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Research Paper

Hybrid molecules inhibiting myeloperoxidase activity and serotonin reuptake: a possible new approach of major depressive disorders with inflammatory syndrome

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Keywords
atherosclerosis; depression; irreversible inhibitor; myeloperoxidase; serotonin

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Abstract

Objectives Major depressive disorder (MDD) is accompanied with an imbalance in the immune system and cardiovascular impairments, such as atherosclerosis. Several mechanisms have been pointed out to underlie this rather unexpected association, and among them the activity of myeloperoxidase (MPO). The aim of our study was to find compounds that inhibit both MPO and serotonin transporter (SERT) for treating MDD associated with cardiovascular diseases.

Methods SERT inhibition was assessed with measuring of [^{3}H]-serotonin uptake using HEK-293 MSR cells. MPO inhibition was determined by taurine chloramine test on 3-(aminoalkyl)-5-fluoroindole derivatives and on clinically relevant antidepressants. All kinetic measurements were performed using a temperature-controlled stopped-flow apparatus (model SX-18 MV). Promising lead compounds were docked onto SERT 3D structure modelled using the LeuT structure complexed to tryptophan (PDB code 3F3A). Their toxicological profile was also assessed.

Key findings 3-(aminoalkyl)-5-fluoroindole derivative with 5 carbons on the side chain and paroxetine showed the best activity on both MPO and SERT at the nanomolar range. Paroxetine was found to be the first irreversible MPO inhibitor at nanomolar concentrations.

Conclusions Our results put forward the first hybrid molecule (compound **25**) and drug (paroxetine) that can be especially used in MDD associated with inflammatory syndrome.

Introduction

Atherosclerosis and depression are quite different pathological entities. The former one is a cardiovascular disease caused by several metabolic disorders, including inflammation, accumulation of fatty compounds in arterial walls and oxidative events.[1] This may lead to fatal complications, such as myocardial infarction and stroke. Depression is clinically characterized by continuous negative mood and anhedonia.[2] The aetiology of depression is very complex and involves several components. In the central nervous system (CNS), the decrease or imbalance of

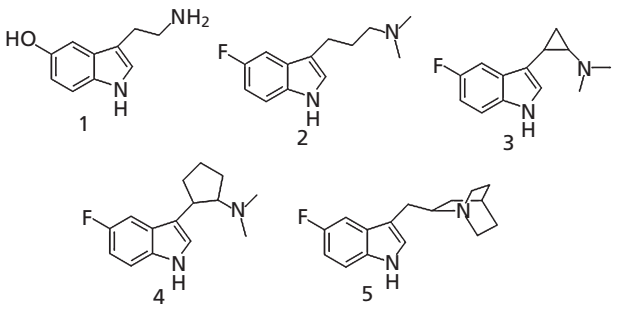


Figure 1 Structure of serotonin and examples of 3-(aminoalkyl)-5-fluoroindoles derivatives described in the literature that are structurally related to compounds **6**–**25**. Compound **2** has been included in the present work.

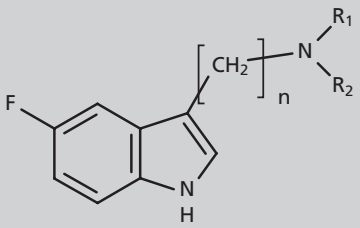
neurotransmitters in the synaptic cleft, in particular the monoamines norepinephrine, serotonin (5-HT, compound **1**, see Figure 1), and dopamine have been considered for the last 50 years as a main cause of depression. Although it cannot explain all cases and stages of the disease, this biogenic amine hypothesis has been the most widely studied mechanism so far.[3] Most interestingly, research papers have shown a rather unexpected relationship between both major depression and atherosclerosis. Among the numerous hypotheses about the correlation between depression and human brain dysfunction, inflammation has become an important issue in understanding some depressive diseases, especially major depressive disorders (MDD). First, clinical investigations have put forward that dysfunction of the immune system could foster the emergence of MDD along with (i) activation and increased production of immune system-related cells, including leucocytes and neutrophils,[4] as well as (ii) increased levels of interleukin-6 (IL-6) and IL-1Rα.[5] This phenomenon could be partially reversed by antidepressant therapy.[6] Second, it should be noted that, among the numerous elicitors of oxidative stress, the heme enzyme myeloperoxidase (MPO, EC 1.11.2.2) has attracted significant attention due to its possible link with MDD symptoms. MPO has been detected in the serum of depressive patients, as observed by Hazen and colleagues.[7] Furthermore, MPO is also directly involved in the modification of biomolecules in the blood of depressive patients, mainly through its ability to generate hypochlorous acid (HOCl) and derived oxidation products, including organic peroxides, nitrotyrosine and oxidized low-density lipoproteins (LDLs).[8] MPO-derived oxidants have also been reported to be involved in the development of neurodegenerative as well as cardiovascular diseases (especially coronary artery disease), which have a high incidence in patients with MDD.[9]

