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N-methyl-citalopram: A quaternary selective serotonin reuptake inhibitor

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ABSTRACT

We describe the synthesis and the pharmacological characterization of a new quaternary selective serotonin reuptake inhibitor (SSRI) N-methyl-citalopram (NMC) with periphery restricted action due to its inability to cross the blood brain barrier. NMC recognized and blocked the human platelet serotonin transporter (SERT) with similar affinity to that of citalopram as was evident from competition binding studies with [^{3}H]citalopram and uptake studies with [^{3}H]5-HT. In contrast, the affinity of NMC to rat brain SERT was 10-fold lower than its parent compound citalopram. Similarly to citalopram, NMC did not inhibit dopamine and noradrenaline uptake in rat brain synaptosomes at 10^{-7} M as well as [^{3}H]ketanserin binding to rat brain membranes at 10^{-5} M, demonstrating its SSRI profile. A comparison of radioactivity retained in perfused mice brain following in vivo intraperitoneal injections of tritium-labeled NMC or citalopram showed that unlike citalopram, NMC did not penetrate the brain. Taken together, our observations suggest that N-methyl-citalopram is a selective serotonin reuptake inhibitor that does not penetrate the mouse brain. Epidemiological studies have suggested that chronic use of SSRI drugs may confer a protective effect against myocardial infarction (MI) apparently reflecting reduced platelet aggregation secondary to reduced platelet serotonin levels. N-methyl-citalopram may therefore have a potential as a new anti-platelet drug that does not cross the blood brain barrier and is thus devoid of the adverse CNS effects of SSRI drugs.

1. Introduction

Serotonin (5-HT) is known to be implicated in platelet aggregation in humans [1–3]. Epidemiological studies have suggested that the use of selective serotonin reuptake inhibitors (SSRI), which are known to block the serotonin transporter (SERT), may be beneficial for myocardial infraction (MI) patients, reducing subsequent cardiac events by 40% [4–7]. It has been suggested that the benefit is secondary to reduced platelets 5-HT storage capacity in patients taking SSRI drugs chronically, and thus reduced platelet aggregation [4–6,8]. Among the lines of evidence pointing towards potential beneficial effects of reduced peripheral 5-HT is the observation that transgenic mice deficient in TPH1 (tph1−/−) and therefore in 5-HT synthesis exhibit reduced thrombosis risk [9]. The beneficial effects of SSRI drugs in cardiovascular disease (CVD) were recently reviewed by Atar et al. [10], Halperin and Reber [11] and Malinin et al. [12]. A randomized study compared sertraline (SSRI drug) versus placebo in depressed post-acute coronary syndromes (ACS) patients, administered in addition to the standard anti-platelet agents aspirin and clopidogrel [13]. In that study, plasma markers of platelet activation were monitored, and the results showed that sertraline treatment was associated with substantially attenuated release of platelet/endothelial biomarkers as compared to the standard treatment with anti-platelet agents. Use of SSRI drugs, but not other types of antidepressants, was associated with a 41% reduction of MI risk during a 3-year follow-up period [6]. Notably, aspirin and clopidogrel were reported to reduce MI risk by only 20% and 10% respectively [14]. Recent studies by Galan et al. [3] have shown that SSRI drugs such as citalopram potently inhibit the in vitro aggregation of human platelet induced by ADP or collagen in the presence of serotonin. Moreover, it was found that serotonin promoted platelet activation, potentiated procoagulant responses on human blood and increased thrombogenesis on damaged vascular surfaces – effects which were all attenuated by SSRI drugs [3].

However, prescribing SSRI drugs to MI patients who do not have depressive symptoms is not a feasible option, as all current SSRI drugs readily penetrate the blood–brain barrier (BBB) and thus have CNS activities, including undesired CNS effects, in addition to their desired peripheral action of reducing platelet 5-HT content. Indeed SSRI drugs are reserved in the clinic only for treating psychiatric conditions such as major or bipolar depression, obsessive-compulsive disorder and additional mood disorders. Due to the beneficial effects of current SSRI drugs in CVD,

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and considering their CNS adverse effects, there is a rational for developing novel peripherally restricted SSRI compounds as added therapeutics to aspirin and clopidogrel for various CVD disorders.

Here we report on the chemical modification of citalopram, one of the most widely prescribed SSRIs, so as to include a positively charged group, which is selected such that the modified SSRI compound retains its charge at any pH, making it brain-impermeable while substantially retaining its selective serotonin reuptake inhibition activity. In the present study we describe the synthesis and the pharmacological characterization of a new SSRI, N-methyl-citalopram (NMC), with periphery restricted effect due to its inability to cross the blood brain barrier (BBB).

2. Materials and methods

2.1. Materials

Citalopram, serotonin, fluoxetine, mazindol, dopamine and noradrenaline were of highest purity obtainable from Sigma–Aldrich Israel Ltd. Mazindol was purchased from Sandoz Pharma AG Basel/Switzerland.

