Pindolol is a potent scavenger of reactive nitrogen species

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Abstract

Pindolol is an indolic drug that has been shown to enhance and/or accelerate selective serotonin specific reuptake inhibitors (SSRI)-induced antidepressant (AD) effect, even though the respective mechanism is still unclear. It has been demonstrated that inhibition of nitric oxide (NO) synthesis in CNS produces anxiolytic and AD-like behavioural effects in a variety of animal paradigms. On the other hand, sustained high levels of NO may be deleterious to CNS, predominantly due to the formation of peroxynitrite anion (ONOO−), which is generated via reaction of NO with superoxide radical (O2−). Therefore, the purpose of the present study was to characterize the putative pindolol scavenging effect on NO, ONOO−, and O2−, using in vitro non-cellular systems. The obtained results clearly show that pindolol is a potent scavenger of NO (IC50 of 449 ± 33 μM) and ONOO− (IC50 of 131 ± 24 μM). Additionally, the scavenging effect of pindolol increased almost 8 times in the presence of 25 mM NaHCO3 (IC50 of 17 ± 3 μM), which indicates that pindolol efficiently scavenges reactive species that are produced from the ONOO−/CO2 reaction such as the nitrogen dioxide radical (NO2•) and the carbonate radical anion (CO3•−). These effects may contribute for the reduction of SSRI antidepressant latency that has been attributed to pindolol and may also constitute an additional value for this drug when depression is associated with pro-oxidant neurodegenerative diseases.

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Keywords: Pindolol; Scavenging activity; Nitric oxide; Peroxynitrite anion; SSRI antidepressant latency
Introduction

Pindolol is an indolic drug (Fig. 1) that has been shown to enhance and/or accelerate selective serotonin specific reuptake inhibitors (SSRI)-induced antidepressant (AD) effect. The prevailing theory concerning the latency of the AD effect is based on the finding that exposure to selective serotonin (5-hydroxytryptamine, 5-HT) specific reuptake inhibitors (SSRI) initially decreases the firing of serotonergic neurons due to an enhanced stimulation of the inhibitory somatodendritic 5HT_{1A} autoreceptors in the dorsal raphe nuclei and 5-HT_{1B} autoreceptors in both the terminal and cell body regions (Dawson and Nguyen, 2000). This decrease in the firing of serotonergic neurons reduces serotonergic activity in the projection areas. With continued administration of SSRI, 5-HT_{1A} and 5-HT_{1B} autoreceptors become desensitised, resulting in enhanced 5-HT transmission after 2–4 weeks of treatment, a time period that correlates with the time of onset of the AD effect (Plenge and Mellerup, 2003). These findings prompted the proposal that 5-HT_{1A} and 5-HT_{1B} autoreceptor antagonists could accelerate (and perhaps augment) the clinical effects of antidepressants by preventing this negative feedback, which would enable a more rapid increase of synaptic 5-HT (Artigas et al., 1994, 1996).

Pharmacologically, pindolol is a β-adrenergic receptor antagonist, which also displays a significant affinity and antagonistic effect on the 5-HT_{1A} and 5-HT_{1B} receptors (Newman-Tancredi et al., 1998). Clinical investigations, based upon the above-mentioned rationale, have demonstrated an enhanced and/or accelerated SSRI-induced AD action by co-administration of pindolol (Artigas et al., 1994, 1996, 2001). Consequently, since 1994, pindolol has been used to accelerate or enhance the clinical effects of antidepressant drugs (Artigas et al., 2001). However, some authors argue that the pharmacokinetics of pindolol following therapeutical doses of this drug does not fully support this rationale. Indeed, the dose of pindolol used in most clinical trials (3 × 2.5 mg/day), which yields a mean plasma concentration of (±) pindolol of about 40 nM, might be insufficient to induce a substantial occupancy of 5-HT_{1A} autoreceptors (in Artigas et al., 2001). Actually, it was shown that pindolol (7.5 mg/day, given during 1 week) occupied 40 ± 29% of dorsal raphe nucleus (DRN) 5-HT_{1A} receptors and only 18 ± 19% of 5-HT_{1A} receptors in cortico–limbic structures (i.e. neocortex, hippocampus and amygdala) of humans (Martinez et al., 2000). Thus, other factors might also be involved in the therapeutical effect of pindolol.

