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Antioxidant activity of β-blockers: An effect mediated by scavenging reactive oxygen and nitrogen species?

Ana Gomes, David Costa, José L. F. C. Lima and Eduarda Fernandes*

REQUIMTE, Departamento de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal

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Abstract—The therapeutic effects of β-blockers are normally explained by their capacity to block the β-adrenoceptors, however, some of the beneficial cardiovascular effects shown by this group of compounds have already been associated with the antioxidant properties that some of them seem to possess. The β-blockers atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol, timolol, and carvedilol were tested for their putative scavenging activity for ROS ($O_2^{\cdot -}$, H_2O_2, $HO^{\cdot}$, HOCl, and $ROO^{\cdot}$) and RNS ($^{\cdot}NO$ and $ONOO^-$). Some of the studied compounds are effective ROS and/or RNS scavengers, these effects being possibly useful in preventing oxidative damage verified in hypertension as well as in other cardiovascular diseases that frequently emerge in association with oxidative stress.

1. Introduction

β-Blockers (or β-adrenergic antagonists) are a group of drugs widely used in the treatment of cardiovascular diseases, namely arterial hypertension, cardiac arrhythmias, and angina pectoris as well as other types of pathologies such as anxiety or glaucoma.[1,2] The therapeutic effects of β-blockers are normally explained by their capacity to block the β-adrenoceptors, hindering the access of the endogenous agonists noradrenaline and adrenaline.[1] However, part of the beneficial cardiovascular effects shown by this group of compounds has already been associated with the antioxidant properties that some of them seem to possess. Some of the earlier observations indicating antioxidant activities for β-blockers were made by Mak and Weglicki,[3] who demonstrated a concentration-dependent membrane anti-peroxidative activity for propranolol, pindolol, metoprolol, atenolol, and sotalol. Of the five β-blockers examined, propranolol was the most potent agent, and the activities seemed to correlate with the drugs' hydrophobicity.[3] Further studies indicated that the antioxidant activity of propranolol was independent of its pharmacological activity and was related to its intrinsic chemical properties rather than to its quinidine-like membrane stabilization effect.[3–5] The antioxidant properties of propranolol were later corroborated using membrane and cellular models.[6,7] More recently, propranolol's antioxidant-related cardioprotective effects were studied in rats, showing that the chronic treatment with this β-blocker was found able to provide protection against ischemia–reperfusion injury.[8] In that study, tissue lipid peroxidation products, both before and after the ischemia–reperfusion episodes, were significantly reduced by propranolol.[8] Thus, it is now accepted that propranolol is an important therapeutic tool against oxidative stress by stabilizing membranes, including lysosomes, inducing the activity of antioxidant and other beneficial enzymes, and increasing endothelial nitric oxide production, and directly protecting isolated membranes, cardiovascular cells, and tissues against oxidative injury.[9] Perhaps the best example of a β-blocker with potent antioxidant effects is carvedilol.[10] Recent studies have shown that carvedilol acting as both a metal chelator and a radical scavenger[11] reduces the lipid peroxidation level as evidenced by changes in plasma thiobarbituric reactive substance,[12,13] plasma oxidized low-density lipoprotein,[11] and myocardial 4-hydroxy-2-nonenal-modified protein[14] in patients with heart failure. Carvedilol was also shown to inhibit mitochondrial permeability transition in isolated heart mitochondria and protect mitochondria against the oxidative damage induced by

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* Corresponding author. Tel.: +00351222078968; fax: +00351222004427; e-mail: egracas@ff.up.pt

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the xanthine oxidase/hypoxanthine pro-oxidant system.[15] Carvedilol was recently shown to reduce total cellular oxidative stress and myocyte apoptosis and cell hypertrophy in congestive heart failure.[16] In that study, qualitatively similar effects were produced by metoprolol. Propranolol exerted a smaller protective effect on myocyte apoptosis and progression of ventricular remodeling.[16] Other β-blockers have also been shown to possess antioxidant properties. Metoprolol, timolol, sotalol, and carvedilol were shown to protect red blood cells against phenazine methosulfate (PMS) (which promotes the formation of superoxide radicals) induced toxicity to red blood cells, within therapeutic concentrations, carvedilol being the most potent.[17] In other studies, pindolol was shown to be a potent scavenger of the peroxyl radical[18,19] and of reactive nitrogen species (RNS).[20] Labetalol was as well shown to inhibit superoxide anion production during normal leukocyte oxidative metabolism.[21]

While β-blockers are effective in the therapeutic treatment of cardiovascular diseases such as angina pectoris, myocardial infarction, arrhythmia, hypertension, and cardiomyopathy, the real contribution of the putative antioxidant effects for their efficacy is still unclear, but may constitute a valuable contribution for the final healing outcome. Most importantly, although a number of studies have shown that some β-blockers are scavengers of reactive oxygen species (ROS) and RNS, a thorough screening about the scavenging of these therapeutic drugs for the different ROS and RNS that would allow us to determine their relative potency in this particular aspect is yet to be performed.

ROS are produced in the cardiovascular system by different cell types, namely the endothelial cells, vascular smooth muscle cells (VSMC), adventitial fibroblasts, and macrophages, primarily through the production of superoxide radical ($O_2^{\bullet -}$). In the cardiovascular system, $O_2^{\bullet -}$ is especially produced by the enzymes NAD(P)H oxidase and xanthine oxidase but also by the 'uncoupled' nitric oxide synthase (NOS), cytochrome P450, and during the mitochondrial respiration.[22–28] $O_2^{\bullet -}$ is quickly converted to hydrogen peroxide (H_2O_2) either spontaneously or by the enzyme superoxide dismutase (SOD). H_2O_2 is not an inherently reactive compound. However, H_2O_2 can be transformed into highly reactive and deleterious products: (i) the interactions of H_2O_2 with $O_2^{\bullet -}$ or with trace levels of transition metals can lead to the formation of hydroxyl radicals ($HO^{\bullet}$), and consequently to the formation of peroxyl radicals ($ROO^{\bullet}$) due to the well-known reactivity of $HO^{\bullet}$ with polyunsaturated fatty acids;[29] (ii) myeloperoxidase (MPO), a hydrogen peroxide oxidoreductase that is specifically found in mammalian granulocytic leukocytes, including neutrophils, monocytes, basophils, and eosinophils, contributes considerably to the bactericidal capabilities of these cells via formation of hypochlorous acid (HOCl) from H_2O_2 and chlorine ions.[30] Thus, beyond $O_2^{\bullet -}$, the other ROS produced in the cardiovascular system are H_2O_2, $HO^{\bullet}$, HOCl, and $ROO^{\bullet}$.[24,26,28]

Nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$) are the most relevant reactive nitrogen species (RNS) in the vasculature. When produced by endothelial NOS, $^{\bullet}NO$ plays an important role in the regulation of vascular tone and inhibition of platelet aggregation.[31] However, in the presence of $O_2^{\bullet -}$, it rapidly originates the highly reactive and harmful species $ONOO^-$.[32–35]

In physiological conditions, ROS and RNS are produced in the vasculature at low concentrations, in a controlled manner, and act as second messengers, being involved in the growth and migration of VSMC and in the regulation of endothelial function. However, under pathological conditions, the overproduction of these species may contribute to vascular damage.[23,24,27] In fact, ROS and RNS have been implicated in various cardiovascular diseases namely hypertension, atherosclerosis, ischemia–reperfusion injury, congestive cardiac failure, and diabetes.[26,36] In the particular case of hypertension, ROS and $ONOO^-$ can directly, or indirectly, by reducing the $^{\bullet}NO$ levels, contribute to the development of the disease through a number of processes like vasoconstriction and proliferation of VSMC, resulting in vascular and myocardial hypertrophy, endothelial cell damage with the consequent impairment of endothelial function, decreased kidney function, and increased sympathetic efferent activity from the central nervous system.[22,24,26,28,37]

Taking into account the above-mentioned rationale, the aim of this work was to investigate and compare the scavenging activity for ROS and RNS of the β-blockers atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol, timolol, and carvedilol.

2. Results

2.1. Superoxide radical scavenging activity

No scavenging activity was observed for any of the tested β-blockers at concentrations up to 5 mM, although an IC_{50} of 3.7 ± 0.7 μM (mean ± SEM) was found for the positive control, tiron.

2.2. Hydrogen peroxide scavenging activity

At concentrations up to 5 mM, only labetalol and timolol were able to inhibit H_2O_2-dependent lucigenin's chemiluminescence (Fig. 1). Labetalol caused a 35% inhibition of chemiluminescence at its maximum concentration (5 mM), whereas timolol showed a 90% inhibition at the same concentration, with an IC_{50} of 1642 ± 145 μM (mean ± SEM). The antioxidant ascorbic acid scavenged H_2O_2, showing an IC_{50} of 860 ± 80 μM (mean ± SEM) (Table 1). The specificity of the method was confirmed by using catalase, which provided an IC_{50} of 50 ± 5 U/mL (mean ± SEM), while SOD, tested at the concentrations 250 and 1000 U/mL, had no preventive effect on the H_2O_2-induced oxidation of lucigenin (data not shown).

Atenolol, carvedilol, and pindolol promoted a chemiluminescence increase in a concentration-dependent manner. This effect only occurred in the presence of H_2O_2 so it did not result from a direct oxidation of lucigenin by

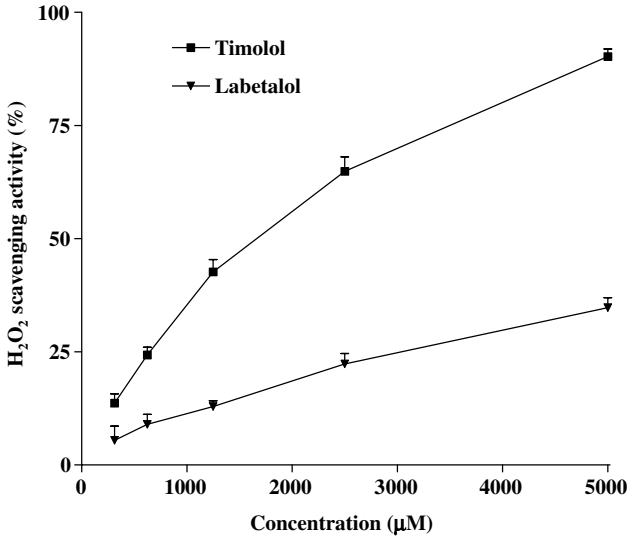


Figure 1. H_2O_2 scavenging activity of timolol and labetalol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

the referred compounds. This indicates that these compounds probably react with H_2O_2, possibly leading to the formation of products that are also able to oxidize lucigenin. Consequently, this methodology is not appropriate to evaluate the H_2O_2 scavenging activity of these three compounds.

2.3. Hydroxyl radical scavenging activity

All the tested compounds were able to prevent HO·-dependent chemiluminescence of luminol (Fig. 2). Carvedilol was not tested due to dissolution problems in the assay system. The scavenging activity order was: labetalol > pindolol > propranolol > sotalol > timolol > atenolol > metoprolol, with IC_{50}s of 94 ± 15, 313 ± 48, 649 ± 68, 1287 ± 140, 3140 ± 851, 4118 ± 36, and >5000 μM (mean ± SEM), respectively (Table 1). Melatonin provided an IC_{50} of 153 ± 32 μM (mean ± SEM) (Table 1).

2.4. Hypochlorous acid scavenging activity

Figure 3 shows the HOCl scavenging effects of the studied β-blockers. All the tested compounds were able to scavenge HOCl in a concentration-dependent manner. The order of potencies found was: metoprolol ≈ propranolol > atenolol ≈ pindolol ≈ sotalol > labetalol ≈ timolol > carvedilol, with IC_{50}s of 7.3 ± 0.9, 9.0 ± 2.0, 14.5 ± 0.4, 15.2 ± 3.1, 15.7 ± 1.8, 19.6 ± 1.8, 20.1 ± 1.4, and 32.8 ± 3.7 μM (mean ± SEM), respectively (Table 1). The IC_{50} obtained for lipoic acid was 6.0 ± 0.5 μM (mean ± SEM) (Table 1).