[^{3}H]Serotonin (20.3 Ci/mmol), [^{3}H]citalopram (79 Ci/mmol), [^{3}H]dopamine (55.1 Ci/mmol) and [^{3}H]ketanserin (76 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). [^{3}H]Noradrenaline (35 Ci/mmol) was purchased from Amersham, GE Healthcare, UK.

2.2. Animals

Male Wistar rats and Imprinting Control Region (ICR) mice (Harlan laboratories, Israel) were maintained under controlled light and temperature conditions, with food and water provided ad libitum.

Animal procedures were approved by the Animal Care Committee of Tel-Aviv University (Approval numbers: M-06-105, M-07-027).

2.3. Preparation of N-methyl-citalopram

N-methyl-citalopram (NMC) was synthesized from the free-base form of the parent compound citalopram via methylation by methyl iodide. Saturated solution of sodium bicarbonate (20 ml) was added to a solution of citalopram (1 g) in dichloromethane (20 ml). The reaction was mixed for a few minutes, and then was allowed to separate to two phases. The organic phase was concentrated under reduced pressure to give 0.5 g of the free-base form of citalopram that was used in the next step without further purification. Methyl iodide (4.2 g, 31 mmol) was added to a cool solution of the free-base form of citalopram (1 g, 3.08 mmol) in acetone. The mixture was heated to reflux overnight, and thereafter the solvent was removed under reduced pressure to produce N-methyl-citalopram. The purity of NMC and the absence of citalopram were verified by HPLC and by mass spectrometry.

2.4. Preparation of [^{3}H]N-methyl-citalopram

[^{3}H]N-methyl-citalopram was prepared by Vitrax (CA, USA). [^{3}H]Methyl iodide (C[^{3}H]$_3$I), at specific activity of 70 Ci/mmol, was vacuum transferred into a tritiation production vessel containing the free base form of citalopram. Following several hours of incubation all volatiles were removed via distillation under reduced pressure. The crude residue was purified by HPLC to yield [^{3}H]N-methyl-citalopram, at more than 99% radiochemical purity.

2.5. Human platelet membrane preparation

Human venous blood samples (25 ml) were collected in the morning (between 8 and 10 a.m.) from healthy volunteers into plastic tubes containing 3.8% sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was separated from blood cells by low-speed centrifugation (350 × *g* for 10 min), diluted in 20 ml buffer A (50 mM Tris–HCl buffer pH 7.4 containing 120 mM NaCl and 5 mM KCl) and centrifuged at 1700 × *g* for 20 min. The supernatant was discarded and the final membrane pellet was kept frozen at −70 °C until use. On the day of use, the pellet was disrupted with Brinkman polytron in 20 ml of buffer A and centrifuged twice at 27,000 × *g* for 20 min. It was then resuspended in 9 ml buffer A to yield a final protein concentration of about 0.8 mg/ml.

2.6. [^{3}H]citalopram binding to human platelets

[^{3}H]Citalopram binding to 5-HT transporter (SERT) was determined using a method modified from Plenge and Mellerup [15]. A standard binding assay contained 200 μl of platelet membranes, 100 μl [^{3}H]citalopram (2 nM) and 50 μl buffer A or test drug. After a 60 min incubation period at 25 °C, homogenates were diluted in 3 ml ice-cold buffer and filtered through Whatman GF/C glass fiber filters. Filters were washed three times with 3 ml ice-cold buffer, and the radioactivity was measured in scintillation liquid in a β-counter (Packard, Tri-Carb 2100TR). Specific binding was defined as the difference between total [^{3}H]citalopram binding (triplicate samples) and the binding in the presence of 10 μM fluoxetine (duplicate samples).

2.7. [^{3}H]citalopram binding to rat brain membranes

Rats were decapitated and cerebral cortex was dissected on ice. Rat brain cortex was disrupted with Brinkman polytron in 50 volumes of buffer A and centrifuged (three times) at 30,000 × *g* for 10 min. It was then resuspended in the same buffer to yield a final concentration of about 21 mg/ml (wet weight). [^{3}H]Citalopram binding was determined using a method modified from Plenge and Mellerup [15]. A standard binding assay contained 100 μl of brain homogenate, 100 μl [^{3}H]citalopram (1 nM) and 300 μl buffer or 250 μl buffer and 50 μl test drug. Incubation and filtration were conducted as described in Section 2.6.

2.8. [^{3}H]5-HT uptake into human platelets

Human blood samples (25 ml) were collected in the morning (between 8 and 10 a.m.) from healthy volunteers into plastic tubes containing 3.8% sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was separated by low-speed centrifugation (350 × *g* for 10 min). The PRP was diluted 1:1 in buffer B (19 mM phosphate buffer, 119 mM NaCl, 3.9 mM KCl, 0.65 mM $MgSO_4$, 0.51 mM $CaCl_2$, 2 mg/ml glucose, 0.2 mg/ml ascorbic acid, 0.16 mM EDTA and 50 μM pargyline, pH 7.4) and centrifuged at 1700 × *g* for 20 min. The platelet pellet was gently resuspended in buffer B (10^8–10^9 platelets/ml) and used immediately to measure uptake. The uptake assays were carried out as described previously [16]. A standard assay contained 200 μl washed platelets, 50 μl radiolabelled drug and 50 μl buffer B or test drug. The tubes were preincubated at 37 °C for 10 min, at which time [^{3}H]5-HT (50 nM) was added. After a 2 min incubation period at 37 °C the reaction was stopped by rapidly cooling the tubes on ice and the mixture was filtered under vacuum on glass fiber filters (GF/C). The filters were washed with ice-cold buffer B and the radioactivity was counted in scintillation liquid in a β-counter. Specific uptake was defined as the difference between total uptake at 37 °C (triplicate samples) and the uptake measured at 0 °C (duplicate samples).