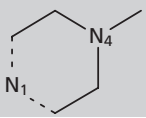
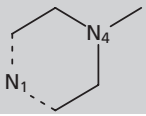
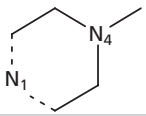
As far as atherosclerosis is concerned, MPO has been identified as a factor contributing to its development.[10] Furthermore, a recent review shed light on the relationship between this cardiovascular disease, depression and the drugs used to treat the latter.[11] Among the biological processes at the origin of atherosclerosis, oxidative stress[12] together with platelet activation has been pointed out.[13] Regarding blood homeostasis, 5-HT is one of the molecules involved in platelet activation,[14] and 5-HT-specific reuptake inhibitors (SSRIs) have been suggested to decrease this process,[11,15–18] yielding favourable cardiovascular effects in patients with MDD.[19] The effect of antidepressants and SSRIs on the oxidative stress remains unclear but is rather well described at least for fluoxetine,[20] escitalopram[21,22] and paroxetine.[23] Furthermore, it is known that 5-HT is one of the best substrates of MPO and is transformed into the following oxidation products: 5-HT dimer and tryptamine-4,5-dione. The latter is known to be neurotoxic[24] as well as implicated in cardiovascular disease.[25] In addition to the production of toxic compounds, the interaction of 5-HT with MPO significantly decreases the concentration of the former, indicating how inflammation might interfere with depression.[26]

A molecule able to act on both pathologies could be valuable to improve the depressive state along with protecting the cardiovascular system in patients with MDD. In modern medicinal chemistry, such molecules are called hybrid drugs, and their discovery has become a great challenge since the 1990s.[27] Designing molecules that include at least two pharmacophores and act at rather low concentrations on at least two different biological systems still remains a very difficult task since both binding sites must accommodate and interact effectively with a single structure. However, the substantial advantage of treating one or even two diseases by acting on two targets is very attractive since drug abuse is a major public health problem due to the cumulative side effects or (dangerous) drug interactions that could follow.[28] Principally, two general methods can be used in the design of hybrid drugs: (i) either linking two different molecules endowed with different activities or (ii) designing one molecule that shares different structural features of several pharmacophores.

In recent papers, we described for the first time a series of 3-(aminoalkyl)-5-fluoroindoles as MPO inhibitors, some of them acting at nanomolar concentrations (see Table 1).[29,30] Analysis of data from the literature showed that one of these compounds and some closely related structural analogues (Figure 1, compounds **2–5**) are potent 5-HT reuptake inhibitors (SRIs), leading to the serendipitous discovery of potential dual-targets acting molecules.[31–33] We have then further investigated the pharmacology of 3-(aminoalkyl)-5-fluoroindole derivatives, by (i) assessment of the 5-HT reuptake inhibition, (ii) analysis of the potential binding modes predicted by docking experiments on a 3D modelled structure of the 5-HT transporter and (iii) assessment of

Table 1 Synthesized 3-(aminoalkyl)-5-fluoroindoles and SSRIs



Compound	*n*	R_1	R_2	K_i (mean ± SD in nM)	IC50 (mean ± SD in nM)[29]	Ratio K_i/IC50
6	1	H	H	995 ± 136	900 ± 300	1.1
7	1			41 ± 11	1000 ± 100	0.04
8	2	H	H	47 ± 11	200 ± 30	0.2
9	2	CH_3	CH_3	53 ± 11	90 ± 60	0.6
10	2	H	CH_2CH_3	39 ± 17	300 ± 100	0.1
11	2	CH_2CH_3	CH_2CH_3	22 ± 5	160 ± 80	0.1
12	2			21 ± 8	40 ± 30	0.5
13	2	H	$CH_2CH_2CH_3$	19 ± 8	800 ± 20	0.02
14	2	H	$CH_2CH_2CH_2CH_3$	148 ± 70	1030 ± 80	0.1
15	2			15 ± 4	200 ± 100	0.07
16	3	H	H	3.9 ± 0.8	50 ± 8	0.05
17	3	H	CH_3	11.2 ± 6.6	200 ± 200	0.06
2	3	CH_3	CH_3	1.2 ± 0.6	130 ± 90	0.009
18	3	H	CH_2CH_3	21 ± 13	300 ± 100	0.07
19	3	CH_2CH_3	CH_2CH_3	5.0 ± 0.6	350 ± 90	0.01
20	3			3.7 ± 1.8	320 ± 10	0.01
21	3	H	$CH_2CH_2CH_3$	214 ± 83	170 ± 80	1.3
22	3	H	$CH_2CH_2CH_2CH_3$	41 ± 5	1500 ± 500	0.03
23	3			2.7 ± 1.1	350 ± 60	0.08
24	4	H	H	5.6 ± 1.3	15 ± 4	0.4
25	5	H	H	2.6 ± 0.7	8 ± 2	0.3
Escitalopram oxalate				0.39 ± 0.35	Not active	–
Fluoxetine hydrochloride				3.4 ± 2.6	Not active	–
Paroxetine hydrochloride hemihydrate				0.3 ± 0.2	22 ± 2	0.02
Fluvoxamine maleate				3.7 ± 2.9	Not active	–

Results for 5-HT reuptake inhibition expressed as K_i, previous results obtained with myeloperoxidase inhibition expressed as IC50, and the calculated ratios between K_i and IC50.[29] The compounds are first classified according to alkyl side chain length and then increasing size of the substituents on the amino group. SSRI, selective serotonin reuptake inhibitor.

the toxicological effects in rats. In addition, MPO inhibition of SSRIs widely used in clinical practice (escitalopram, paroxetine, fluoxetine and fluvoxamine, see Figure 2) was studied to investigate their potential beneficial effects in cardiovascular disorders accompanying severe depression. Finally, transient kinetics experiments were undertaken to analyse the mechanism of action of the possible MPO-inhibiting SSRIs.

