicotine and epibatidine were purchased from Sigma-Aldrich.

[^{125}I] α-Bungarotoxin binding

[^{125}I] α-Bungarotoxin with a specific activity of 100-120 Ci/mmol was radiolabeled by us using Na^{125}I purchased from Perkin Elmer (Boston MA) and was used for the saturation and competition experiments.

Pharmacological experiments

The inhibition of [^{3}H]-epibatidine and [^{125}I]-αBungarotoxin binding by the test compounds was measured by preincubating zebrafish membrane homogenates with increasing concentrations (10 pM - 10 mM) of the drug to be tested for 30 min at room temperature, followed by overnight incubation with a final concentration of 0.25 nM of [^{3}H]-epibatidine at 0°C or 2 nM [^{125}I]-αBungarotoxin overnight at room temperature .

Statistical analysis

All behavioural data were expressed as mean ± SEM. Data were analysed using one-way analysis of variance (ANOVA) for multiple comparisons followed by Tukey's post-hoc test or two-factor ANOVA, with treatment and time as between-subjects factors followed by Bonferroni's test. Data from VORT experiment were expressed as discrimination index [(time spent exploring novel shape−time exploring familiar shape) /(time spent exploring novel shape + time exploring familiar shape)], as previously described (Braida et al. 2014b). Data from fish receiving saline 20 or 30 min before T1 were pooled after making sure that there was no statistical difference between the two groups. Data from radioligand binding were evaluated by one-site competitive binding curve-fitting procedures In the saturation binding assay, the maximum specific binding (Bmax) and the equilibrium binding constant (Kd) values were calculated using one site–specific binding with Hill slope – model. The K_i values were obtained by fitting three independent competition binding experiments, each performed in duplicate for each compound on each subtype. The level of

significance was taken as $p<0.05$. All statistical analyses were done using software Prism, version 6 (GraphPad, SanDiego, CA, USA) 3.

RESULTS

Effect of nicotinic full agonists on spatial memory

The effect of NIC and the nicotinic full agonists is shown in Fig. 2. MCL-11 (Fig. 2a) showed a significant treatment effect ($F_{(6,63)}=21.14$, $p<0.0001$). Tukey's test revealed that it induced a significant cognitive enhancement at a dose of 0.00001 mg/kg. MCL-28 also had a significant treatment effect ($F_{(5,54)}=7.83$, $p<0.0001$) (Fig. 2b). Post hoc analyses showed that MCL-28 was maximally active at a dose of 0.001mg/kg. Both the compounds showed an inverted U-shaped dose–response function. There was a significant effect of treatment when nicotine, MCL-11 and MCL-28 were combined with the antagonist (MCL-117) ($F_{(7,72)}=17.02$, $p<0.0001$). Post hoc analysis revealed that MCL-117, which *per se* was ineffective, significantly antagonized memory improvement at the lowest dose (Fig2c).

Nicotinic full agonists enhance attention in VORT

In Fig. 3 the effects of different nicotinic full agonists on VORT, in terms of discrimination index (Fig. 3a-c) and total exploration time respectively (Fig. 3d-f), are shown. One-way ANOVA showed a significant difference among groups in the discrimination index after MCL-11 treatment ($F_{(5,54)}=20.22$, $p<0.0001$) (Fig.3a). Post-hoc analysis revealed that the dose as 0.00001 mg/kg increased attention at a degree similar to nicotine. High doses progressively reduced the memory improvement. Similar results were obtained with MCL-28 ($F_{(6,63)}=4.34$, $p=0.001$) (Fig 3b). Post-hoc analysis revealed that 0.1 mg/kg was the maximal effective dose. Findings obtained with MCL-117 are reported in Fig.3c. There was a difference among groups on discrimination index highlighted by one-way ANOVA ($F_{(7,72)}=8.65$, $p<0.0001$). Post hoc analysis indicated that the antagonist, *per se*, had a discrimination not different from vehicle groups but it significantly reduced nicotine-, MCL-11- and MCL-28-induced memory improvement. Concerning the total exploration time (Fig 3d-f), two-way ANOVA revealed no difference among groups (effect of treatment: $F_{(7,144)}= 0.84$, $p = 0.54$; effect of time: $F_{(1,144)}= 2.77$, $p = 0.09$; treatment x time interaction: $F_{(7,144)}= 0.08$, $p = 0.99$).