Alternatively, it has been demonstrated that inhibition of nitric oxide (NO) synthesis in CNS produces anxiolytic and AD-like behavioural effects in a variety of animal paradigms (Jefferys and Funder, 1996; Silva et al., 2000; Yildiz et al., 2000; Harkin et al., 2003). Some of these effects are reverted by administration of the NO synthase substrate, L-arginine, consistent with an

Fig. 1. Chemical structure of pindolol.
involvement of \(^{\text{NO}}\) in this behavioural response. Of note, the depletion of 5-HT in the frontal cortex by p-chlorophenylalanine prevents the AD-like effects of nitric oxide synthase inhibitors (Harkin et al., 2003). Therefore, a lessening effect on the \(^{\text{NO}}\) levels in the CNS would play a relevant role in AD therapies. Nevertheless, the direct interaction of pindolol with \(^{\text{NO}}\) has yet to be studied.

Importantly, sustained high levels of \(^{\text{NO}}\) may also be deleterious to CNS, predominantly due to the formation of peroxynitrite anion (ONOO\(^{-}\)), which is generated via reaction of \(^{\text{NO}}\) with superoxide radical (O\(_2^{-}\)) (Radi et al., 1991a; Rubbo et al., 1996). These reactive radicals have been implicated in neurotoxic effects (Bolanos et al., 1997; Lipton, 1999). Thus, a putative scavenging effect of pindolol against \(^{\text{NO}}\), ONOO\(^{-}\), or O\(_2^{-}\), might also be of therapeutical relevance, in what concerns the neuroprotection against sustained high levels of reactive nitrogen species (RNS) in the CNS.

The purpose of the present study was to characterize the putative pindolol scavenging effect on \(^{\text{NO}}\), ONOO\(^{-}\), and O\(_2^{-}\), using in vitro non-cellular systems, taking into account the possible relevance of this specific type of effect for pindolol-induced reduction in antidepressant latency for SSRIs and its activity as a neuroprotective agent.

**Materials and methods**

**Materials**

Pindolol (1-(1H-indol-4-yloxy)-3-(isopropylamino)-2-propanol), dihydorhodamine 123 (DHR 123), diethylenetriaminepentaacetic acid (DTPA), ebselen, 4,5-diaminofluorescein (DAF-2), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), rutin, xanthine, xanthine oxidase grade I from buttermilk (EC 1.1.3.22), nitroblue tetrazolium chloride (NBT), ethylenediaminetetraacetic acid (EDTA) disodium salt, and manganese dioxide were purchased from Sigma Chemical Co. (St. Louis, USA). Trolox and potassium chloride were purchased from Fluka Chemie GmbH (Steinheim, Germany). Hydrogen peroxide (30% solution), sodium bicarbonate, sodium nitrite, sodium hydroxide, hydrochloric acid and sodium chloride were obtained from Merck (Darmstadt, Germany).