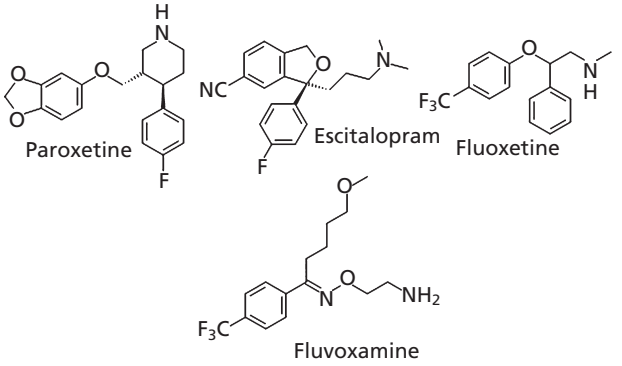


Figure 2 Structures of the most clinically relevant selective serotonin reuptake inhibitors used in the present work. Compounds are drawn with the most important structural features of the 5-HT reuptake pharmacophore in similar positions.

Materials and Methods

Chemicals

The 3-(aminoalkyl)-5-fluoroindoles used in this work were obtained by chemical synthesis (see Table 1). The synthetic and biological procedures (including MPO inhibition, LDL oxidation inhibition and docking experiments of MPO), as well as the MPO inhibition data, were described elsewhere.[29] Escitalopram oxalate and fluoxetine hydrochloride were purchased from Sigma-Aldrich (Saint-Louis, MI, USA). Paroxetine hydrochloride hemihydrate and fluvoxamine maleate were available from Fagron (Boom, Belgium). 2-chloro-5,5-dimethyl-1,3-cyclohexanedione (MCD) was purchased from Alfa Aesar (Karlsruhe, Germany). Highly purified MPO was purchased from Planta Natural Products (Vienna, Austria).

Assessment of 5-HT reuptake inhibition

The mammalian expression plasmid pcDNA3.1 containing human 5-HT transporter (hSERT) cDNA was previously described by Kristensen *et al.*[34] HEK-293 MSR cells (Invitrogen, Carlsbad, NM, USA) were cultured as monolayers in Dulbecco's modified Eagle's medium (BioWhittaker, Basel, Switzerland) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (BioWhittaker) and 6 μg/ml Geneticin (Invitrogen) at 95% humidity, 5% p(CO_2) and 37°C. Cells were detached from the culture flasks by Versene (Invitrogen) and trypsin/EDTA (BioWhittaker) treatment for subculturing or seeding into white TC-microtiter plates (Nunc, Roskilde, Denmark). Transfection and measurement of [^{3}H]5-HT (PerkinElmer Life Sciences, Waltham, MA, USA) uptake were performed as described by Larsen *et al.* (2004), except that HEK-293 MSR cells (Invitrogen) were used instead of COS-1 cells.[35]

Docking experiments

Docking of 5-HT and of other compounds was performed on hSERT 3D structure. No high resolution three-dimensional (3D) structure of the transporter has been determined so far. A BLAST search performed using the PDB sequences identified the bacterial Aquifex aeolicus leucine transporter LeuT as homologous to hSERT.[36] Therefore, a 3D model was built with MODELLER,[37] using the LeuT structure complexed to tryptophan (PDB code 3F3A) as a template and a sequence alignment produced by Promals3D,[38] which features about 24% sequence identity. The 3D model of hSERT superimposes on the LeuT structure with a root mean square deviation of 1.5 Å for the Cα positions, which is a fairly low value. A Ramachandran plot showed that the model features a stereochemistry for the large proportion of residues consistent with allowed areas in the conformational space, except for six residues that are located in loops and distal to the binding site. The transmembrane (TM) segments, TM1, TM3, TM6 and TM8, which shape the binding pocket, feature a high sequence identity and similarity to LeuT, which reinforces the reliability of the model in this region. Compounds were docked in the binding site of the hSERT 3D model that corresponds to the primary binding pocket of LeuT. The underlying assumption that these molecules bind at this site is sustained by a study reporting that tryptamine analogues are competitive inhibitors of hSERT transport.[39] Protein flexibility was included in the docking process (Induced-fit protocol from Schrödinger Inc., New York, NJ, USA) via flexible side chain residues selected within a 4 Å sphere surrounding the tryptophan ligand, which was modelled together with the hSERT protein.[40] To be exhaustive, it is worth to mention that two new models of 5-HT and dopamine transporters have been published after this manuscript had been submitted.[41,42]

Transient-state kinetics

All kinetic measurements were performed using a temperature-controlled stopped-flow apparatus (model SX-18 MV) from Applied Photophysics (Leatherhead, UK) with either a diode-array detector (Applied Photophysics PD.1) or a monochromator attached to the stopped-flow machine. In a typical sequential-mixing stopped-flow experiment the enzyme solution (2 μM heme) was premixed with a 10-fold excess of H_2O_2. After a delay time of 20 ms, Compound I (50% hypochromicity at 430 nm) was formed and allowed to react with varying concentrations of substrate in 100 mM phosphate buffer (pH 7.0). The reactions were followed at the Soret maximum of Compound II (456 nm). To determine the kinetics of reduction of Compound II, following of formation and reduction of Compound II was performed in one measurement. The

resulting biphasic curves at 456 nm showed the initial formation of Compound II (exponential increase at 456 nm) and then its subsequent transition to ferric MPO (exponential decrease at 456 nm).