Nicotinic full agonists do not alter swimming behaviour

NIC, MCL-11, MCL-28 and MCL-117 were injected at pro-mnesic and at the highest dose used in cognitive tasks (Fig. 4). No change in swimming activity, compared to vehicle group ($F_{(6, 63)} = 0.55$, $p=0.76$,One-way ANOVA) was found for each compound.

Nicotinic partial agonists decrease nicotine-induced conditioned place preference

The development of a CPP by nicotine and different doses of the partial agonist MCL-54 injection is shown in Fig. 5**.** Statistical analysis revealed a significant difference among groups when comparing the time in the drug-paired compartment during the pre- and post- conditioning period ($F_{(7, 72)} = 21.99$, $p < 0.0001$). Tukey's test showed that MCL-54 induced a progressive increase in the time spent in the drug-paired side during the post-conditioning phase in a range of doses between 1 and 10 mg/kg, in comparison with vehicle group. This reinforcing effect, induced by MCL-54, was similar to that exhibited by nicotine (0.001 mg/kg). The dose of 5 mg/kg resulted in the greater reinforcing effect, whereas the doses of 0.5 and 20 mg/kg were ineffective. Combined treatment with nicotine and the partial agonist, given at the maximal used dose, significantly blocked the reinforcing effect of nicotine.

Pharmacological characterisation of the compounds to the zebrafish nAChRs by binding studies

Saturation binding experiments using the two radioligands [^{125}I]-αBungarotoxin and [^{3}H]-epibatidine, confirmed that zebrafish brain expresses two classes of nicotinic receptors: a class of receptors that contains the α7 subunit and bind [^{125}I]-αBungarotoxin with high affinity (Bmax receptors 134 ±4.7 fmol/mg of brain protein; Kd 1.6 nM; n=3) and a class of receptors that bind [^{3}H]- epibatidine with high affinity (Bmax 164.3±2 fmol/mg of protein; Kd 89 pM; n=3).

Competition binding affinity studies determined the affinity (Ki) of compounds MCL-11, MCL-54, MCL-28 and MCL-117 for native zebrafish brain [^{3}H]- epibatidine and [^{125}I]-αBungarotoxin receptors and in order to obtain a complete pharmacological profile we also tested nicotine as a reference compound. As shown in Table 1, the order of affinity for [^{3}H]- epibatidine receptors was nicotine>MCL-28>MCL-54>MCL-11>>MCL-117 and the order of affinity for [^{125}I]-αBungarotoxin receptors was MCL-28>nicotine>MCL-54 >MCL-11>>MCL-117. The affinity of all the compounds for [^{125}I]-αBungarotoxin receptors was always less (higher Ki values) than that for [^{3}H]- epibatidine receptors. Although compound MCL-11 showed less affinity for both classes of receptors than MCL-28 and MCL-54, it had the highest selectivity ratio (36.9 fold) for the native [^{3}H]- epibatidine receptors.

DISCUSSION

Over the last ten years, the zebrafish model has been widely used in bio-behavioural studies and, as zebrafish have many similar behaviours to those of rats or mice, they have been increasingly used to study the mechanisms of nicotine addiction and as a means of screening nicotinic drugs

affecting behaviour (Boyd, 2013; Fontana et al. 2018).

Our laboratories have been involved in synthesising and pharmacologically characterising a number of benzodioxane derivatives and prolinol aryl ethers as potent $\alpha4\beta2$ agonists and antagonists with diversified selectivity profiles and interactions. In order to study their *in vivo* effects on zebrafish behavior, we tested two compounds that have been previously functionally characterised as full agonists of human $\alpha4\beta2$ nAChRs (MCL-11 and MCL-28) and the antagonist (MCL-117) using the T-maze and a VORT, which respectively evaluate spatial memory and attention.

The full agonists MCL-11 and MCL-28 both improved attention and spatial memory in a dose-dependent manner: the inverted U-shaped dose-response curves were similar to that of nicotine, and indicated that moderate doses improved cognitive functions, whereas low and high doses were ineffective (Braida et al. 2014a). However, it is important to note that the maximally effective doses expressed as molar concentration of MCL-11 (4.8×10^{-11}) and MCL-28 (4.1×10^{-9}) were respectively 882 and 10.5 times lower than those of nicotine (4.32×10^{-8}). We have previously shown that $\alpha4\beta2$ receptors play a major role in the cognitive performance of zebrafish. Our binding studies, show that MCL-11 is highly selective of heteromeric [^{3}H]-epibatidine receptors, and this may explain the lower effective dose of MCL-11 in comparison with nicotine or MCL-28 in this test.