2.9. [^{3}H]5-HT uptake into rat brain synaptosomes

Rat brain cortex sample was homogenized in 10 volumes of 0.32 M sucrose using a Teflon-glass homogenizer. The sample was centrifuged at 1000 × *g* for 10 min and the resulting supernatant (S1) containing synaptosomes was used to measure [^{3}H]5-HT uptake.

[^{3}H]5-HT uptake assay was carried out as described previously [17]. A standard assay contained 50 μl synaptosomes, 50 μl [^{3}H]5-HT and 900 μl buffer B or 850 μl buffer B and 50 μl test drug. The tubes were preincubated at 37 °C for 10 min, at which time [^{3}H]5-HT (50 nM) was added. After a 4 min incubation period at 37 °C the reaction was stopped, the tubes washed and the radioactivity counted as described in Section 2.8.

2.10. [^{3}H]ketanserin binding to rat brain membranes

[^{3}H]ketanserin binding experiment was performed according to Takao et al. [18]. In brief, frozen rat brain cortex was homogenized in 10 volumes of ice-cold 0.32 M sucrose using a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C and the resulting supernatant was centrifuged at 34,000 × *g* for 20 min at 4 °C. The obtained pellet was homogenized with polytron in 40 volumes 50 mM Tris–HCl buffer pH 7.4 before incubated for 15 min at 37 °C. The homogenate was finally centrifuged at 38,000 × *g* for 20 min and pellet resuspended in buffer (10 mg protein/ml) and kept at −70 °C until further use.

A standard binding assay contained 200 μl of brain membranes, 100 μl [^{3}H]ketanserin (0.4 nM) and 700 μl buffer or 600 μl buffer and 100 μl test drug. [^{3}H]ketanserin was added to the test tubes after a 3-min preincubation period at 37 °C preceding the 15-min incubation at 37 °C. The homogenates were diluted in 3 ml ice-cold buffer and filtered through Whatman GF/C glass fiber filters preincubated in 0.05% polyethyleneimine. Specific binding was defined as the difference between total [^{3}H]ketanserin binding (triplicate samples) and the binding in the presence of 10 μM mianserin (duplicate).

2.11. Determination of [^{3}H]N-methyl-citalopram BBB penetration in vivo

[^{3}H]N-methyl-citalopram was prepared by Vitrax (CA, USA) to a specific radioactivity of 70 Ci/mmol and shown by HPLC to be chemically identical to non-labeled N-methyl-citalopram. In comparison, [^{3}H]citalopram had a specific radioactivity of 79 Ci/mmol. Each of the radioactive compounds, [^{3}H]N-methyl-citalopram and [^{3}H]citalopram, was diluted with the same non-radioactive compound so that the dose of the injected compound was 1.5 μg/g of body weight. The amounts of radioactivity injected i.p. to mice were 318,000 and 174,000 dpm/g weight for [^{3}H]N-methyl-citalopram and [^{3}H]citalopram, respectively. This protocol allowed the dose of injected citalopram to be similar to the higher end of the human clinical recommended dosage of 1.5 mg citalopram/kg body weight.

Mice were given 20 mg/kg pentobarbital (i.p.) 10 min prior to being perfused with saline for about 2 min. The perfusion is required for removing residual blood from the brain tissues and the use of pentobarbital is obligatory due to animal welfare considerations. Saline/heparin perfusion was started at 5, 10, 20 or 40 min from the time of intraperitoneal injections of the radioactive compounds. At the end of the perfusion, mice heads were cut off and their dissected brains were washed with ice-cold saline for 30 s. The cerebral cortex tissues were dissected according to the Atlas of the Mouse Brain [19] and immediately weighed (weight range: 132–205 mg). The tissues were thereafter homogenized in 10 volumes of distilled water using a Teflon/glass grinder. The radioactivity, expressed as cpm per 500 μl homogenate samples, was counted in a liquid scintillation counter (Packard, 2100TR, USA) and corrected for dpm using an internal standard.

2.12. Statistics

The independent samples *t*-test, using SPSS software (SPSS Inc., Chicago, IL), was used as appropriate. Results are expressed as means ± S.E.M.

3. Results

3.1. Preparation of N-methyl-citalopram

N-methyl-citalopram (NMC) was synthesized via methylation of citalopram, using methyl iodide. The structure of NMC is shown in Fig. 1. The purity of NMC and the absence of citalopram were verified by HPLC as well as by mass spectrometry (calculated formula weight 339.4 versus mass spectrometry value of 339.1).

