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Original article

Arylnitroalkenes as scavengers of macrophage-generated oxidants

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ABSTRACT

Oxygen and nitrogen derived molecules mediated oxidation and nitration have been involved in several pathological conditions. Conversely, nitric oxide and hydrogen peroxide are important signalization intermediates, whose concentrations are tightly regulated by specialized enzyme repertoires and should remain undisturbed by the addition of exogenous antioxidant molecules, as already demonstrated by intervention studies with antioxidant vitamins. Our goal was to develop specific antioxidants able to scavenge peroxynitrite anion, as well the radicals derived from the homolytic decomposition of its conjugated acid, nitrogen dioxide and hydroxyl radical. Fourteen substituted nitroalkenes, seven 4-substituted 1-(2-nitro-1*Z*-ethenyl)benzene, and seven 4-substituted (2-nitro-1*Z*-propenyl)benzene, with different stereochemical and electronic characteristics were synthesized and tested. Compounds with the electron donor group *N*,*N*-dimethylamino showed the highest reaction rates against peroxynitrite, and also reacted with its homolytic decomposition products, •OH and $•NO_2$. While 1,1-dimethylamino-4-(2-nitro-1*Z*-ethenyl)benzene came up as a lead for future developments without the risk of interfering with signalization pathways, since it was highly specific for peroxynitrite and peroxynitrite derived radicals, its methylated analogous 1,1-dimethylamino-4-(2-nitro-1*Z*-propenyl)benzene was less specific and also reacted with •NO and $O_2•^-$, the biological precursor of H_2O_2.

1. Introduction

Reactive species (RS, it will be used in general to account for both oxygen and nitrogen species) are continuously produced by normal metabolism, and its deleterious effects on biological molecules are kept under control by an organized array of antioxidant defences. However, an imbalance in the normal relationship between oxidants and physiological antioxidants is in the basis of several pathologies.

Although macrophage respiratory burst with the consequent generation of RS is a key weapon against pathogens, if excessive or unresolved could cause organ dysfunction. In particular, peroxynitrite the product of the diffusion-controlled reaction between two radicals, nitric oxide and superoxide, is a relevant biological oxidant and a nitrating agent [1].

Several human diseases including rheumatoid arthritis [2], types 1 and 2 diabetes [3,4], cardiovascular disease [5–8], and metabolic syndrome [9], involve activation of the immune system and the associated oxidative stress as contributors to their etiology and progression. However, therapeutically targeting oxidative stress in various diseases has proven more problematic than first anticipated. Many natural and synthetic antioxidants have been tested as potential therapeutic tools with variable results [10]. Among them, nitro-fatty acids (NO_2-FA), that are electrophilic Michael acceptors, react with nucleophiles such as cysteine thiolate, imidazole ring of histidine and ε-amino group of lysine residues [11]. *In vitro* studies showed that NO_2—FAs inhibit neutrophil and platelet function, vascular smooth muscle cell proliferation,

Abbreviations: RS, reactive species; NO_2-FA, nitro-fatty acids; LPS, lipopolysaccharide; Fe^{2+}-Cyt c, Fe^{2+}-cytochrome c; Fe^{3+}-Cyt c, Fe^{3+}-cytochrome c; DHR, dihydrorhodamine; RH, rhodamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAF-FM, 4-amino-5-methylamino-2′,7′ difluorofluorescein diacetate; IFNγ, interferon-gamma; NOS, nitric oxide synthase; 1400W, *N*-(3-(aminomethyl)benzyl) acetamidine; MetHb, methemoglobin; Hb, hemoglobin; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide; HbO_2, oxyhemoglobin; DMEM, Dulbecco's modified Eagle medium; dPBS, Dulbecco's phosphate buffer saline; SOD, superoxide dismutase.

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expression of endothelial adhesion molecule, lipopolysaccharide (LPS)-induced macrophage activation and transmigration [12–14]. Additionally, we found that nitroalkene (**I**) (Fig. 1) was able to release •NO in the absence and the presence of cysteine at 37 °C [15].

Knowing that the biological activity of this nitroalkene was due to the nitroethenyl moiety, we developed a molecular simplification programme analyzing herein simpler nitroalkene derivatives. Consequently, we synthesized and tested different nitroethenyl derivatives containing substituents in the 4-position of the phenyl ring, with different stereoelectronic properties. In this kind of systems, the nitro group is distant from the aromatic ring and conjugated via the ethylene system, allowing long-distance transmission of electronic effects [16]. In addition, the bioisoster of the phenyl moiety, 2-thienyl group, was also studied (Fig. 1). The reactivity of these nitroalkenes as scavengers of different RS, peroxynitrite and its derivatives radicals, was assayed chemically. *In vitro* analysis in murine macrophages was performed to determine the capacity to scavenge peroxynitrite derived or precursor radicals.

2. Results

2.1. Chemistry

2.1.1. Synthesis

Fourteen nitroalkenes were obtained via Henry reaction. The process took place in one pot using the corresponding aromatic aldehyde and nitromethane or nitroethane in the presence of catalytic amounts of *n*-butylamine (Scheme 1) [15,17,18]. All nitroalkenes were characterized by ^{1}H NMR, COSY experiments, MS, and UV spectroscopy (Table 1). They were obtained as a unique isomer around the double bound. In all cases the *Z* isomer was obtained, this was determined by NOE experiments (see Supplementary Material). The purity of the synthesized compounds was established by TLC and microanalysis. Only compounds with analytical results for C, H and N, within ±0.4 of the theoretical values were considered pure enough.