Reactions were analysed by fitting the monophasic time traces using the single-exponential equation, provided by the Applied Photophysics software programme. From the slope of the linear plot of the k_{obs} values vs substrate concentrations, the apparent second-order rate constant was estimated. At least three determinations (2000 data points) of pseudo-first-order rate constants (k_{obs}) were performed for each substrate concentration (pH 7.0, 25°C), and the mean value was used to calculate the second-order rate constant. All reactions were analysed using the Pro-K simulation programme from Applied Photophysics, which allows the synthesis of artificial sets of time-dependent spectra as well as spectral analysis of enzyme intermediates.

Inactivation of myeloperoxidase

These experiments are described in the Supporting Information.

Toxicology

All animal experiments were approved by the local ethical committee for animal care of the institution (University of Mons, Mons, Belgium). The most active compound **25** was administered by intraperitoneal injection to Wistar Han male rats (mean weight: 553 ± 41 g). The following concentrations were administered in a solution of ethanol (33%): 1, 10, 100 and 300 mg/kg body weight. Each dose was administered to groups of two rats (1 mg/kg R1 and R2, 10 mg/kg R3 and R4, 100 mg/kg R5 and R6, and 300 mg/kg R7 and R8). Only animals treated with 100, 10 and 1 mg/kg, respectively, were placed in metabolism cages for 144 h. The first two days were used for acclimatization of the animals. The animals received a single dose of the tested compound at time '0 h'. Urine was collected daily and blood tests were performed 24 and 96 h after treatment.

Results

5-HT reuptake inhibition

The 3-(aminoalkyl)-5-fluoroindoles previously reported as MPO inhibitors were tested for their 5-HT reuptake inhibition potency (see Table 1).[29] Variation of the alkyl chain length on unsubstituted compounds resulted in the following observations: (i) homotryptamines from $n = 3$–5 (compounds **16**, **24,** and **25**) are more potent inhibitors than fluorotryptamine (**8**), (ii) compound **6**, with one carbon atom chain length between the indole ring and the amino group, features a dramatically reduced inhibition potency.

The structure activity relationships (SAR) can be inferred from inspection of the data presented in Table 1. For identical substituents, compounds with $n = 3$ are in most cases more active than those with $n = 2$, except $R_2 = -CH_2CH_2CH_3$ (compare compounds **21** and **13**). Regarding compounds with $n = 2$, substitution with either one $-CH_2CH_2CH_3$ (**13**) or two $-CH_2CH_3$ (**11**) groups, and the two alicyclic molecules (**12** and **15**), generated the most potent inhibitors in the series. Compound **14** had the lowest inhibition, perhaps as a result of its longer aliphatic chain substituent. In the series with $n = 3$, compounds with no substituents, two identical substituents or alicyclic substituents tend to have higher inhibition potency than their homologs with one alkyl chain substitution. In contrast to the series with $n = 2$, an improved inhibition was observed for the compound with a butyl substituent relative to one with a propyl moiety (compare compounds **22** and **14**). SAR showed that in the piperazine series (**7**, **15** and **23**), all of the compounds are among the most active ones compared with molecules containing similar alkyl side chains.

Comparison of 5-HT reuptake and myeloperoxidase inhibition potencies of synthesized compounds

The SAR of the MPO inhibition potency of the compounds has been already discussed in a previous report (see Table 1).[29] Comparison of inhibition of SERT and MPO activity was made for each compound by calculating the ratio K_i (SERT inhibition)/IC50 (MPO inhibition). Compounds having a ratio >0.1 and nanomolar activity on both targets retained our attention, and consequently molecules **24** and **25** were selected as hybrid lead candidates.

To find other candidates as SERT/MPO inhibitors, we extended our investigation to the most efficient SSRIs used in clinical practice (Table 1 and Figure 2). MPO inhibition screening was done for escitalopram, fluoxetine, paroxetine and fluvoxamine (Figure 2) using the taurine chloramine test (see Supporting Information). Only paroxetine inhibited MPO with an IC50 value at 22 ± 2 nM. The other drugs had no effect on the chlorination activity of MPO. Additionally, these SSRIs were probed for their interference with MPO-mediated LDL oxidation. Again, only paroxetine showed a potent inhibition an IC50 value of 35 ± 3 nM.

Molecular docking

The binding mode of 5-HT has been investigated by molecular docking in the modelled 3D structure of hSERT. The best-scored predicted positions for 5-HT in the binding pocket feature a salt bridge formed between the amine group and Asp98 as an important interaction for binding