3.2. [^{3}H]citalopram binding and [^{3}H]5-HT uptake in human platelets

The affinity of N-methyl-citalopram (NMC) to the 5-HT transporter (SERT) in human platelets was compared to the affinity of citalopram. Fig. 2 is a representative experiment, of [^{3}H]citalopram binding inhibition to human platelet membranes

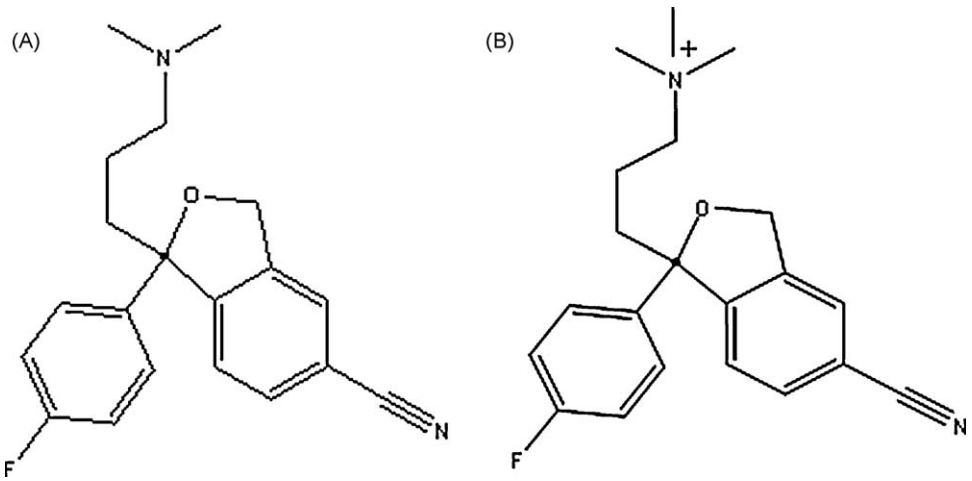

Fig. 1. Structure of citalopram (A) and N-methyl-citalopram (B).

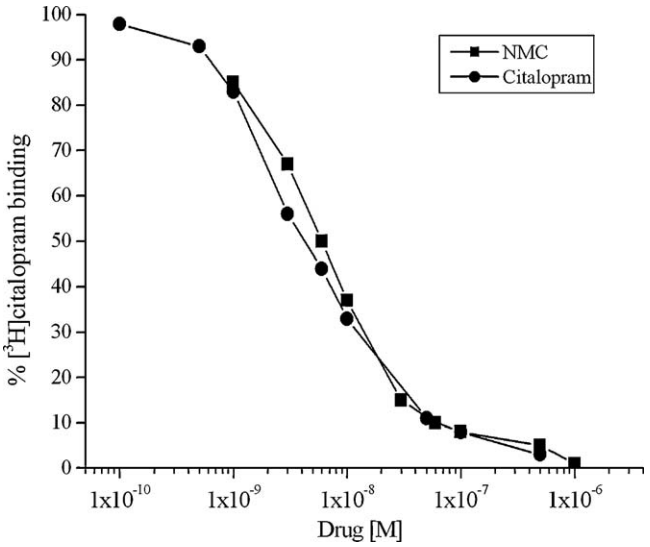

Fig. 2. Inhibition of [^{3}H]citalopram binding (2 nM) to human platelet membranes by citalopram or N-methyl-citalopram. Membranes were incubated with [^{3}H]citalopram for 60 min at 25 °C in the presence of increasing concentrations citalopram or NMC. Specific binding was defined as the difference between total binding (without competitor, performed in triplicate) and non-specific binding determined in the presence of 10 μM fluoxetine (triplicate). Three separate experiments were performed. Data shown are from a representative experiment.

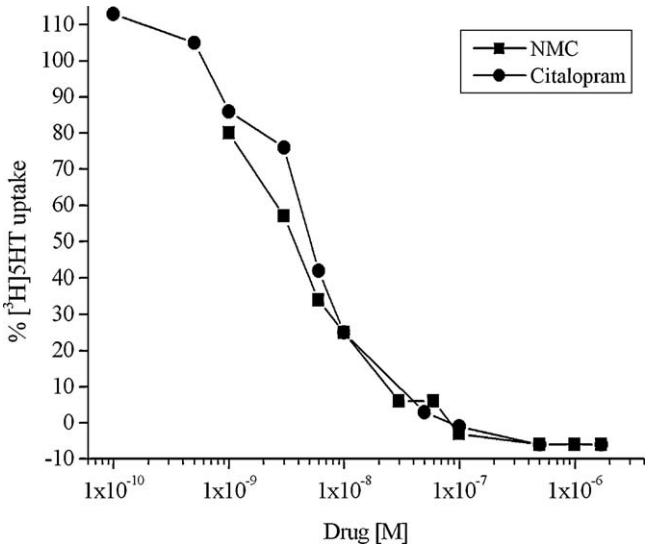

Fig. 3. Inhibition of [^{3}H]5-HT uptake (50 nM) to intact human platelets by citalopram or N-methyl-citalopram. Platelets were preincubated for 10 min at 37 °C at which time [^{3}H]serotonin was added for 2 min. The reaction was stopped by rapid cooling on ice. Specific uptake was defined as the difference between total uptake at 37 °C (triplicate samples) and the uptake measured at 0 °C (duplicate samples). Three separate experiments were performed. Data shown are from a representative experiment.