2.5. Peroxyl radical scavenging activity

Only pindolol and propranolol were able to delay, in a concentration-dependent manner, the loss of fluorescence due to ROO·-dependent fluorescein oxidation. The ORAC values for pindolol and propranolol, obtained from the decline of the linear regressions, were 0.62 ± 0.05 and 0.35 ± 0.03, respectively. The positive controls GSH and ascorbic acid provided ORAC values of 0.34 ± 0.10 and 0.25 ± 0.07, respectively.

2.6. Nitric oxide scavenging activity

The results obtained in the ·NO scavenging assay are shown in Figure 4. Pindolol, carvedilol, atenolol, and propranolol presented ·NO scavenging activity, pindolol being the most effective compound. The IC_{50}s provided by pindolol and atenolol were 496 ± 40 and 1646 ± 154 μM (mean ± SEM), respectively, while carvedilol and propranolol did not reach a 50% effect at the maximum tested concentrations (500 and 5000 μM, respectively) (Table 1). Rutin provided an IC_{50} of 0.52 ± 0.02 (mean ± SEM) (Table 1).

It was not possible to evaluate labetalol, sotalol, and timolol using this screening test because these compounds interfered with the methodology, provoking an increase in the fluorescence signal in a concentration-dependent manner, in the presence of ·NO.

Table 1. Scavenging activities for H_2O_2, HO·, HOCl, ·NO, and $ONOO^-$ (IC_{50}, mean ± SEM) presented by the studied β-blockers and by the respective positive controls

Tested compounds	IC_{50} (μM)					
	H_2O_2	HO·	HOCl	·NO	$ONOO^-$ without $NaHCO_3$	$ONOO^-$ with $NaHCO_3$
Atenolol	INT	4118 ± 36	14.5 ± 0.4	1646 ± 154	2415 ± 278	>5000
Carvedilol	INT	—	32.8 ± 3.7	>500	>400	27 ± 3
Labetalol	>5000	94 ± 15	19.6 ± 1.8	INT	INT	INT
Metoprolol	NA	>5000	7.3 ± 0.9	NA	>5000	4515 ± 167
Pindolol	INT	313 ± 48	15.2 ± 3.1	496 ± 40	122 ± 20	18 ± 2
Propranolol	NA	649 ± 68	9.0 ± 2.0	>5000	1112 ± 232	299 ± 32
Sotalol	NA	1287 ± 140	15.7 ± 1.8	INT	49 ± 3	32 ± 5
Timolol	1642 ± 145	3140 ± 851	20.1 ± 1.4	INT	INT	INT
Ascorbic acid	860 ± 80	—	—	—	—	—
Lipoic acid	—	—	6.0 ± 0.5	—	—	—
Melatonin	—	153 ± 32	—	—	—	—
Rutin	—	—	—	0.52 ± 0.02	—	—
Ebselen	—	—	—	—	2.5 ± 0.1	16 ± 2

NA, No activity was found within the tested concentrations. INT, Interference with the methodology.

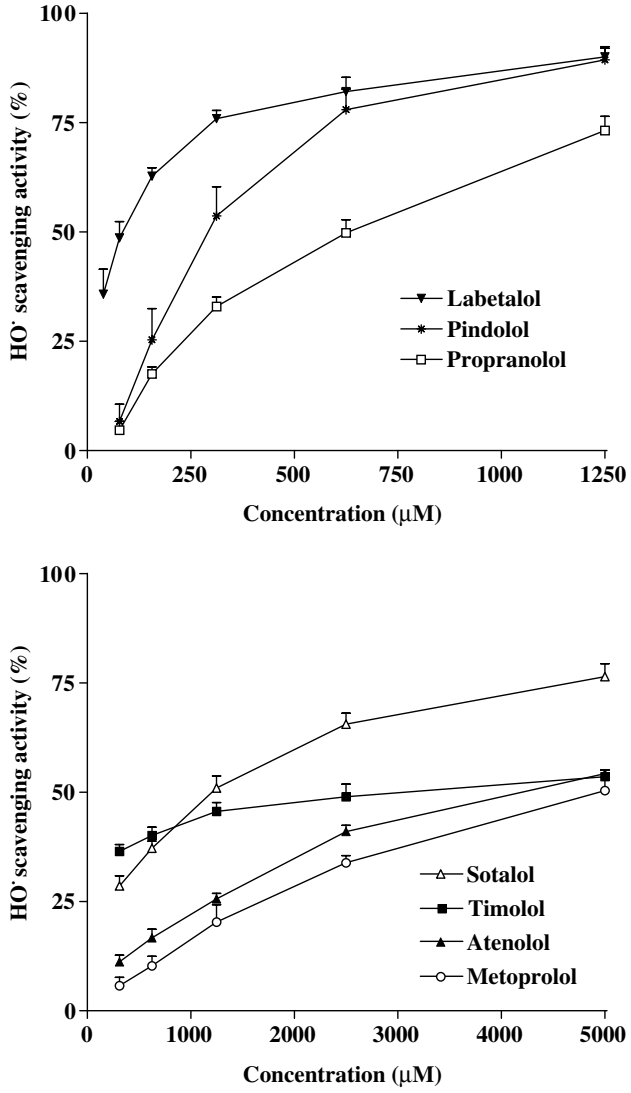


Figure 2. HO· scavenging activity of labetalol, pindolol, propranolol, sotalol, timolol, atenolol, and metoprolol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