**Measurement of \(^{\text{NO}}\) scavenging activity**

The \(^{\text{NO}}\) scavenging activity was measured by monitoring the oxidation of the non-fluorescent 4,5-diaminofluorescein (DAF-2) to the fluorescent triazolofluorescein by \(^{\text{NO}}\), using the method of Nagata et al. (1999), with modifications. A stock solution of 2.76 mM DAF-2 in DMSO was purged with nitrogen and stored at \(-20^\circ\)C. Working solutions of DAF-2 diluted with buffer (1.3 mM NaH\(_2\)PO\(_4\), 5.4 mM KCl, 5.6 mM glucose, 24 mM NaHCO\(_3\), 120 mM NaCl, 1 mM MgCl\(_2\) and 2 mM CaCl\(_2\), pH 7.4) to 1/368-fold from the stock solution were placed on ice in the dark immediately before the determinations. The reaction mixtures contained, in a final volume of 300 \(\mu\)L, the following reagents at the indicated final concentrations: DAF-2 (5 \(\mu\)M), NOC-5 (10 \(\mu\)M) and tested compound dissolved in DMSO, at various concentrations (0, 313, 625, 1250 and 2500 \(\mu\)M). The mixtures were incubated for 30 min at 37 \(^\circ\)C in a microplate reader (Synergy HT, BIO-
The fluorescence signal caused by the reaction of DAF-2 with \( \cdot \)NO was measured using the microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. The effects are expressed as the percentual inhibition of the \( \cdot \)NO-induced DAF-2 oxidation. Rutin was used as a positive control. Each study corresponds to four experiments, performed in triplicate.

**Synthesis of ONOO\(^{-} \)**

Synthesis of ONOO\(^{-} \) was essential as described before by Beckman et al. (1994). Briefly, an acidic solution (HCl 0.7 M) of H\(_2\)O\(_2\) 0.6 M was mixed with NaNO\(_2\) (0.66 M) on ice for 1 s and the reaction quenched with ice-cold NaOH 3 M. Residual H\(_2\)O\(_2\) was removed by mixing with granular MnO\(_2\) pre-washed with NaOH 3 M. The stock ONOO\(^{-}\) solution was filtered and then frozen (\(-80^\circ\)C), and the top layer of the solution collected for the experiment. The concentration of ONOO\(^{-}\) was determined by measuring the absorbance at 302 nm (\( \varepsilon = 1670 \) M\(^{-1}\) cm\(^{-1}\)). The typical yield of freshly prepared ONOO\(^{-}\) ranged 60–80 mM. Higher concentrations (>200 mM) of ONOO\(^{-}\) can be obtained by freeze fractionation. However, in the present study only freshly prepared ONOO\(^{-}\) solution was used to minimise nitrite ion contamination.

**Measurement of ONOO\(^{-}\) scavenging activity**

The ONOO\(^{-}\) scavenging activity was measured by monitoring the oxidation of the non-fluorescent dihydrorhodamine 123 (DHR 123) to the fluorescent rhodamine 123 by ONOO\(^{-}\) using the method of Kooy et al. (1994), with modifications. A stock solution of 2.89 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at \(-20^\circ\)C. Buffer (90 mM NaCl, 50 mM Na\(_3\)PO\(_4\) and 5 mM KCl, pH 7.4 with HCl) was purged with nitrogen and placed on ice before use. At the beginning of the experiments, 100 \( \mu \)M DTPA was added to the buffer. Working solutions of DHR 123 were diluted from the stock solution, using this buffer, and were placed on ice in the dark immediately before the determinations. Reaction mixtures contained, in a final volume of 300 \( \mu \)L, the following reagents at the indicated final concentrations: DHR 123 (5 \( \mu \)M), tested compound dissolved in DMSO, at various concentrations (0, 39, 78, 156, 312 and 625 \( \mu \)M) and ONOO\(^{-}\) (600 nM). The mixtures were incubated, in a microplate reader (Synergy HT, BIO-TEK), for 5 min at 37 \( ^\circ \)C. The fluorescence signal caused by the reaction of DHR 123 with ONOO\(^{-}\) was measured using the microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. Ebselen was used as a positive control. The effects are expressed as the percentual inhibition of the ONOO\(^{-}\) induced DHR 123 oxidation.