The data obtained from the VORT test indicate that the administration of these compounds improved attention in zebrafish, which showed a marked discrimination of shapes that would otherwise not be distinguished (Braida et al. 2014b). Both MCL-11 and MCL-28 were more potent than nicotine. The effects of the full agonists were completely blocked by MCL-117, a compound previously identified as an antagonist in patch clamp electrophysiological studies (Bolchi et al. 2016). In particular, the dose of 0.001 mg/kg proved to be efficient in antagonizing the maximally effective doses of nicotine and the full agonists (MCL-11 and MCL-28). The memory test data were not influenced by changes in swimming activity as the total number of lines crossed did not significantly change following the administration of the most effective doses.

Braida et al. (2014a) have used mecamylamine and dihydro beta erythroidine (DhβE) in the T-maze test to block the positive effect that the maximally active nicotine dose (0.02 mg / kg) has on the memory of the zebrafish. The results obtained with MCL-117 are qualitatively comparable with those of mecamylamine and DhβE.

The results that we obtained with MCL-11 and MCL-28 are comparable with those found in some previous studies of other nicotinic compounds. The effects of some new partial cytisine-derived agonists (CC4 and CC26) have also been evaluated using the T-maze task which revealed similar inverted U-shaped dose-response curves. They are also comparable with previous findings obtained using varenicline (Braida et al. 2014a), which significantly enhanced the time rats spent exploring the novel object and improved the animals' capacity to attenuate the impairment of

performance under challenging distractor conditions in a sustained attention task (Rollema et al. 2009; Howe et al. 2010).

In mammalian brain, $\alpha4\beta2$ receptors, are expressed at high levels in memory related areas such as the hippocampus and cerebral cortex. Zebrafish have no cortex or defined hippocampus, but have analogous brain structures containing heteromeric receptors (Panula et al. 2010; Parker et al. 2013) with which MCL-11 and MCL-28 can interact to enhance zebrafish memory and cognition.

MCL-54 was the only compound tested in the CPP task. Its maximal reinforcing dose was 5 mg/kg although the 1 and 10 mg / kg doses were also significantly effective. The lowest dose used (0.5 mg / kg) was ineffective, as was the 20 mg/kg dose, (although the latter was effective in attenuating nicotine-induced CPP and showed a typical antagonistic behavior). This is qualitatively comparable with the results obtained using partial agonists, such as cytisine, varenicline, CC4 and CC26 (Ponzoni et al. 2014), although these were more potent in inducing Our previous electrophysiological experiments have shown that MCL-54 is a partial agonist acting of human $\alpha4\beta2$ nAChRs and increases dopamine release in rat striatal slices by acting through $\alpha4\beta2$ and $\alpha6\beta2$ nAChRs. Moreover, when co-incubated with nicotine, MCL-54 prevents the maximal effect of nicotine on this response (Bolchi et al. 2011). The highly conserved nature of the reward pathways and the ability of drugs of abuse to stimulate them has made it possible to study the effect of many drugs of abuse in zebrafish and to test new compounds that can decrease or block their rewarding properties.

The findings of this study show that MCL-54 antagonises the reinforcement properties of nicotine, and are in line with those of previous studies of other partial agonists carried out in our laboratory (Sala et al. 2013). This antagonistic activity maybe due to the intrinsic pharmacological properties of partial agonists, which may decrease the rewarding effects of nicotine by reducing the release of dopamine at the level of the mesolimbic circuit.

In conclusion, the results of these *in vivo* studies,(which are significantly consistent with our previous functional data), have identified potential new lead molecules for improving learning and memory (MCL-11, MCL-28) or for inducing smoking cessation (MCL-54). However concerning MCL54 there is still the possibility that this compound can be addictive and this may restrict its therapeutic use.

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FIGURE LEGENDS

Figure 1

Chemical structures of the tested compounds.