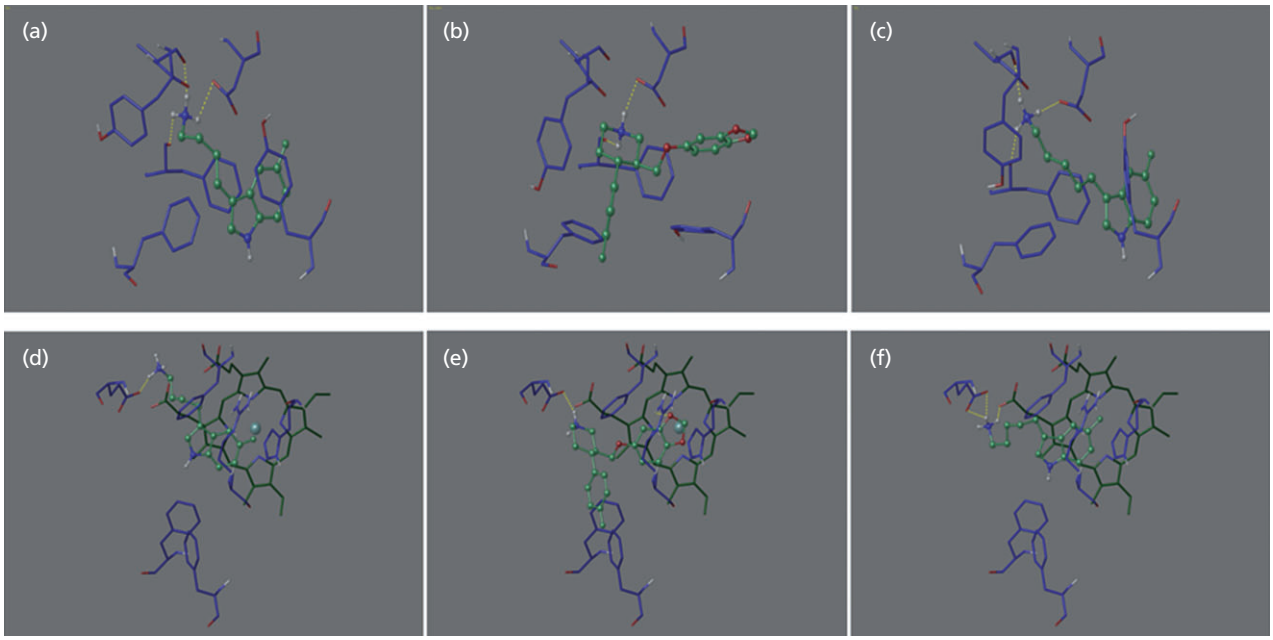


Figure 3 The best-scored binding mode predicted by docking in the hSERT 3D modelled structure for (a) compound **24**, (b) paroxetine and (c) compound **25**. The best-scored binding mode predicted by docking in the myeloperoxidase structure for (d) compound **24**, (e) paroxetine and (f) compound **25**. The ligands are shown as green ball-and-sticks, the neighbouring residues as blue sticks. Hydrogen bonds and salt bridges between the ligand and the protein are depicted as yellow broken lines.

the transporter. A weak cation–π interaction is also observed with Tyr95. The 5-HT amine hydrogen bonds with the backbone of Ala96 and Phe335. These latter interactions are remarkably similar to those of the tryptophan-bound LeuT crystal structure. A T-shape aromatic interaction is observed between 5-HT and Tyr176.

Molecular docking of the most active compounds both on hSERT and MPO (compounds **24** and **25**) was performed in a 3D model of the transporter. It is indeed essential to get insight into the binding mode of these molecules to implement rationale pharmacomodulation. The predicted binding modes on MPO were described in a previous study.[29] The binding modes of compounds **24** and **25** predicted on hSERT are similar (Figure 3a and 3c): the amine side chain forms a salt bridge with Asp98, a weak cation–π interaction with Tyr95, and two hydrogen bonds with Ala96 and Phe335 backbone. Aromatic interactions are also observed with Tyr176 and Phe335. The indole NH also hydrogen bonds with the Tyr175 main chain. Hydrophobic interactions are formed with Leu99, Ile172, Ile179, Phe335 and Phe341.

The importance of Asp98 and Tyr95 for the transport function in monoamine transporters was underscored in mutagenesis experiments.[39,43] Given the difficulty in predicting binding affinity using the scoring functions available in the docking programmes, it is often challenging to quantitatively discriminate the potency of different ligands. Nevertheless, it is interesting to note that compound **24** and **25** are predicted to bind with a similar affinity score (about −11 kcal/mol) in agreement with the measured K_i values.

Noticeably, the binding mode of paroxetine in hSERT features similarities with compound **24** and **25** (Figure 3b). In particular, its amino group forms a salt bridge with Asp98, a cation–π interaction and one hydrogen bond with Phe335. The fluorophenyl moiety makes interactions with other aromatic residues: Tyr95, Tyr176 and Phe341, and also with Val343. The benzodioxole moiety forms a π–π interaction with Tyr176, and also interacts with Leu99, Ile179 and Phe335. The affinity score of paroxetine ($\Delta G = -13.7$ kcal/mol) is about 2 kcal/mol higher than that of compounds **24** and **25** ($\Delta G = -11.8$ and -11.4 respectively).

The binding mode of paroxetine in MPO active site presents strong similarities with that of compound **24** and **25**, and with fluorotryptamines (Figure 3e). The best pose of paroxetine features a stacking of the six-membered ring benzodioxole onto the pyrrole ring D. Its five-membered ring positions itself between the iron pocket and His95, allowing one of its oxygen to hydrogen bond to Gln91 and Arg239 side chains. In addition to the stacking, one salt bridge is formed by the amino group with Glu102. The fluorophenyl moiety makes interactions with surrounding residues in a pocket at the entrance of the binding site: Phe99, Phe366 and Phe407. The affinity score of