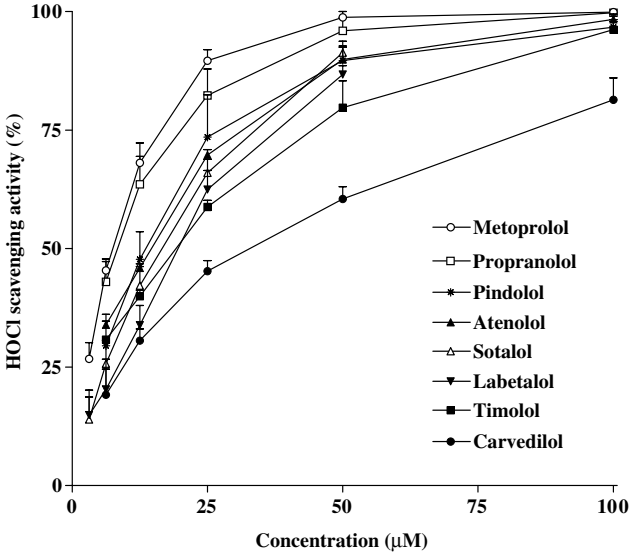


Figure 3. HOCl scavenging activity of labetalol, sotalol, timolol, atenolol, propranolol, pindolol, carvedilol, and metoprolol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

2.7. Peroxynitrite scavenging activity

As seen in Figures 5 and 6, all the assayed compounds, except timolol and labetalol, were able to scavenge $ONOO^-$ in a concentration-dependent manner. Pindolol and sotalol were the most effective compounds, providing IC_{50} values of 122 ± 20 and 49 ± 3 μM (mean ± SEM) (Table 1). Carvedilol also showed to be a potent scavenger. However, due to its low solubility in the assay system, the maximum concentration of this compound that could be tested was 400 μM, which did not achieve a 50% effect. Propranolol and atenolol provided IC_{50}s of 1112 ± 232 and 2415 ± 278 μM (mean ± SEM), respectively (Table 1). Metoprolol did not achieve a 50% effect at the maximum tested concentration (5000 μM). The IC_{50} obtained for the positive control ebselen was 2.5 ± 0.1 μM (mean ± SEM) (Table 1).

The same compounds that presented scavenging activity in the absence of bicarbonate also behaved as scavengers in the presence of 25 mM $NaHCO_3$ (Figs. 7 and 8). However, the concentration–effect relationship changed. In fact, all the compounds except atenolol have shown to be more powerful scavengers in the presence of bicarbonate. The order of potencies obtained was pindolol > carvedilol > sotalol > propranolol > metoprolol > atenolol, with IC_{50}s of 18 ± 2, 27 ± 3, 32 ± 5, 299 ± 32, 4515 ± 167, and >5000 μM (mean ± SEM), respectively (Table 1). The IC_{50} found for ebselen was 16 ± 2 μM (mean ± SEM) (Table 1).

It was not possible to evaluate labetalol and timolol using this screening test because these compounds interfered with the methodology, provoking an increase in the fluorescence signal in a concentration-dependent manner, in the presence of $ONOO^-$.

3. Discussion

The present study indicates that the ROS and RNS scavenging effect of the tested β-blockers should be taken into account when these compounds are used in the therapy of cardiovascular diseases. It has been previously shown that a diet rich in antioxidants can contribute to the prevention or improvement of cardiovascular diseases.[38] However, most of the available clinical trials employing antioxidant vitamins, especially supplements of vitamins C and E, have been inconclusive, probably because these compounds can achieve better effects when acting in synergy with other antioxidants than when used alone.[38–41] In addition, when used in high doses, antioxidant vitamins can paradoxically behave as pro-oxidants, intensifying the oxidative damages.[39,40] Recently, other studies using different antioxidants such as SOD mimetics, xanthine oxidase inhibitors or thiol containing compounds revealed their apparent contribution to the decrease of the oxidative stress associated

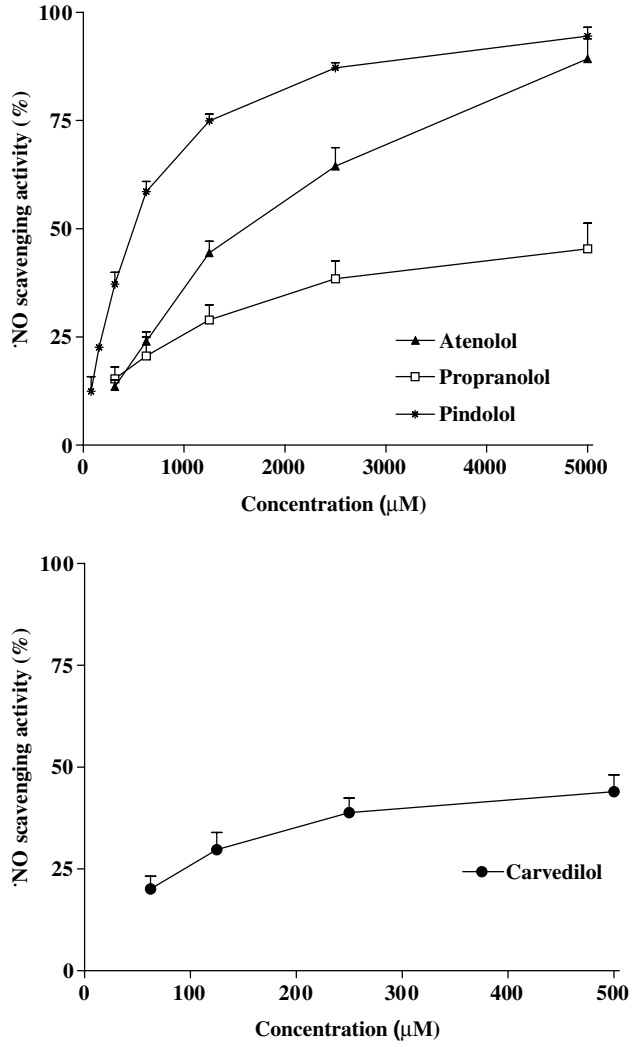


Figure 4. ·NO scavenging activity of pindolol, atenolol, propranolol, and carvedilol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