by increasing concentrations of NMC or citalopram. N-methyl-citalopram recognized the human platelet SERT with similar affinity as compared to that of citalopram (mean K_i values of 7.2 ± 1.3 nM and 3.9 ± 0.3 nM for NMC and citalopram, respectively) (Table 1).

Inhibition of [^{3}H]serotonin uptake into intact freshly prepared human platelets by NMC or citalopram is presented in Fig. 3. NMC inhibited [^{3}H]5-HT uptake in freshly isolated human platelets with mean inhibition constant (K_i) value of 5.2 ± 0.8 nM, a value almost similar to that determined for citalopram (4.2 ± 0.6 nM) (Table 1).

3.3. [^{3}H]citalopram binding and [^{3}H]5-HT uptake in rat brain

A representative experiment of [^{3}H]citalopram binding inhibition to rat brain membranes is depicted in Fig. 4. The K_i values were 53 ± 15 nM for NMC and 3 ± 1 nM for citalopram (Table 1). The inhibitory effect of citalopram and NMC on [^{3}H]serotonin uptake to freshly prepared rat brain synaptosomes is presented in Fig. 5. The K_i values were 44 ± 6.6 nM for NMC and 4.5 ± 0.7 nM for citalopram (Table 1). Thus, for both binding to the serotonin transporter and inhibition of its serotonin uptake activity NMC shows a similar affinity to its parent compound citalopram in human platelets while in rat brain it exhibits about 10-fold lower affinity compared with citalopram.

3.4. [^{3}H]Dopamine and [^{3}H]noradrenaline uptake to rat brain synaptosomes

The inhibitory effect of citalopram and NMC on [^{3}H]dopamine and [^{3}H]noradrenaline uptake into rat brain synaptosomes is compared to the inhibitory effect of mazindol in Fig. 6. Mazindol, an inhibitor of dopamine and noradrenaline uptake, inhibited [^{3}H]dopamine uptake by 50% at 45 nM. At this concentration citalopram did not inhibit the uptake of dopamine while NMC exhibited less then 10% inhibition. The uptake of [^{3}H]noradrenaline was inhibited by 50% at 8.5 nM mazindol whereas at this concentration citalopram did not have any effect and NMC inhibited less then 20% of the uptake.

3.5. [^{3}H]ketanserin binding to rat brain membranes

The affinity of N-methyl-citalopram to rat brain membranes serotonin 5-HT_{2A} receptors was evaluated in [^{3}H]ketanserin binding assay. IC_{50} of the 5-HT_{2A} antagonist mianserin was 3.3 nM while up to 10 μM of either citalopram or NMC did not inhibit [^{3}H]ketanserin (data not shown).

3.6. Determination of [^{3}H]N-methyl-citalopram BBB penetration in vivo

[^{3}H]N-methyl-citalopram and [^{3}H]citalopram were injected (i.p.) to mice at a clinically relevant dose of 1.5 mg/kg body weight and the detected radioactivity in the mouse brain tissues following perfusion at different time points and expressed as dpm are shown in Fig. 7. As can be seen, [^{3}H]citalopram readily entered the brain shortly after its i.p. injection, reaching levels of about 3350 dpm per 500 μl cerebral cortex homogenate sample at 40 min, corresponding to about 13,400 dpm for the entire cerebral cortex.

In sharp contrast, only very low amounts of radioactivity were detected in the brain homogenate samples of mice following

Table 1
[^{3}H]citalopram binding and [^{3}H]5-HT uptake inhibition constants in rat brain and human platelets. Values represent the means ± S.E.M. obtained from three independent experiments each conducted in triplicate. See Section 2 for further details. A significant difference of K_i values was observed only in rat brain: [a] $p = 0.029$, [b] $p = 0.001$ versus citalopram.