In a parallel set of experiments the assays were performed in the presence of 25 mM NaHCO\(_3\) in order to simulate the physiological conditions with high CO\(_2\) concentrations in vivo. In this set of experiments, the concentrations of pindolol used (0, 5, 10, 20, 39 and 78 \( \mu \)M) were lower than those in the absence of NaHCO\(_3\). This evaluation is important because, under physiological conditions, the reaction of ONOO\(^{-}\) with CO\(_2\) is predominant, with a very fast rate constant of reaction of CO\(_2\) with ONOO\(^{-}\) being very fast (\( k_2 = 3 - 5.8 \times 10^4 \) M\(^{-1}\) s\(^{-1}\)) (Whiteman et al., 2002). Thus, the reactivity of the putative scavengers for ONOO\(^{-}\) should be able to match or exceed that of CO\(_2\), in order to avoid any loss in its effectiveness. On the other hand, a putative high scavenging effect on the reactive species that are produced from the ONOO\(^{-}\)/CO\(_2\) reaction such as...
the nitrogen dioxide radical (\(\cdot\)NO\(_2\)) and the carbonate radical anion (CO\(_5^-\)), may increase its efficacy.

Each study corresponds to four experiments, performed in triplicate.

**Measurement of \(O_2^-\) scavenging activity**

The \(O_2^-\) scavenging activity was measured by monitoring reduction of nitroblue tetrazolium chloride (NBT) to the blue chromogen diformazan by \(O_2^-\) accordingly to a described procedure (Fernandes et al., 2003). \(O_2^-\) was generated by a xanthine/xanthine oxidase system. The reaction mixtures contained, in a final volume of 300 \(\mu\)L, the following reagents at the indicated final concentrations: NBT (50 \(\mu\)M), xanthine (44 \(\mu\)M), tested compound dissolved in DMSO, at various concentrations (0, 0.625, 1.25, 2.5 and 5 mM) and xanthine oxidase (0.05 U/mL). Xanthine was dissolved in NaOH 1 mM, and subsequently in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8. Xanthine oxidase was dissolved in EDTA 0.1 mM, and the other components in phosphate buffer 50 mM with EDTA 0.1 mM, pH 7.8. The reaction was conducted at 37 °C for 2 min. The \(O_2^-\) induced reduction of NBT to diformazan was determined spectrophotometrically at 560 nm, using a microplate reader (Synergy HT, BIO-TEK). The effects are expressed as the percentual inhibition of the \(O_2^-\) induced NBT reduction. Trolox was used as a positive control. Each study corresponds to four experiments, performed in triplicate.

**Results**

**Nitric oxide scavenging activity**

Fig. 2 shows the results obtained in the \(\cdot\)NO scavenging assay. Pindolol showed to be a potent inhibitor of \(\cdot\)NO-induced oxidation of DAF-2 to triazolofluorescein in a concentration dependent manner. The resulting IC\(_{50}\) was 449 ± 33 \(\mu\)M (Mean ± SEM) for pindolol and the positive control rutin, originated an IC\(_{50}\) of 0.52 ± 0.02 \(\mu\)M (Mean ± SEM) (Table 1).

![Fig. 2. \(\cdot\)NO scavenging activity of pindolol (represented as a percentual decrease of \(\cdot\)NO-induced DAF-2 oxidation). Each point represents the values obtained from four experiments, performed in triplicate (Mean ± SEM).](image-url)
Peroxynitrite scavenging activity

Fig. 3 shows the results obtained in the ONOO⁻ scavenging assay in the absence (A) and in the presence of 25 mM NaHCO₃ (B). In the absence of NaHCO₃, pindolol showed to be a potent inhibitor of ONOO⁻ induced oxidation of DHR 123 in a concentration dependent manner. The resulting IC₅₀ was 131 ± 24 μM (Mean ± SEM), and the positive control ebselen, provided an IC₅₀ of 2.5 ± 0.1 μM (Mean ± SEM) (Table 1).

In the presence of 25 mM NaHCO₃, pindolol exhibited a dramatic increase of activity (IC₅₀ = 17 ± 3 μM) (Mean ± SEM) while the activity observed for the positive control ebselen was shown to be decreased, revealing an IC₅₀ of 16 ± 1 μM (Mean ± SEM) (Table 1).