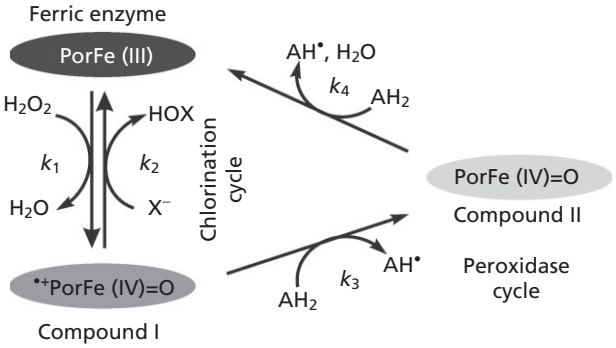


Figure 4 Scheme of the chlorination and peroxidation pathway of myeloperoxidase. Reaction (1), ferric myeloperoxidase is oxidized by hydrogen peroxide to Compound I (i.e. oxoiron(IV) porphyrin radical cation). Reaction (2), Compound I is directly reduced back to the resting state by halides, thereby releasing hypohalous acid. Reaction (3), Compound I is reduced to Compound II (i.e. protonated oxoiron(IV)) by a one-electron donor. Reaction (4), Compound II is reduced to native state thereby oxidizing a second substrate molecule. Reactions (1), (3) and (4) constitute the peroxidation pathway. Reactions (1) and (2) constitute the halogenation pathway.

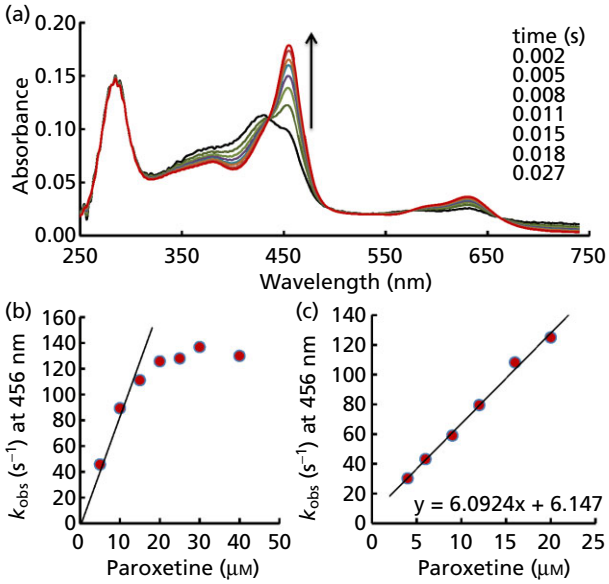


Figure 5 Reaction of myeloperoxidase Compound I with paroxetine. (a) Spectral changes upon reaction of 2-μM myeloperoxidase Compound I with 10-μM paroxetine: first spectrum after mixing corresponds to Compound I and subsequent spectra were recorded after the indicated times. Arrow indicates spectral changes at 456 nm. (b) Pseudo-first-order rate constants for myeloperoxidase Compound I: reduction was plotted against paroxetine concentration. Saturation behaviour is observed. (c) Pseudo-first-order rate constants for Compound I reduction plotted against paroxetine concentrations (<20 μM).

paroxetine $\Delta G = -9.1$ kcal/mol is significantly higher than that of compounds **24** and **25**, which amounts to −6.6 and −6.4 kcal/mol, respectively.[29]

Transient kinetics

We recently reported that 3-(aminoalkyl)-5-fluoroindole derivatives are reversible inhibitors of MPO. They react with both redox intermediates of MPO, i.e. Compound I and Compound II, but at significantly different rates.[29] The reaction is fast with Compound I but very slow with Compound II. This causes accumulation of Compound II, which is outside the chlorination cycle (Figure 4). The latter needs Compound I for the two-electron oxidation of chloride to HOCl. Alternatively, Compound I can be reduced by one-electron donors to Compound II as do these MPO inhibitors very efficiently. Upon mixing Compound I with paroxetine, a fast one-electron reduction occurred, indicated by a red shift of the Soret band to 456 nm, clearly suggesting formation of Compound II (Figure 5a). The reaction was concentration-dependent but showed a saturation behaviour (Figure 5b). The bimolecular rate constant (k_3) of this reaction (Figure 4) was calculated from the linear correlation between the k_{obs} values and low paroxetine concentrations (<20 μM) to be $(6.1 \pm 0.2) \times 10^6$ M/s (Figure 5c). Compound II was not stable but was slowly converted to the resting state. However, while direct reaction of Compound II with 3-(aminoalkyl)-5-fluoroindole derivatives gives full recovery of native MPO (underlining the reversible nature of these inhibitors), reaction of

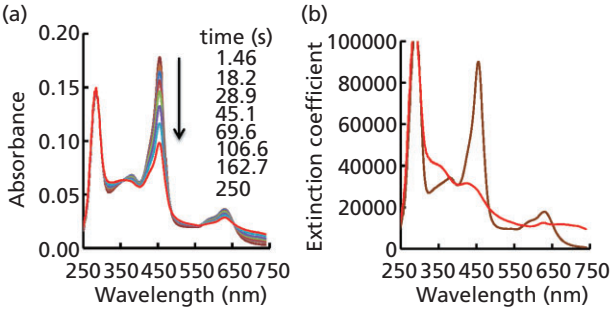


Figure 6 Reaction of myeloperoxidase Compound II with paroxetine. (a) Spectral changes upon reaction of 2-μM myeloperoxidase Compound I with 50-μM paroxetine. First spectrum after mixing corresponds to Compound II and subsequent spectra were recorded after the indicated times. Arrow indicates spectral changes at 456 nm. (b) Fitted spectra of the decay. The orange spectrum corresponds to Compound II and the red spectrum corresponds to the last spectrum.