Figure 2. Cognitive ability of zebrafish in terms of running time (sec) to reach the reservoir in a T-maze task after treatment with different drugs: a) MCL-11; (b) MCL-28 and (c) MCL-117 compared to nicotine (NIC).The agonist and the antagonist was given in combination. All the drugs were injected i.p. 20 min before the test. Running time was calculated as difference of pre-training running time at 0 h minus post-training running time evaluated at 24h. Each value represents the mean± SEM of 10 observations per group. *p<0.05, ** p<0.01,***p<0.001 ,****p<0.0001 vs Vehicle (Veh) ; $$ p<0.01 vs corresponding agonist (Tukey's test).

Figure 3 Effect of different drugs: (a) MCL-11, (b) MCL-28 and (c) compared to nicotine (NIC) on discrimination index (a,b,c) and exploration time (d,e,f) in VORT. Performance was assessed using poorly discriminated shapes. All the drugs were injected i.p. 20 min before T1. N= novel shape, F=familiar shape. Each agonist and the antagonist was given in combination. Each value represents the mean± SEM of 10 observations per group.** p<0.01,***p<0.001 ,****p<0.0001 vs Vehicle (Veh); && p<0.01,&&& p<0.001 vs MCL-11 (0.00001); $$ p<0.01, $$$ p<0.001 vs corresponding agonist (Tukey's test).

Figure 4. Swimming activity evaluated in zebrafish after treatment with different drugs:(a) MCL-11, (b) MCL-28, (c) MCL-117 compared to nicotine. Animals were individually recorded by counting the number of line crossings in a 30-sec observation period every 5 min over 30 min. All the drugs were injected i.p. 20 min before T1. N= novel shape, F=familiar shape. Each agonist and the antagonist was given in combination. Each value represents the mean± SEM of 10 observations per group. *p<0.05, ** p<0.01,***p<0.001 ,****p<0.0001 vs Vehicle (Veh); $$ p<0.01, vs corresponding agonist (Tukey's test).

Figure 5. Effect of MCL-54 on CPP in comparison with nicotine (NIC). MCL-54 elicited CPP following an inverted U shape. The drugs were given i.p. 20 min before the conditioning session. Preference was calculated by subtracting the time (mean ± SEM) spent in the drug-paired compartment before drug conditioning from the time spent after drug conditioning. Each value represents the mean± SEM of 10 observations per group. ** p<0.01,***p<0.001 ,****p<0.0001 vs Vehicle (Veh) (Tukey's test).