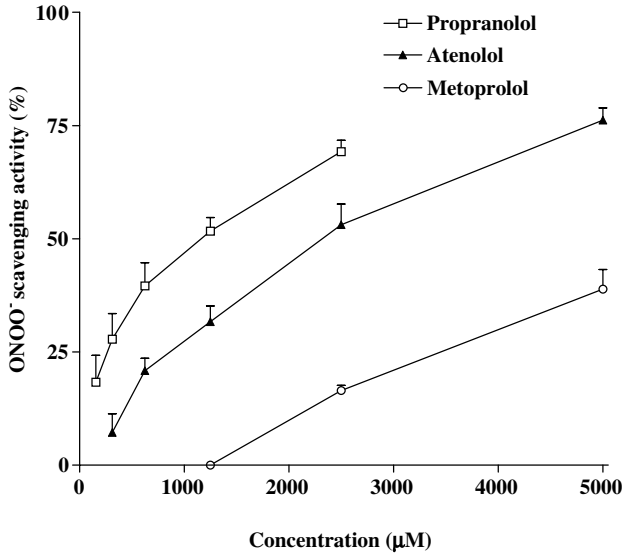


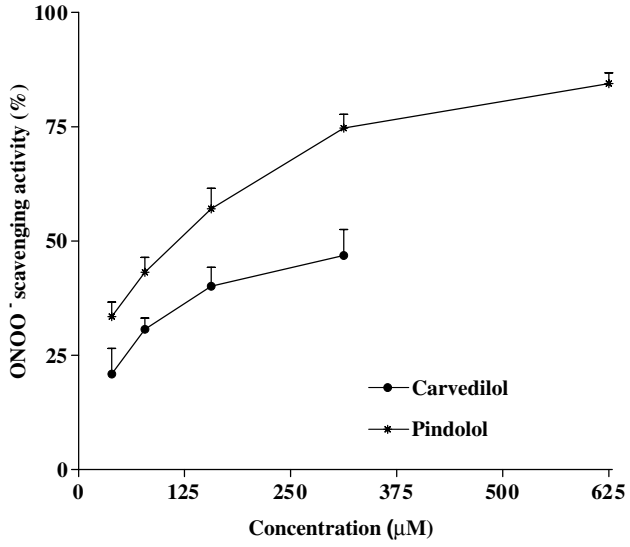
Figure 5. $ONOO^-$ scavenging activity of propranolol, metoprolol, and atenolol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

with cardiovascular diseases and to the improvement of blood pressure or endothelial function.[38,42,43] It is thus expected that a scavenging effect against ROS and RNS mediated by cardiovascular drugs has additional benefits far beyond their receptor-related activities as referred in Section 1.

The results described in the present study showed that none of the studied compounds were able to scavenge $O_2^{\cdot -}$, generated by the hypoxanthine/xanthine oxidase system. Other authors had already referred the inability of some β-blockers to scavenge $O_2^{\cdot -}$, using different methodologies,[6,7,19,44] while Yue et al.[45] have shown that carvedilol was capable of scavenging $O_2^{\cdot -}$, generated by metal catalyzed oxidation of dihydroxyfumaric acid (DHF). Tadolini and Franconi[44] suggested that this discrepancy of results might be related with the $O_2^{\cdot -}$ generation system used by Yue et al.,[45] considering that these authors did not study the influence of carvedilol on the rate of DHF oxidation. As mentioned before, metoprolol, timolol, sotalol, and carvedilol were shown to protect erythrocytes against PMS-produced $O_2^{\cdot -}$, within therapeutic concentrations, carvedilol being the most potent.[17] Nevertheless, erythrocyte SOD may rapidly convert $O_2^{\cdot -}$ into H_2O_2, with a possible subsequent conversion of this ROS into HO·. Labetalol was as well shown to inhibit $O_2^{\cdot -}$ production during normal leukocyte oxidative metabolism[21] but this effect may be mediated by a NADPH oxidase inhibition, a possible cause that needs to be confirmed in the future.

All the studied β-blockers were able to scavenge HO·, except carvedilol, which could not be tested by this methodology due to its low solubility in the tested system. However, it is noteworthy that other works refer carvedilol as a strong HO· scavenger,[44,46] a characteristic that has been considered important in the control of some of myocardial disorders involving oxidative-related damage.[47] It was also suggested that the HO· scavenging effect of carvedilol could explain its confirmed ability to inhibit lipid peroxidation.[46] Taking this rationale into account, it is possible that the HO· scavenging effects observed for the other β-blockers may also contribute for the prevention of cardiovascular diseases promoted by this species.

The studied compounds showed to be strong HOCl scavengers. It is well known that HOCl strongly reacts with amines.[48] Thus, it is possible that the secondary amine group, which is a common feature in the chemical structure of β-blockers, is responsible for their HOCl-scavenging effects. However, the confirmation of this hypothesis needs further structure–activity studies. HOCl can be produced in the cardiovascular system during an ischemia–reperfusion injury, by the enzyme myeloperoxidase present in activated neutrophils that infiltrate a reoxygenated tissue.[49,50] The ability of β-blockers to scavenge HOCl may contribute to avoid some of the damages associated with this cardiovascular disorder. In fact, Aruoma[51] had already focused this aspect based on his own study in which carvedilol presented a strong HOCl-scavenging effect.