Superoxide radical scavenging activity

No scavenging activity was observed for pindolol at concentrations up to 5 mM, although an IC₅₀ of 1.0 ± 0.1 mM (Mean ± SEM) was found for the positive control, trolox (Table 1).

Table 1
Scavenging activities (IC₅₀, Mean ± SEM) of pindolol, rutin, ebselen and trolox against ·NO, ONOO⁻ (in the absence or presence of NaHCO₃) and O₂⁻

<table>
<thead>
<tr>
<th>Tested compound</th>
<th>·NO Absence of NaHCO₃</th>
<th>ONOO⁻ Presence of 25 mM NaHCO₃</th>
<th>O₂⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC₅₀ (μM)</td>
<td>IC₅₀ (μM)</td>
<td>IC₅₀ (μM)</td>
</tr>
<tr>
<td>Pindolol</td>
<td>449 ± 33</td>
<td>131 ± 24</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.52 ± 0.02</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ebselen</td>
<td>–</td>
<td>2.5 ± 0.1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Trolox</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NA — no activity was found within the tested concentrations (up to 5 mM).

Fig. 3. ONOO⁻ scavenging activity of pindolol in the absence of NaHCO₃ (A), and in the presence of 25 mM NaHCO₃ (B) (represented as a percentual decrease of ONOO⁻ induced DHR 123 oxidation). Each point represents the values obtained from four experiments, performed in triplicate (Mean ± SEM).
Discussion

The results obtained in the present study clearly show that pindolol is a potent scavenger of $\cdot$NO and ONOO$^-$, while no activity was found against $O_2^-$. These effects are possibly of therapeutical relevance since they were shown to be concentration dependent at the micromolar range. It is important to stress that although the concentrations of pindolol shown to be active against the RNS in the present non-cellular in vitro assays are higher than those expected to be attained during the therapeutical use of this drug, the scavenging efficacy depends on the RNS levels, which are normally lower in vivo than in the present in vitro assays. Of note, the non-cellular scavenging assays are designed in such a way that the levels of radicals are adequately high to assure a good signal in the detection systems. In vitro, we always verify that, when the levels of $\cdot$NO are lower, the IC$_{50}$ of the scavenger species is decreased (Sousa et al., 2005). Consequently, the levels of scavengers that are needed in vitro, are higher than those needed for an effective activity in vivo.

The role of $\cdot$NO in depression is still not entirely understood. Nevertheless, in addition to the mentioned anxiolytic and AD-like behavioural effects, in a variety of animal paradigms, this type of effect has also been observed in humans, after the administration of a $\cdot$NO scavenger (methylene blue). Indeed, treatment of 24 patients, who were refractory to lithium therapy, with methylene blue (100 mg b.i.d. or t.i.d.) resulted in a 63% positive response (Narsapur and Naylor, 1983). Of note, it has also been shown that paroxetine, in addition to its selective inhibition of serotonin re-uptake, also inhibits the production of $\cdot$NO by nitric oxide synthase (NOS) (Finkel et al., 1996). Whether the paroxetine- or pindolol-induced $\cdot$NO depletion in the CNS contributes or not for the AD effect still needs and is worthy of being further evaluated in vivo.

Structurally, pindolol is an indole derivative (see Fig. 1). Importantly, the chemical structure of this compound is similar to that of endogenous indole derivatives like melatonin, 5-HT, and tryptophan, which have already been shown to be scavengers of RNS (Gilad et al., 1997; Blanchard et al., 1997; Alvarez and Radi, 2003; Zhang et al., 1999; Blanchard et al., 2000; Alvarez et al., 1996). Computational studies with indole derivatives, calculating the relative free energy of radical reactions, indicated that the carbon atoms at positions 2, 3, 4, 6 and 7 of the indole heterocycle are suitable sites for nitrogen radical reactions (Tan et al., 2002; Turjanski et al., 2001). This chemical reactivity has been confirmed by several studies, namely for tryptophan (Alvarez et al., 1996), 5-HT (Blanchard et al., 1997) and melatonin (Zhang et al., 1998). Although the evaluation of the structure-activity relationship was not the aim of the present study, it is possible that the positions N-1, C-2, C-3, C-6 and C-7 of this compound are involved in the observed scavenging activity by pindolol.