	K_i (nM) rat brain		K_i (nM) human platelets	
	[^{3}H]citalopram binding	[^{3}H]5-HT uptake	[^{3}H]citalopram binding	[^{3}H]5-HT uptake
Citalopram	3 ± 1	4.5 ± 0.7	3.9 ± 0.3	4.2 ± 0.6
NMC	53 ± 15[a]	44 ± 6.6[b]	7.2 ± 1.3	5.2 ± 0.8

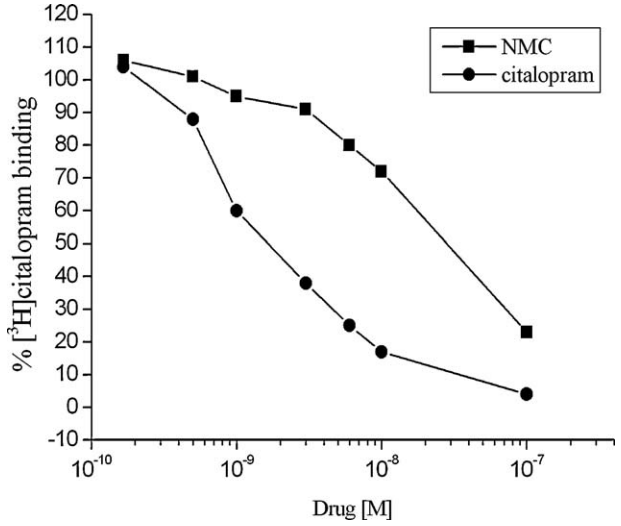

Fig. 4. Inhibition of [^{3}H]citalopram binding (1 nM) to rat brain membranes by citalopram or N-methyl-citalopram membranes were incubated with [^{3}H]citalopram for 60 min at 25 °C in the presence of increasing concentrations of citalopram or NMC. Specific binding was defined as the difference between total binding (without competitor, performed in triplicate) and non-specific binding determined in the presence of 10 μM fluoxetine (triplicate). Three separate experiments were performed. Data shown are from a representative experiment.

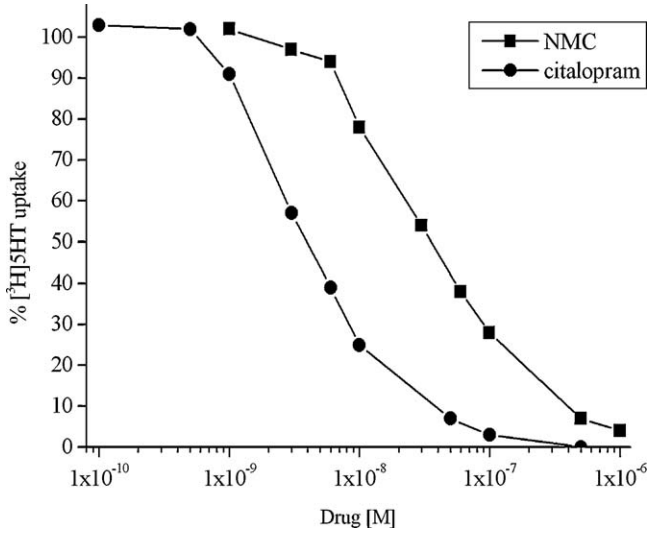

Fig. 5. Inhibition of [^{3}H]5-HT uptake (50 nM) to rat brain synaptosomes by citalopram or N-methyl-citalopram. Uptake assays were performed in synaptosomes prepared from rat brain cortex. After a 10 min preincubation period at 37 °C, [^{3}H]serotonin was added to the tubes containing synaptosomes and tested drugs for 2 min. The reaction was stopped by rapid cooling on ice. Specific uptake was defined as the difference between total uptake at 37 °C (triplicate samples) and the uptake measured at 0 °C (duplicate samples). Three separate experiments were performed. Data shown are from a representative experiment.

injections of a similar dose of [^{3}H]N-methyl-citalopram (Fig. 7). The radioactivity levels, expressed in dpm, in these brain homogenate samples were about 55-fold lower compared with the radioactivity levels in mice injected with [^{3}H]citalopram. These dpm counts, ranging from 50 dpm at 5 min to 110 dpm at 40 min were only slightly above the background radioactivity of about 20 dpm.

4. Discussion

We have demonstrated that addition of a methyl group to the nitrogen atom of citalopram, thereby making it a quaternary nitrogen compound, leads to a new compound that retains its capacity to interact with the human platelet serotonin transporter