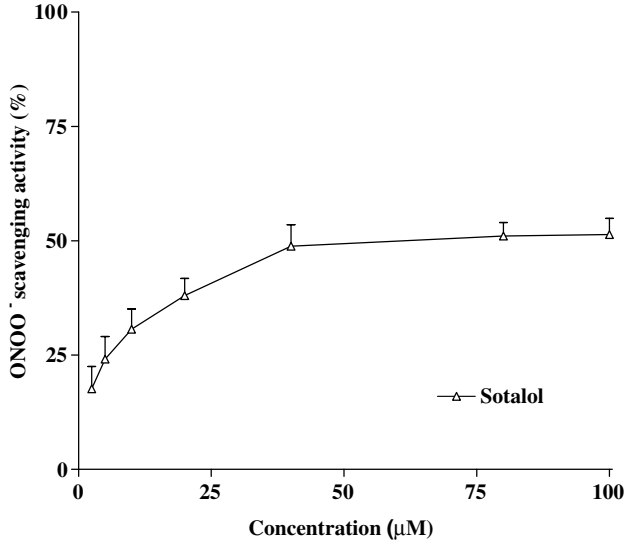


Figure 6. $ONOO^-$ scavenging activity of carvedilol, pindolol, and sotalol. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

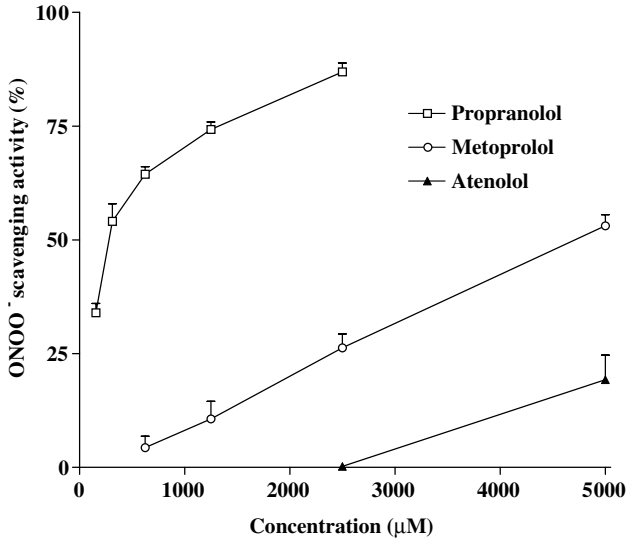


Figure 7. $ONOO^-$ scavenging activity of propranolol, metoprolol, and atenolol in the presence of 25 mM $NaHCO_3$. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

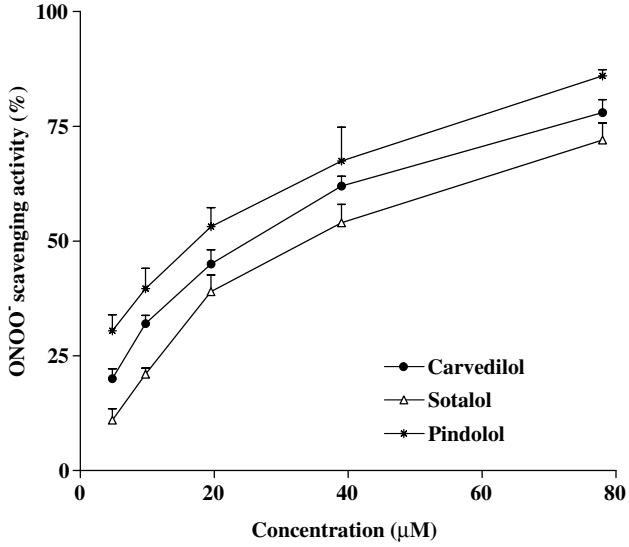


Figure 8. $ONOO^-$ scavenging activity of carvedilol, pindolol, and sotalol, in the presence of 25 mM $NaHCO_3$. Each point represents the values obtained from four experiments, performed in triplicate (mean ± SEM).

In the ORAC assay, propranolol and pindolol showed to be $ROO^{\bullet}$ scavengers, providing higher ORAC values than the endogenous antioxidants GSH and ascorbic acid. $ROO^{\bullet}$ is directly involved in the propagation phase of lipid peroxidation, which results from the oxidation of polyunsaturated fatty acids (PUFA) by highly reactive species such as $HO^{\bullet}$ or $ONOO^-$.[52,53] The low-density lipoproteins (LDL) present in the vasculature can be a target for lipid peroxidation, resulting in oxidized LDL particles which are captured by macrophages originating the 'foam cells.' This is the first step for the formation of atherosclerotic plaques, which, in an advanced stage, can result in circulatory obstruction.[54] The ability of β-blockers to prevent lipid peroxidation has already been evaluated by other investigators through different in vitro assays. In fact, some authors refer that compounds like pindolol, propranolol or carvedilol are capable to inhibit lipid peroxidation,[3,6,19,44] although the results seem to depend on the type of methodology used. While carvedilol's effect on lipid peroxidation is probably related to its capacity to chelate iron,[44] pindolol and propranolol seem to exert their effects by scavenging $ROO^{\bullet}$ or alkyl radicals ($R^{\bullet}$). Nevertheless, it is important to consider that both compounds are lipophilic and might accumulate within membranes, which may allow them to exert an antioxidant effect in vivo.[6]

From the studied compounds, only pindolol, carvedilol, atenolol, and propranolol were able to scavenge $^{\bullet}NO$. The low $^{\bullet}NO$ scavenging activity of the other tested β-blockers can be seen as an advantage because these compounds act in the cardiovascular system where

·NO plays an important role as vasodilator and inhibitor of platelet aggregation.[31] Nevertheless, the overproduction of ·NO has been involved in certain pathological conditions including, endotoxin shock, inflammation, and several neurodegenerative disorders such as, brain ischemia, Alzheimer's disease, and Parkinson's disease.[31,55,56] Thus, in these cases, the ·NO scavenging activity might be of some therapeutic value.