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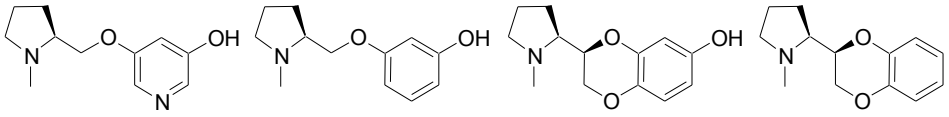
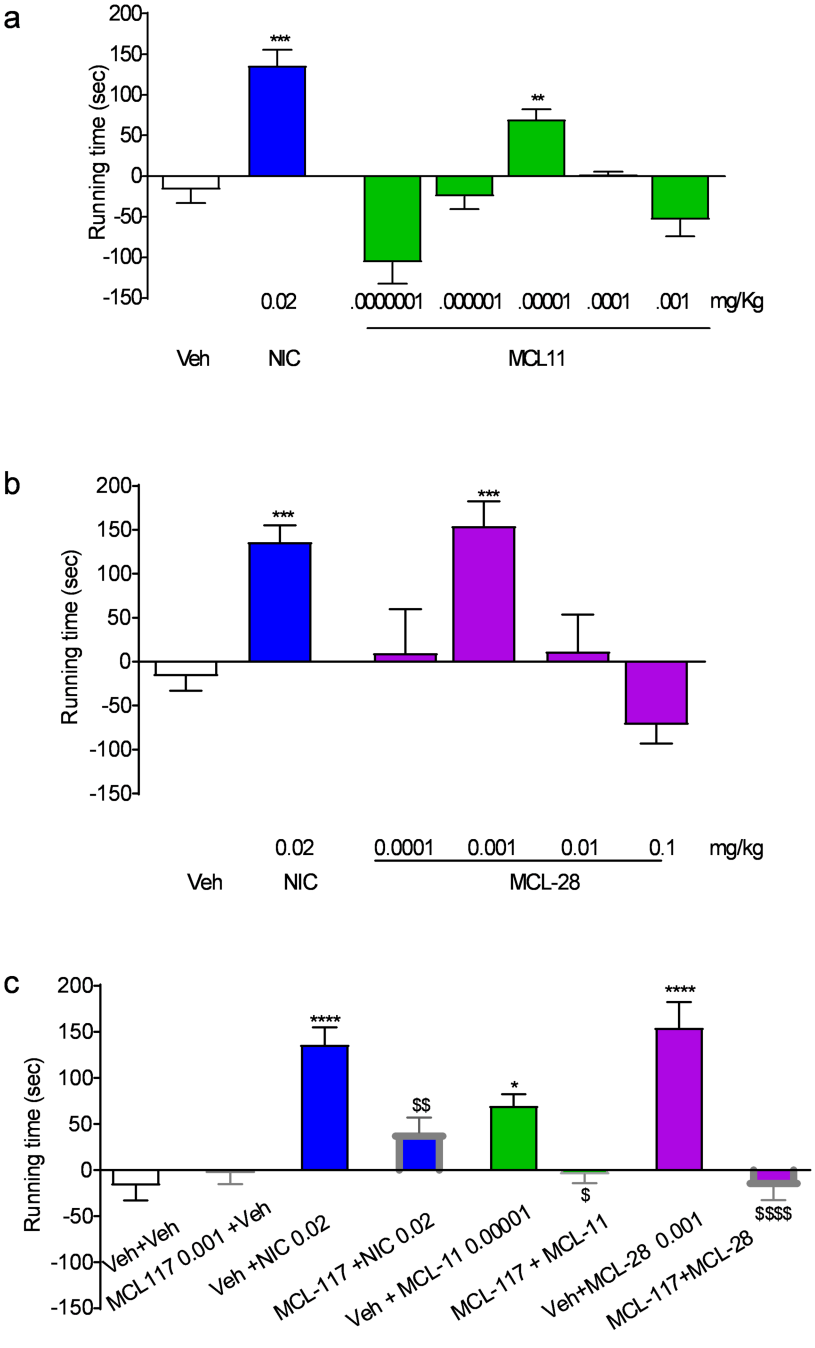