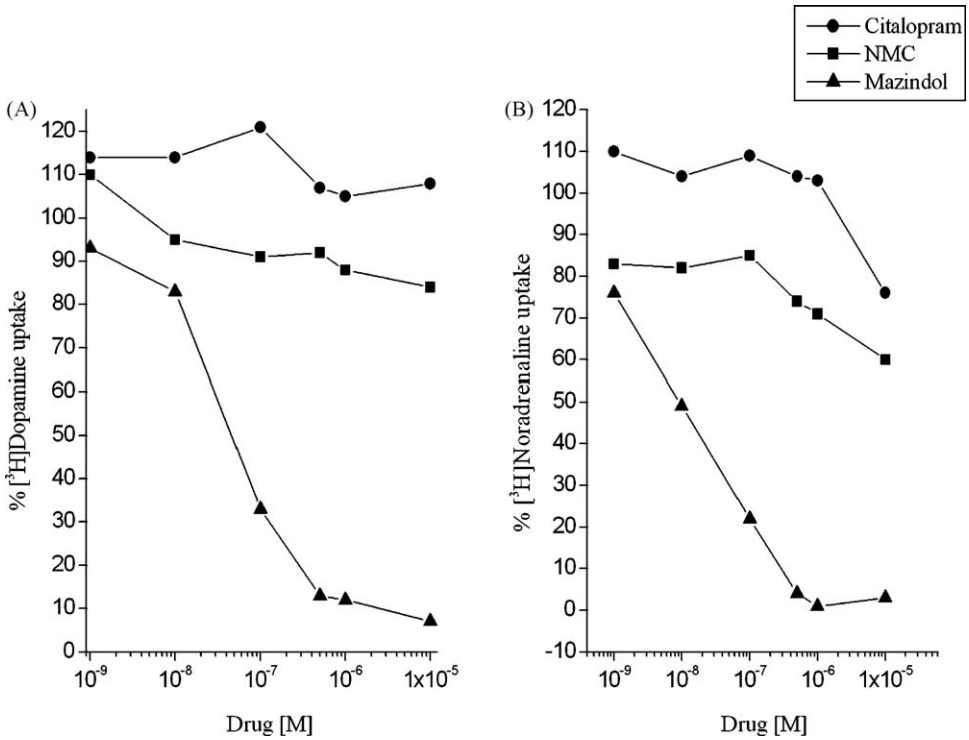

Fig. 6. Inhibition of [^{3}H]dopamine (A) and [^{3}H] noradrenaline (B) uptake (1 μM) by mazindol, citalopram and N-methyl-citalopram. [^{3}H]dopamine and [^{3}H]noradrenaline uptake assays were carried out in synaptosomes from rat brain cortex. After a 10 min preincubation period at 37 °C, [^{3}H]dopamine or [^{3}H] noradrenaline were added to the tubes containing synaptosomes and tested drugs. The reactions were stopped after 4 min by rapid cooling on ice. Specific uptake was defined as the difference between total uptake at 37 °C (triplicate samples) and the uptake measured at 0 °C (duplicate samples). Three separate experiments were performed. Data shown are from a representative experiment.

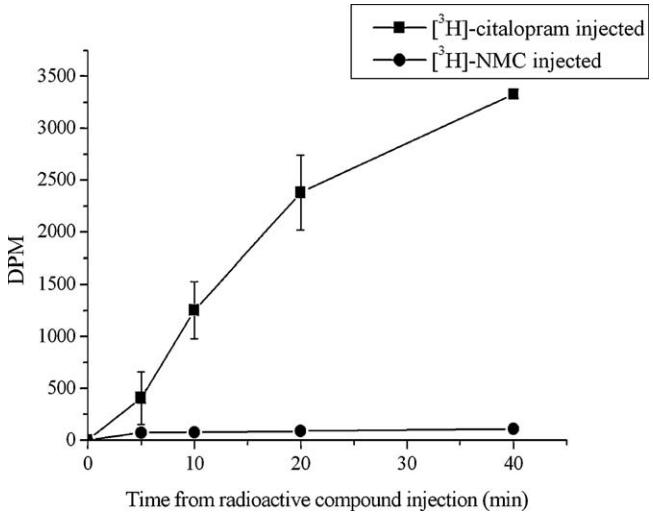

Fig. 7. Lack of brain penetration of [^{3}H]N-methyl-citalopram following i.p. injections to mice as compared with similar injections of [^{3}H]citalopram. Data for each time point represents the mean of three injected mice. See Section 2 for further details. Error bars for [^{3}H]N-methyl-citalopram injected mice are too small to be represented.

and inhibits its capacity to transport serotonin with similarly high affinity as its parent compound citalopram. NMC recognized the human platelet SERT with similar affinity to citalopram (7.2 nM and 3.9 nM respectively), as shown by competition binding experiments with [^{3}H]citalopram (Fig. 2 and Table 1). NMC was also shown to inhibit [^{3}H]5-HT uptake by freshly isolated human platelets with a similar affinity as determined for citalopram (K_i values of 5.2 nM and 4.2 nM respectively; Fig. 3 and Table 1). In sharp contrast, NMC was about 10-fold less potent than citalopram for binding to rat brain membranes SERT and inhibiting its function in rat synaptosomes. To our knowledge, such marked differences between the affinity of a SSRI drug to brain compared with platelet SERT have never been reported. The reasons for the lower affinity of NMC compared with citalopram for brain but not for platelet SERT are puzzling, as the brain and the platelet SERT are coded by a single gene, *SLC6A4*, which is not known to undergo alternative splicing. Although the molecular weight of the full length transporter (68 kDa) was detected in most immunoblot analysis of both brain and platelet tissues, other forms with higher and lower molecular weight were also reported [20–22]. This may reflect different glycosylations or cleavage due to different extraction methods or post extraction degradation as suggested by Dmitriev et al. [21]. In a study performed in rat brain and platelets [22], the molecular weight of the platelet transporter appeared to be 94 kDa which represents a highly glycosylated form compared to the lower molecular weight detected in brain, 76–80 kDa. According to these authors both MW forms of SERT are formed from identical polypeptides (the full-size SERT molecules), differing significantly in their N-linked glycosylation. Furhtermore Rasmussen et al. reported a correlation between the number of glycosylation sites and the level of SERT activity [23]. It could be that such glycosylation differences underlie the lower affinity of NMC to the brain compared with the platelet transporter, while they do not affect the affinity of citalopram or other SSRI drugs for this protein. In addition, it was reported that the serotonin transporter functions as an oligomeric glycoprotein, probably a dimer that may associate into a higher order complex [24,25]. It was also suggested that glycosyl modification may contribute to the correct folding and oligomeric properties of SERT proteins [26]. Further studies are needed to clarify if differences in glycosylation or oligomerization states of brain and platelet SERT differentially affect their affinities to citalopram and NMC.