Compound II with paroxetine (Figure 6a) did not allow full recovery of the native state but showed loss of heme absorbance. The extent of recovery of ferric MPO and heme degradation strongly depended on the amount of paroxetine used as electron donor, suggesting that it might act as a suicide inhibitor (Figure 6b). Finally, 500 μM paroxetine

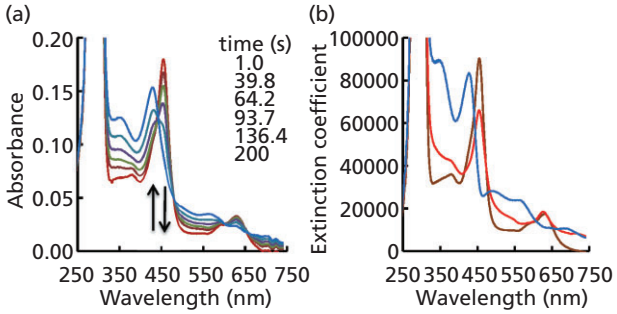


Figure 7 (a) Spectral changes upon reaction of 2-μM myeloperoxidase Compound II with 500-μM paroxetine. First spectrum after mixing corresponds to Compound II and subsequent spectra were recorded after the indicated times. Arrows indicate spectral changes at 456 nm and 430 nm. At this concentration, partially ferric myeloperoxidase is formed. (b) Fitted spectra of the reaction of Compound II with paroxetine (orange for Compound II, red for decayed intermediate and blue for ferric myeloperoxidase).

were added to Compound II, which was slowly and partially converted to the resting state (Figure 7a). As k_4 (Figure 4) is very low, its exact value could only be estimated using the Pro-K simulation programme (around 20 M/s). The ratio of k_3/k_4 (~305 000) suggests that paroxetine acts as reversible inhibitor promoting the accumulation of Compound II. However, after reaction of MPO with paroxetine, the MPO activity was irreversibly inhibited, suggesting that paroxetine irreversibly interacted with the prosthetic group of MPO during turnover (see Supporting Information).

Toxicology

The two animals (R7 and R8) treated with 300 mg of **25** died within 20 min after administration. At necropsy, gross observations showed an early bleeding in the lungs in two individuals. Rats treated with the high dose of compound **25** (R5, R6, 100 mg/kg) stopped eating and drinking until death. Rats treated with low dose (R1 and R2, 1 mg/kg) showed a normal behaviour. Rats treated with 10 mg/kg (R3 and R4) showed different eating habits. During the first 20 min after treatment, all animals seemed prostrate, and had a gasping breath and a swollen nose. Twenty-four hours after treatment, rats treated with low dose behaved normally. Individuals treated with 100 mg/kg appeared severely affected. They were prostrate, breathing quickly and suffered from hypothermia. Four hours after treatment, only rats treated with 100 mg/kg exhibited abnormal behaviour similar to that described above. Seventy-two hours after treatment, the two individuals treated with 100 mg/kg died. The behaviour of other rats showed no sign of abnormality. The surviving rats were killed. Blood and organs (liver, kidney, heart and muscle) were collected and no abnormality was observed. In summary, no observable adverse effects were seen in animals receiving compound **25** at 1 mg/kg, animals treated at 10 mg/kg showed effects but recovered within 48 h, whereas at the high dose (100 mg/kg) the animals died several days after receiving a single dose. Finally, compound **25** has been assessed with the predictive programme Percepta (QSAR approach of toxic effects), and only cardiovascular problems have been reported as possible side effects.

Discussion

The major contribution of this study is the discovery of a new class of hybrid molecules acting on two targets: SERT and MPO. Hybrid drugs are not well represented in the armamentarium, with the noticeable exception of drugs treating CNS diseases, such as antipsychotics and antidepressants, that act on several neurotransmitter receptors and transporters, and also some anticancer agents (for instance tyrosine kinases inhibitors). However, the ability of these compounds to act on different targets is generally considered at a first sight more as a lack of selectivity than a real endeavour to design efficient hybrid drugs. As a matter of fact, the targets of hybrid drugs now used in clinical practice have very similar 3D structures or belong to the same protein or enzyme/receptor subfamily (in the above-given examples monoamines receptors and kinases using ATP as cofactor). The case of compounds **24** and **25**, and paroxetine, is different: these molecules inhibit two very different proteins, i.e. a heme-containing enzyme (MPO) and a TM transporter (SERT). So it is crucial to understand the molecular characteristics that could explain this double activity.

A comparison of the binding modes predicted by docking in hSERT and MPO active sites shows common structural features in molecules **24**, **25** and paroxetine critical for interactions with both target proteins. The pharmacophores derived from these three compounds are summarized in Table 2. Two chemical groups, i.e. the amino group and an aromatic moiety, and their interactions provide a rationale for the nanomolar activity of these compounds in the two binding sites. A protonated amino group on the side chain and an aromatic or heteroaromatic cycle play critical roles in binding to both SERT and MPO. It also appears that the voids around the nitrogen atom are smaller in the MPO than in the SERT binding pocket as shown by the overall low activity obtained in MPO inhibition for compounds featuring substitutions on the amino group, whereas the effect of the same substitution pattern is in most cases less dramatic on SERT inhibition (see compounds with low K_i/IC50 ratios, such as **2**, **7**, **13**, **15** to **20**, **22** and **23**). Some homotryptamines, however, appear as exceptions to this rule (compare compounds **13** and **14**, as well as **21** and **22**).