MCL-11 **MCL-28** **MCL-54** **MCL-117**

FIGURE 1

Figure 2


a
Running time (sec)
200
150
100
50
0
-50
-100
-150
***
**
0.02
.0000001
.000001
.00001
.0001
.001
mg/Kg
Veh
NIC
MCL11
b
Running time (sec)
***
***
0.02
0.0001
0.001
0.01
0.1
mg/kg
Veh
NIC
MCL-28
c
Running time (sec)
****
$$
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$
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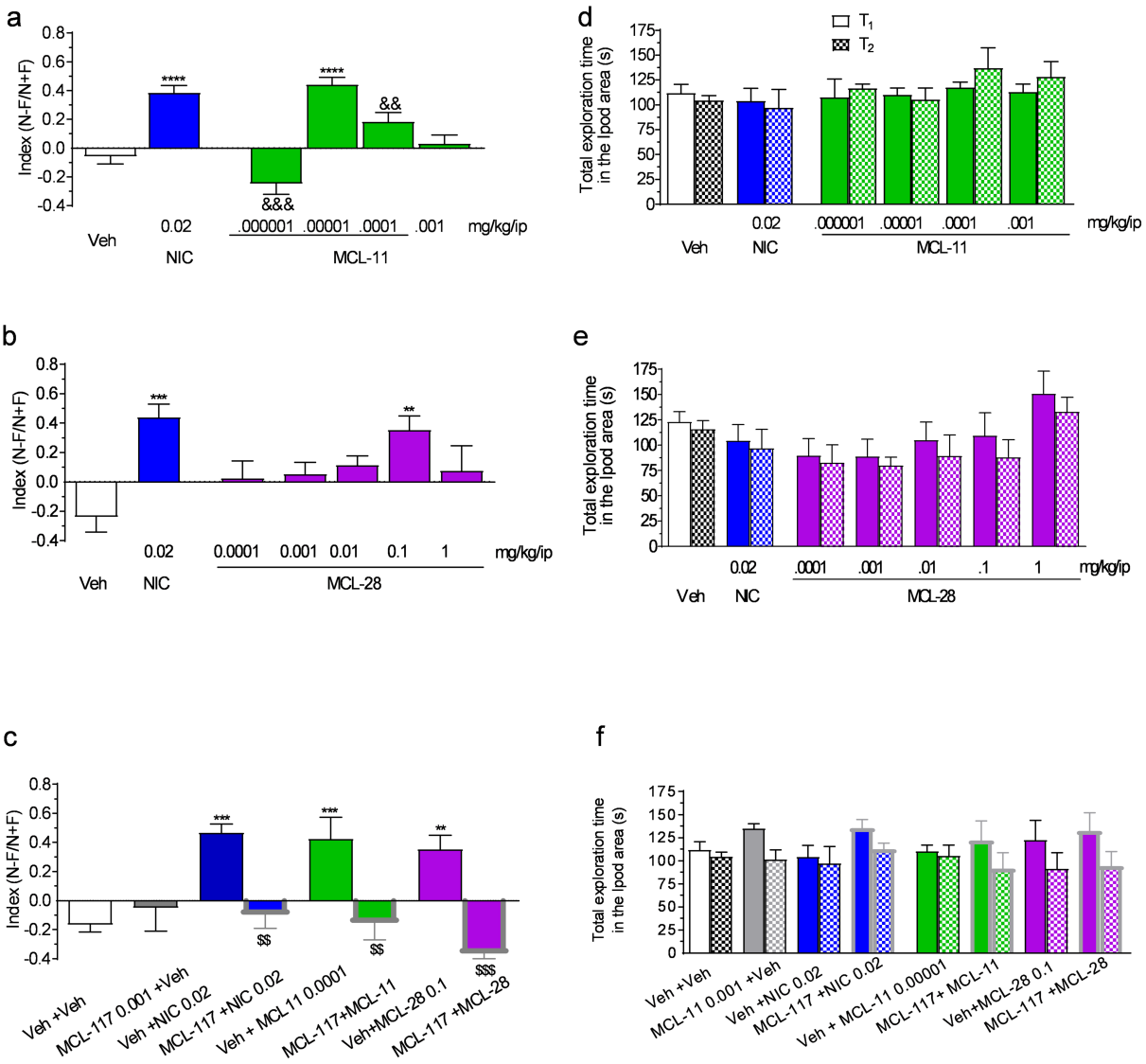
$$$$
Veh+Veh
MCL117 0.001 +Veh
Veh +NIC 0.02
MCL-117 +NIC 0.02
Veh + MCL-11 0.00001
MCL-117 + MCL-11
Veh+MCL-28 0.001
MCL-117+MCL-28

Figure 3


a
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0.4
0.2
0.0
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-0.4
****
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&&
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Veh
0.02
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.001
mg/kg/ip
NIC
MCL-11
d
Total exploration time in the Ipod area (s)
175
150
125
100
75
50
25
0
T1
T2
Veh
0.02
NIC
.000001
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.0001
.001
mg/kg/ip
MCL-11
b
Index (N-F/N+F)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
***
**
Veh
0.02
NIC
0.0001
0.001
0.01
0.1
1
mg/kg/ip
MCL-28
e
Total exploration time in the Ipod area (s)
175
150
125
100
75
50
25
0
Veh
0.02
NIC
.0001
.001
.01
.1
1
mg/kg/ip
MCL-28
c
Index (N-F/N+F)
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
***
***
**
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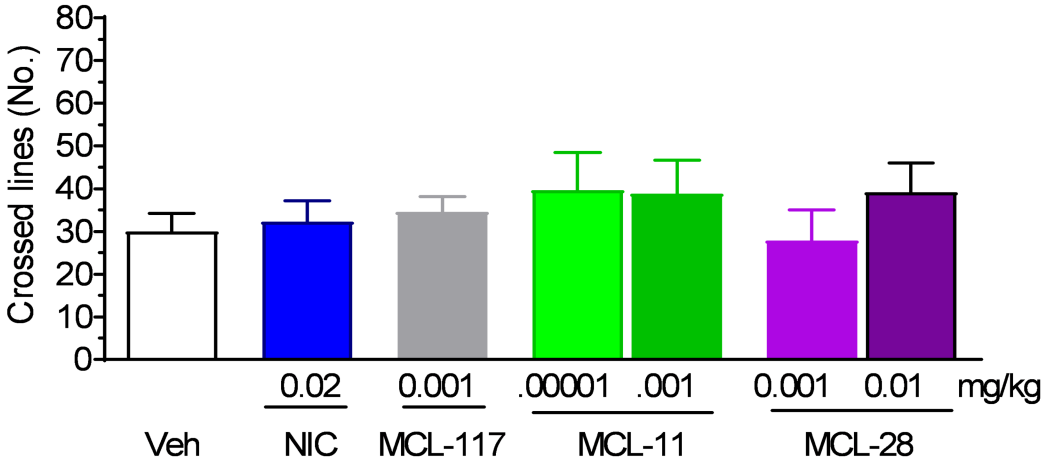
$$
$$$
Veh +Veh
MCL-117 0.001 +Veh
Veh +NIC 0.02
MCL-117 +NIC 0.02
Veh + MCL11 0.0001
MCL-117+MCL-11
Veh+MCL-28 0.1
MCL-117 +MCL-28
f
Total exploration time in the Ipod area (s)
175
150
125
100
75
50
25
0
Veh +Veh
MCL-11 0.001 +Veh
Veh +NIC 0.02
MCL-117 +NIC 0.02
Veh + MCL-11 0.00001
MCL-117+ MCL-11
Veh+MCL-28 0.1
MCL-117 +MCL-28