NMC did not recognize the noradrenaline or the dopamine transporters: its potency for inhibiting [^{3}H]Ddopamine or [^{3}H]noradrenaline uptake, measured in rat brain synaptosomes, was extremely low ($IC_{50} > 10\ \mu M$). The 5-HT_{2A} receptor, found in brain and in platelets is a G protein coupled receptor activated by serotonin. This activation leads to elevation of intracellular calcium concentration and thereby to platelet activation. Since the 5-HT_{2A} receptor participates in platelet aggregation, it was of importance to assess its affinity to NMC. Similarly to citalopram, NMC failed to inhibit [^{3}H]ketanserin binding to the 5-HT_{2A} receptor in rat brain membranes ($IC_{50} > 10\ \mu M$). Our observations therefore classify NMC as an SSRI compound devoid of 5-HT_{2A} receptor binding similarly to its parent compound citalopram.

Using tritium-labeled compounds, we have demonstrated that N-methyl-citalopram does not penetrate the mouse brain following intraperitoneal injections (Fig. 7) as expected from its permanent positive charge. Forty minutes after intraperitoneal injections to mice with tritium-labeled NMC only very minor traces of radioactivity were found in their brains while high accumulation of radioactivity was detected following [^{3}H]citalopram injection. Considering that the amount of injected [^{3}H]N-methyl-citalopram (in dpm) was almost twice compared with that of [^{3}H]citalopram, these data represent more than 55-fold less brain penetration for [^{3}H]N-methyl-citalopram compared with brain penetration for [^{3}H]citalopram. It is well established that compounds behave similarly with respect to penetration of the human or mouse brain following their administration to a peripheral organ [27]. A compound that only minimally enters the mouse brain will show the same pattern for penetrating the human brain. Together with our observations on its SSRI properties, NMC may be considered as a peripherally restricted SSRI. Considering the epidemiological evidence that chronic treatment with SSRI drugs affords beneficial effects in cardiovascular disease patients [4–6,28], N-methyl-citalopram could be a promising new anti-platelet drug of similar potency as citalopram. Its inability to cross the BBB and penetrate the brain suggests that it is devoid of the adverse CNS activities of citalopram (and other SSRI drugs) such as flattened emotions, reduced libido, and aggressiveness [29,30]. Thus, NMC has a clear advantage over current SSRI drugs for development as an anti-platelet agent for chronic treatment of CVD patients.

Furthermore, Pollock et al. [31] have investigated the effect of 1-week treatment with the SSRI drug paroxetine on platelet activation biomarkers. They measured plasma levels of the platelet granule release products, beta-thromboglobulin (BTG) and platelet factor 4 (PF4) in 17 depressed patients with ischemic heart disease (IHD) who were treated for 1 week with paroxetine. This treatment reduced the levels of both biomarkers as compared to their levels before paroxetine treatment as well as compared to the levels in IHD depressed patients treated with nortriptyline. These observations suggest that paroxetine (or other SSRI drugs) may reduce platelet aggregation in vivo and may positively impact IHD-related mortality in this population. In vitro studies of platelet aggregation induced by collagen and arachidonic acid show a decrease in aggregation in platelet from SSRI treated patients versus non-SSRI treated patients [32]. Depletion of platelet serotonin following chronic SSRI treatment [6,8] could underlie the decreased platelet activation. This might contribute to lower rates of cardiovascular events in SSRI treated patients [8,11,12]. In addition, it has been suggested that chronic administration of SSRI downregulates brain and platelet serotonin transporter [33–36], and moreover it might downregulate or desensitize the brain 5-HT_{2A} receptor [37,38]. It was also reported that chronic administration of the tricyclic antidepressant drug imipramine leads to a reduced serotonin-amplified platelet aggregation to ADP [39]. Studies on the effect of NMC on the functional platelet aggregation response mediated by

5-HT_{2A} and other mediators of platelet aggregation are in progress in our lab.

In addition to N-methyl-citalopram we have synthesized two other quarternary analogues of SSRI drugs, N-methyl-fluoxetine and N-methyl-fluvoxamine, using a similar method as described for the preparation of NMC from citalopram. Both N-methyl-fluoxetine and N-methyl-fluvoxamine did not bind to the serotonin transporter and did not inhibit 5-HT uptake in human platelets (data not shown). In conclusion, NMC is shown here to be a new SSRI compound having a potential to be developed as a peripherally restricted anti-platelet drug candidate.

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