Table 2 Common structural features for the pharmacophores of compounds **24** and **25**, and paroxetine related to their binding to hSERT and myeloperoxidase active site

	Interactions with binding sites	
Chemical groups	MPO ([29] and this study)	hSERT
For compounds 24, 25, and paroxetine		
Side chain amino group	Salt bridge with Glu102	Salt bridge with Asp98 Cation–π interaction with Phe335
Aromatic ring : indole or benzodioxole	π–π interaction with the heme	π–π interaction with Tyr176
For compounds 24 and 25 only		
Amino group of the indole ring	–	Hydrogen bond with Tyr175
For paroxetine only		
Oxygen of the benzodioxole moiety	Hydrogen bond with Gln91 and with Arg239	–

The interaction of paroxetine with MPO is twofold. On the one hand, it acts as electron donor for Compound I and Compound II. But because of the huge differences in the apparent bimolecular rate constants ($k_3 >> k_4$), it promotes Compound II accumulation (Figure 4). Since this redox intermediate does not participate in the chlorination cycle, paroxetine behaves as a reversible MPO inhibitor[44,45] like 5-fluoroindole derivatives.[29] On the other hand, oxidation of paroxetine by MPO depends on the presence of H_2O_2 and causes heme bleaching and irreversible inhibition (see Supporting Information). This reactivity is typical of suicide inhibitors. It is indeed known that paroxetine is metabolized by the heme enzyme cytochrome 2D6. The major metabolization pathway is oxidation of the -CH_2- of the benzodioxole group and release of the methylene group as a carbene, which reacts irreversibly with the heme group of the cytochrome.[46] A similar suicide mechanism might take place with MPO. Recently, 2-thioxanthines were described as potent mechanism-based inactivators of MPO.[47,48] Kinetic analysis, mass spectrometry and X-ray crystal structures revealed that these inhibitors become oxidized by MPO, and finally covalently attached to the heme prosthetic groups, thereby blocking the entrance to the heme cavity. Paroxetine, thus, simultaneously acts as a reversible and irreversible inhibitor of MPO. It significantly decreases the chlorination activity by both promoting Compound II formation as well as – in contrast to the 3-(aminoalkyl)-5-fluoroindoles – irreversibly modifying the heme moiety.

By comparing the acute toxicity of compound **25** (no effect at concentrations below 10 mg/kg) with that of paroxetine, it is found that this compound has almost the same effects as this SSRI. It is reported that paroxetine LD50 values in rats are 27 mg/kg following intravenous administration and 374 mg/kg per os.[49] Giving 120 mg/kg per os paroxetine to rats leads to reduced body weight and decreased food consumption.[50] Furthermore, the concentration of MPO in patients who have coronary heart disease in plasma is 100–300 ng/ml (0.7–2 nM), suggesting that the dose of 10 mg/kg is much higher than the expected required clinical dose, which should be rather around 10 μg/kg (calculated approximately by using the IC50s and molecular masses of compound **25** and paroxetine hydrochloride hemihydrate). Consequently, compound **25** might have wide therapeutic margin.[51,52] To conclude about the toxicity, a major concern could come from the potentially long-term use of MPO inhibitors in patients. Since this enzyme is involved in the immune system, one could suspect that its inhibition might have dangerous effects on human body, causing increased number of opportunistic infections. However, MPO is well protected inside the neutrophils, and the only enzymes targeted by the MPO inhibitors are those that are secreted outside these cells and cause the deleterious effects.[10,53] So we can reasonably assume that the effects of such compounds could be of minor importance.

Finally, the critical question whether the molecule could be active in the CNS will be investigated in future works. However, we can reasonably assume that compound **25** can cross the blood–brain barrier. Indeed, it has a relatively low molecular weight and a positive partition coefficient (logP, estimated using computer simulations to be between 1.5 and 3.1, confirming the rather lipophilic nature of the molecule), both being in the same ranges as other SSRIs. However, the stability towards monoamine oxidases should be also assessed since primary amino groups are sensitive to these catabolic enzymes.

Conclusion

The serendipitous discovery that newly synthesized 3-(aminoalkyl)-5-fluoroindole derivatives and paroxetine are nanomolar inhibitors of both SERT and MPO could open a new field in the treatment of MDD accompanied with inflammatory syndrome. Analogues of 5-fluorotryptamine were shown to be highly active as SSRIs but also to inhibit the chlorination activity of MPO.[29] Derivatives **24** and **25** with, respectively, 4 and 5 carbons on the side chain showed the best activity on both targets

at nanomolar concentrations (MPO: IC50 = 15 ± 4 and 8 ± 2 nM and SRI: K_i = 5.6 ± 1.3 and 2.6 ± 0.7 nM, respectively). Additionally, a new clinical application was demonstrated for the SSRI paroxetine that inhibits the chlorination activity (IC50 = 22 ± 2 nM) and MPO-mediated LDL oxidation (IC50 = 35 ± 3 nM). Thus, these three compounds (compounds **24**, **25** and paroxetine) can be considered as starting compounds to develop other SSRIs as hybrid drugs that could also inhibit MPO, and consequently can be used in the treatment of MDD associated with atherosclerotic events. In our newly synthesized indole series, compound **25** is the most promising one with no significant adverse effect at the clinically relevant concentrations.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Data for inactivation of myeloperoxidase by paroxetine, taurine chloramine test procedure and data for the inhibitory effect of paroxetine on the chlorination activity of MPO are given.