Concerning $ONOO^-$, all the tested compounds, except timolol and labetalol, showed scavenging activity. In the presence of $NaHCO_3$ 25 mM, the $ONOO^-$ scavenging effects of the compounds increased, except for atenolol whose activity decreased at these conditions. It has been reported that physiological concentrations of CO_2 can modulate $ONOO^-$ reactivity due to the fast reaction between these two compounds, yielding $^{\cdot}NO_2$ and $CO_3^{\cdot-}$, which are the main responsible radicals for the nitration and oxidation reactions that are usually observed in vivo.[57] Thus, a scavenger can directly trap $ONOO^-$ only if it reacts faster with the later than does CO_2.[58] On the other hand, a putative scavenging effect on $^{\cdot}NO_2$ and $CO_3^{\cdot-}$ may increase the compound's efficiency.[20] This may be the explanation for the higher scavenging effect shown by some of the studied β-blockers in the presence of CO_2. The potent $ONOO^-$ scavenging effects provided essentially by pindolol, sotalol, and carvedilol may be useful in cardiovascular pathologies like atherosclerosis or the ischemia–reperfusion injury in which the involvement of this species has already been recognized.[37,59–61]

The potential value of pindolol antioxidant effects in other pathologies affecting CNS was also previously reported. Indeed, it was pointed out that the ·NO and $ONOO^-$ scavenging activities of pindolol may contribute for enhancing and/or accelerating selective serotonin specific reuptake inhibitors (SSRI)-induced antidepressant (AD) effect 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.[20]

The scavenging effects presented by the tested compounds occurred at higher concentrations than those reached in plasma following therapeutic doses. Nevertheless, as mentioned above, several of the tested compounds have already been established as excellent antioxidants. Lipophilicity is a major determinant of several aspects of the disposition and biological action of drugs. Indeed, the lipophilicity of some of the tested compounds may also explain their efficacy in protecting membranes against peroxidation, as it happens with propranolol, which accumulates within membranes.[62,63] It is also of note that the scavenging efficacy of antioxidants depends on the amount of reactive species being produced, which is normally higher in vitro than in vivo.[20,64] Indeed, 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 verify that, when the levels of the reactive species are lower, the IC_{50} of the scavenger species is decreased.[64] Consequently, the levels of scavengers that are needed in vitro are higher than those needed for an effective activity in vivo, as it happens with carvedilol and propranolol.

In conclusion, the results obtained in this work show that some of the studied compounds are good ROS and/or RNS scavengers, these effects being possibly useful in preventing the oxidative damages verified in hypertension as well as in other cardiovascular diseases that frequently emerge in association with oxidative stress.

4. Materials and methods

4.1. Chemicals

All the chemicals and reagents were of analytical grade. Dihydrorhodamine 123 (DHR 123), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, ethylenediaminetetraacetic acid disodium salt (EDTA), ascorbic acid, sodium hypochlorite solution, with 4% available chlorine, hypoxanthine, xanthine oxidase grade I from buttermilk (EC 1.1.3.22), superoxide dismutase (SOD) (EC 1.15.1.1), catalase from bovine liver (EC 1.11.1.6), lipoic acid, diethylenetriaminepentaacetic acid (DTPA), reduced glutathione (GSH), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), rutin, lucigenin, luminol, desferrioxamine, ebselen, and the β-blockers, except carvedilol, were obtained from Sigma–Aldrich (St. Louis, USA). α,α′-Azodiisobutyramidine dihydrochloride (AAPH), melatonin, and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt and tiron were obtained from Aldrich (Milwaukee, USA). Carvedilol was an offer from Roche Diagnostics GmbH. All the other reagents were obtained from Merck (Darmstadt, Germany).

Taking into account the low water solubility of some of the tested compounds, organic solvents had to be used in several assays. The choice of solvents was made case by case, depending on the reactive species to be tested since in some cases the solvent gives strong interferences, usually by scavenging the reactive species under assay. For example, DMSO is a strong scavenger of HOCl and $HO^{\cdot}$, and ethanol is a strong inhibitor of $HO^{\cdot}$. Thus, it is not possible to use the same organic solvent for all tested compounds. Another encountered problem in some assays was the insolubilization verified in the reactional mixture for the concentrations used.

4.2. Equipment

A microplate reader (Synergy HT, BIO-TEK), with spectrophotometric, fluorimetric, and chemiluminometric detection, was used for all the scavenging assays for ROS and RNS.

4.3. ROS and RNS scavenging assays

4.3.1. Superoxide radical scavenging assay. The $O_2^{\cdot-}$ scavenging activity was measured by monitoring the $O_2^{\cdot-}$-induced oxidation of lucigenin, using a previously

described chemiluminescence methodology.[65,66] $O_2^{\cdot-}$ was generated by a hypoxanthine/xanthine oxidase system. Reaction mixtures contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 0.5 M carbonate buffer, pH 10, lucigenin (154 μM), hypoxanthine (0.4 mM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in DMSO and the other β-blockers in 0.5 M carbonate buffer, pH 10), and xanthine oxidase (150 mU/mL), dissolved in a 10 mM phosphate buffer, pH 8. The assays were performed at 37 °C. The chemiluminescence signal was monitored for 5 min, using the microplate reader. Tiron was used as positive control. The effects were expressed as the percentual inhibition of the $O_2^{\cdot-}$-induced oxidation of lucigenin. Each study corresponds to four experiments, performed in triplicate.

4.3.2. Hydrogen peroxide scavenging assay. The H_2O_2 scavenging activity was measured by monitoring the H_2O_2-induced oxidation of lucigenin, using a previously described chemiluminescence methodology,[67] with modifications. Reaction mixtures contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 50 mM Tris–HCl buffer, pH 7.4, lucigenin (1.6 mM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in DMSO and the other β-blockers in 50 mM Tris–HCl buffer, pH 7.4), and 2% H_2O_2. The assays were performed at 37 °C. The chemiluminescence signal was detected, after a 10 min incubation period, using the microplate reader. The endogenous antioxidant ascorbic acid was also assayed under the same conditions, for comparison. Catalase was used as positive control. The effects were expressed as the percentual inhibition of the H_2O_2-induced lucigenin's oxidation. Each study corresponds to four experiments, performed in triplicate.

4.3.3. Hydroxyl radical scavenging assay. The $HO^{\cdot}$ scavenging activity was measured by monitoring the $HO^{\cdot}$-induced oxidation of luminol, using a previously described chemiluminescence methodology,[68] with modifications. $HO^{\cdot}$ was generated by a Fenton system ($FeCl_2$–EDTA–H_2O_2). Reaction mixtures in the sample wells contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 0.5 M carbonate buffer, pH 10, luminol (20 μM), $FeCl_2$–EDTA (25 and 100 μM), the β-blockers at various concentrations (labetalol, propranolol, and pindolol were dissolved in HCl 0.1 M, while metoprolol, sotalol, and timolol were dissolved in 0.5 M carbonate buffer, pH 10) and H_2O_2 (3.5 mM). The assays were performed at 37 °C. The chemiluminescence signal was detected, after a 5 min incubation period, using the microplate reader. Melatonin was used as positive control. The effects are expressed as the percentual inhibition of the $HO^{\cdot}$-induced oxidation of luminol. Each study corresponds to four experiments, performed in triplicate.