Although $\cdot$NO is an important messenger molecule, when generated at high concentrations, this radical can initiate a neurotoxic cascade [Bolanos et al., 1997; Lipton, 1999; Dawson and Dawson, 1996; Nicotera et al., 1999]. For example, $\cdot$NO has been implicated in neuronal injury, such as that found in brain ischemia and Parkinson’s disease and in killing of immune cells (Nicotera et al., 1999). However, $\cdot$NO itself is weakly toxic with only a limited number of potential cellular targets (Dawson and Dawson, 1996). Indeed, most of the cytotoxic pathways activated following overproduction of NO have been attributed to ONOO$^-$. ONOO$^-$ is a highly reactive species produced by the near-diffusion limited reaction of $\cdot$NO with $O_2^-$, which nitrates tyrosine residues in proteins, and is also known to be a powerful sulfhydryl oxidant and inducer of lipid peroxidation (Radi et al., 1991a,b; Quijano et al., 1997). The strong scavenging effect against ONOO$^-$ demonstrated in the present study for pindolol possibly gives an additional value to this drug in pro-oxidant neurodegenerative diseases.
It has been reported that physiological concentrations of CO₂ can modulate ONOO⁻ reactivity due to the fast reaction between these two compounds, yielding 'NO₂ and CO₃⁻, which are the main responsible radicals for the nitrination and oxidation reactions that are usually observed in vivo (Squadrito and Pryor, 1998). A scavenger can directly trap ONOO⁻ only if it reacts faster with ONOO⁻ than does CO₂. Thus, CO₂ (25 mM), by competing for ONOO⁻, significantly weakens the ability of several antioxidants to scavenge this RNS (Ketsawatsakul et al., 2000). On the other hand, it has been shown in other studies that CO₂ functions as a catalyst for ONOO⁻ induced nitrination, as shown for tryptophan (Alvarez et al., 1996), and melatonin (Zhang et al., 1999). Therefore, a putative scavenging effect on the reactive species that are produced from the ONOO⁻/CO₂ reaction may also be of therapeutical value. Zhang et al. (1999) demonstrated that the reaction of ONOO⁻ with melatonin in the absence of added bicarbonate produces mainly 6-hydroxymelatonin and 1,2,3,3a,8a-hexahydro-1-acetyl-5-methoxy-8a-hydroxypyrrolo[2,3-b]indole, with some isomeric 1,2,3,3a,8a-hexahydro-1-acetyl-5-methoxy-3a-hydroxypyrrolo[2,3-b]indole. In the presence of added bicarbonate, product yields decrease and 6-hydroxymelatonin is not formed, indicating reactivity of melatonin with other reactive species, probably 'NO₂ and CO₃⁻. It is possible that, due to its indolic structure, pindolol chemical reactivity may be similar to that of melatonin, even though this hypothesis requires further investigation. In line with the findings for the mentioned indole derivatives, in the present study we also observed the catalytic effect of CO₂ on pindolol (almost 8 times more potent in the presence of CO₂), while the activity of the positive control, ebselen, was decreased. It was previously shown that ebselen, reacts with ONOO⁻ nearly 30 times faster than CO₂ (Squadrito and Pryor, 1998). Thus, the presence of high concentrations of CO₂ was expected to lower its activity, which was in fact observed in the present study.

In conclusion, the results obtained in the present study clearly show that pindolol is a potent scavenger of 'NO and ONOO⁻. These effects may contribute to the reduction of SSRI antidepressant latency that has been attributed to pindolol and also may constitute an additional value for this drug when depression is associated with pro-oxidant neurodegenerative diseases.

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