Figure 4


Crossed lines (No.)
80
70
60
50
40
30
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10
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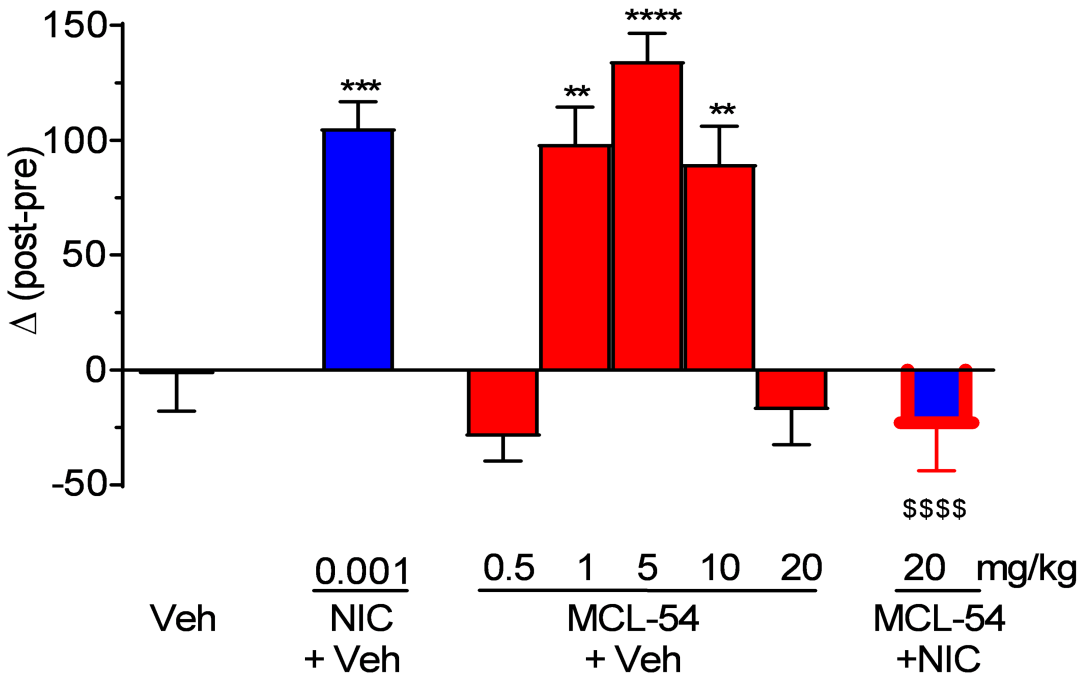
0.001
0.01
mg/kg
Veh
NIC
MCL-117
MCL-11
MCL-28

Figure 5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Δ (post-pre)
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Veh
0.001
NIC
+ Veh
0.5
1
5
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20
MCL-54
+ Veh
20
mg/kg
MCL-54
+NIC