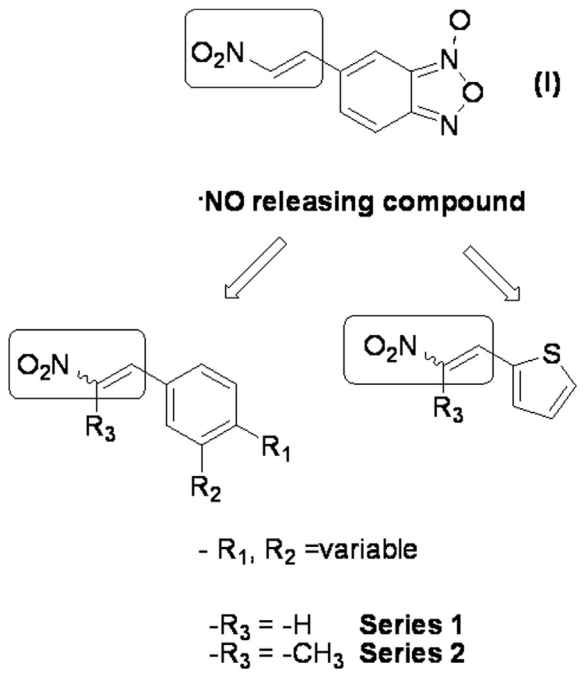

Fig. 1. Design of nitroalkenes based on benzofuroxan containing nitroethenyl moiety releasing •NO.

2.2. •NO release

The ability of the synthesized compounds to release •NO was addressed first measuring hemoglobin oxidation by 100 μM of the compounds. Exclusively nitroalkenes **1b**, **2b**, **1f** and **2f** released measurable amounts of •NO under our experimental conditions (Table 1). The rate constants of •NO release were similar to previously reported values for well known •NO donors [19], ranging from 0.06 to 0.25 × 10^{-3} min^{-1} for the *N*,*N*-dimethylamino derivatives (**1b** and **2b**), and from 0.48 to 1.32 × 10^{-3} min^{-1} for the [1,3]dioxole derivatives (**1f** and **2f**). •NO release was also demonstrated for nitroalkenes **1b**, **2b**, **1f** and **2f** with an electrochemical sensor, with similar results (Fig. 2).

2.3. Reactivity with peroxynitrite

2.3.1. Second order rate constant estimation with peroxynitrite

The reactivity of the studied nitroalkenes as antioxidants was analyzed by their reaction with peroxynitrite, an important biological oxidant that reacts by one or two electron mechanisms. Since the rate constant of Fe^{2+}-cytochrome c (Fe^{2+}-Cyt c) oxidation by peroxynitrite is already known ($k = 2.4 \times 10^4$ M^{-1} s^{-1} at pH 7.4, and 37 °C) [20] the magnitude of this oxidation can be easily determined at 550 nm, through competition experiments performed to determine the second order rate constant for the reaction between peroxynitrite and the panel of nitroalkenes. Given that pseudo-first order conditions were unattainable due to solubility limitations, nitroalkenes were used at the same concentration as Fe^{2+}-Cyt c (360 μM). Under those conditions it can be assumed that ~90% of peroxynitrite reacts directly with the targets instead of undergoing homolysis, which occurs with a rate constant of 0.9 s^{-1}. Since the concentration ratio between the Fe^{3+}-Cyt c generated in the absence and in the presence of the nitroalkene competitor is proportional to the relationship between the kinetic constants, the desired rate constant can be estimated using Eq. (1) [21,22].

$$\frac{k_1}{k_2} = \frac{\ln \frac{[Fe^{2+}-\text{Cyt c}]_0}{[Fe^{2+}-\text{Cyt c}]_0-[Fe^{3+}-\text{Cyt c}]_\infty}}{\ln \frac{[\text{Nitroalkene}]_0}{[\text{Nitroalkene}]_0-[P_{\text{nitroalkene}}]_\infty}} \quad (1)$$

where the concentration of $P_{\text{nitroalkene}}$ was calculated as the difference between Fe^{3+}-Cyt c concentration obtained after peroxynitrite exposure in the absence and in the presence of nitroalkene. The values obtained were ~10^3 M^{-1} s^{-1} (Table 2) one order of magnitude lower than the reported rate constant for the reaction between peroxynitrite and Fe^{2+}-Cyt c. The substituents at the 4-position of the aromatic ring increased in a different extent the reactivity of the compounds with peroxynitrite. In particular, the *N*,*N*-dimethylamino substituted derivatives, **1b** and **2b**, reacted with peroxynitrite more than 2 times faster than the unsubstituted ones, **1a** and **2a**. Regarding the relationship between the rate constants of **series 1** and **2**, except for **2f**, were equal or slightly lower in **series 1**. The kinetic constants obtained for the direct reaction of peroxynitrite with nitroalkenes were in the same order than with glutathione [23] but lower than the reaction with oxyhemoglobin [24] and the amino acid selenocysteine [25]. Higher reaction rates were described for other synthetic scavengers, like manganese porphyrins (10^5 to 10^7 M^{-1} s^{-1}) [26] and iron porphyrins (<10^7 M^{-1} s^{-1}) [27].

2.3.2. Reactivity with peroxynitrite derived radicals

Kinetic results showed that the fourteen studied nitroalkenes reacted directly with peroxynitrite, but in addition they might interact with the radicals derived from it, •NO_2 and •OH.