4.3.4. Hypochlorous acid scavenging assay. The HOCl scavenging activity was measured by monitoring the HOCl-induced oxidation of luminol, using a previously described chemiluminescence methodology,[69] with modifications. HOCl was prepared immediately before use by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with dropwise addition of 10% H_2SO_4. The concentration of HOCl was further determined spectrophotometrically at 235 nm using the molar absorption coefficient of 100 $M^{-1}\,cm^{-1}$.[51] Reaction mixtures in the sample wells contained, in a final volume of 250 μL, the following reagents at the indicated final concentrations: 50 mM phosphate buffer, pH 12, luminol (250 μM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in ethanol and the other β-blockers in 50 mM phosphate buffer, pH 12), and HOCl (25 μM). The assays were performed at 37 °C. The chemiluminescence signal was detected after the HOCl addition using the microplate reader. Lipoic acid was used as positive control. The effects are expressed as the percentual inhibition of the HOCl-induced oxidation of luminol. Each study corresponds to four experiments, performed in triplicate.

4.3.5. Peroxyl radical scavenging assay. $ROO^{\cdot}$ scavenging activity was measured by monitoring the fluorescence decay in result of $ROO^{\cdot}$-induced oxidation of fluorescein, according to a described procedure[70] known as oxygen radical absorbance capacity (ORAC) assay. $ROO^{\cdot}$ was generated by thermodecomposition of AAPH. Reaction mixtures in the sample wells contained, in a final volume of 200 μL, the following reagents at the indicated final concentrations: 75 mM phosphate buffer, pH 7.4, fluorescein (61 nM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in DMSO and the other β-blockers in 75 mM phosphate buffer, pH 7.4), and AAPH (19 mM). The assays were performed at 37 °C. The fluorescence signal was monitored every minute at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm, using the microplate reader, until the total decay of fluorescence. GSH and ascorbic acid were used as positive controls. The scavenging effects are expressed as the relative trolox equivalent ORAC value, which is calculated by the following equation, where AUC represents the area under curve:

$$\begin{aligned}\text{Relative ORAC value} = {} & [(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}) \\ & /(\text{AUC}_{\text{trolox}} - \text{AUC}_{\text{blank}})] \\ & \times (\text{moles of trolox} \\ & /\text{moles of sample}).\end{aligned}$$

Each study corresponds to four experiments, performed in triplicate.

4.3.6. Nitric oxide scavenging assay. The $^{\cdot}NO$ scavenging activity was measured by monitoring the $^{\cdot}NO$-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), according to a described procedure.[20] $^{\cdot}NO$ was generated by NOC-5. A stock solution of 2.76 mM DAF-2 in DMSO was purged with nitrogen and stored at −20 °C. Working solutions of DAF-2 diluted with the buffer (1.3 mM NaH_2PO_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 in the sample wells contained, in a final volume of 300 μL, the following reagents at the indicated final concentrations: DAF-2 (5 μM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in DMSO and the other β-blockers in the buffer solution described above), and NOC-5 (10 μM). The assays were performed at 37 °C. The fluorescence signal was detected after a 30 min incubation period at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm, using the microplate reader. Rutin was used as positive control. The effects are expressed as the percentual inhibition of ·NO-induced oxidation of DAF-2. Each study corresponds to four experiments, performed in triplicate.

4.3.7. Peroxynitrite scavenging assay. The $ONOO^-$ scavenging activity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR 123 to fluorescent rhodamine 123, according to a described procedure.[20] $ONOO^-$ was synthesized as described before.[20] Briefly, an acidic solution (HCl 0.7 M) of H_2O_2 0.6 M was mixed with $NaNO_2$ (0.66 M) in a Y junction and the reaction was quenched with ice-cold NaOH 3 M. Residual H_2O_2 was removed by mixing with granular MnO_2 pre-washed with NaOH 3 M. The obtained $ONOO^-$ solution was filtered and then frozen (−80 °C). Prior to each experiment, the top layer of the stock solution was collected and the concentration of peroxynitrite was determined spectrophotometrically in 0.05 M NaOH ($\varepsilon_{302nm} = 1670\ M^{-1}\ cm^{-1}$). A stock solution of 2.89 mM DHR 123 in dimethylformamide was purged with nitrogen and stored at −20 °C. Working solutions of DHR 123 diluted with buffer (90 mM NaCl, 50 mM Na_3PO_4, and 5 mM KCl, pH 7.4 with HCl) from the stock solution were placed on ice in the dark immediately before the determinations. At the beginning of the experiments, 100 μM DTPA was added to the buffer. Reaction mixtures in the sample wells contained, in a final volume of 300 μL, the following reagents at the indicated final concentrations: DHR 123 (5 μM), the β-blockers at various concentrations (carvedilol, labetalol, propranolol, and pindolol were dissolved in DMSO and the other β-blockers in the buffer solution described above), and $ONOO^-$ (600 nM). The assays were performed at 37 °C. The fluorescence signal was detected after a 5 min incubation period at the emission wavelength 528 ± 20 nm with excitation at 485 ± 20 nm, using the microplate reader. Ebselen was used as positive control. In a parallel set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$ in order to simulate the physiological CO_2 concentrations in vivo. This evaluation is important because, under physiological conditions, the reaction of $ONOO^-$ with bicarbonate is predominant, with a very fast rate constant ($k_2 = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$).[71] The effects are expressed as the percentual inhibition of the $ONOO^-$-induced oxidation of DHR. Each study corresponds to four experiments, performed in triplicate.

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