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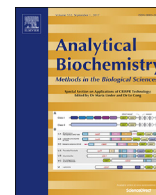

Dansylglycine, a fluorescent probe for specific determination of halogenating activity of myeloperoxidase and eosinophil peroxidase

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ABSTRACT

Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are enzymes present in neutrophil and eosinophil leukocytes, respectively. Here, we present the development of a sensitive and specific assay for determination of the halogenating enzymatic activity of MPO and EPO based on the electrophilic attack of HOCl and HOBr on aromatic ring of dansylglycine (DG). We found that the intrinsic fluorescence of DG was promptly depleted by the action of these acids. In the presence of the enzymes, the fluorescence bleaching was dependent of chloride (Cl^-) and bromide (Br^-), which makes the assay able to distinguish the halogenating from the peroxidase activity. A linear correlation was obtained between the hydrogen peroxide (H_2O_2) concentration and the fluorescent decay. Similarly, the enzyme activity was measured by keeping constant H_2O_2. The method was applied for studding MPO/EPO specific inhibitors as 5-fluortryptamine (reversible inhibitor) and 4-hydroxybenzhydrazide (irreversible inhibitor). Differently of the taurine chloramine/3,3′,5,5'-tetramethylbenzidine assay, which is among the most used technique, the dansylglycine assay was able to differentiate these inhibitors based on their kinetic behavior. In conclusion, this assay can differentiate the peroxidase and halogenating activity of MPO and EPO. Moreover, the method is adequate for real-time measurement of the production of HOCl and HOBr.

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Introduction

Myeloperoxidase (MPO) is an enzyme abundantly present in the azurophilic granules of neutrophils, the predominant leukocyte in the circulation [1]. MPO is engaged in the innate immune defense, acting against invading pathogens by catalyzing the oxidation of chloride (Cl^-) to hypochlorous acid (HOCl). This halogenating and oxidizing agent is a potent microbicidal compound, which is released into the phagosome and/or into the extracellular medium when neutrophils are challenged by bacteria, fungi, etc [1,2]. Similarly, eosinophilic leukocytes are endowed with the structurally related enzyme eosinophil peroxidase (EPO), which is devoted to catalyze the oxidation of bromide (Br^-) to hypobromous acid (HOBr) [3]. Differently of neutrophilia, which are involved in several inflammatory processes, eosinophilia is typically related to the pathogenesis of asthma, allergic and parasitic diseases [4].

The preceding paragraph describes an important and exclusive feature of MPO, because differently from others peroxidases, this enzyme is the unique able to catalyze efficiently at pH 7.0 the oxidation of Cl^- to HOCl [5]. It is noteworthy that peroxidases are usually described as “promiscuous“ enzymes in the sense of their lack of substrate specificity [6]. This is not different for MPO, which acts by two main pathways. One of them is the peroxidase cycle, a two-step oxidation mechanism involving the transient redox active forms compound I and II (MPO-I and MPO-II), which are responsible by the one-electron oxidation of a myriad of phenols and aromatic amines; the other mechanism of catalytic action is the chlorinating cycle, responsible by the two-electron oxidation of Cl^-, Br^- and SCN^- to the respective HOCl, HOBr and HOSCN [5,7,8]. The following chemical reactions describe the peroxidase (1–3) and chlorinating (1 and 4) catalytic cycles of MPO:

$$MPO + H_2O_2 \rightarrow MPO(I) + H_2O \quad (1)$$

$$MPO(I) + AH \rightarrow MPO(II) + A^o \quad (2)$$

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$$MPO(II) + AH \rightarrow MPO + A^{o} \quad (3)$$

$$MPO(I) + Cl^{-}/Br^{-}/SCN^{-} \rightarrow MPO + HOCl/HOBr/HOSCN \quad (4)$$

Where AH and A represent phenols and aromatic amines in its reduced and oxidized forms, respectively.

Besides their roles in the innate immune defense, MPO and EPO are also involved in deleterious processes related to inflammatory, allergic and degenerative diseases [9–12]. This is due to the capacity of HOCl and HOBr as oxidants of several cell constituents leading to their degradation and loss of function [13]. For this reason, to inhibit the halogenating activity of these enzymes is a current hot topic in biomedical research [14,15].

To study the inhibition of these enzymes is necessary analytical procedures able to differentiate peroxidase and halogenating activities of MPO and EPO. In this regard, one of the first substrate used for this propose was the β-diketone, monochlorodimedon. The enol form of monochlorodimedon reacts with HOCl to produce dichlorodimedon and the reaction can be monitored by the absorbance loss at 290 nm [16]. However, this method lacks specificity, since the enol form is also able to react through peroxidase pathway [17]. Other widely used method for measurement of chlorinating activity of MPO is a two steps procedure by which the formation of HOCl is captured by taurine leading to the formation of taurine chloramine. This is a mild and stable oxidizing agent whose concentration is measured in the second step of the method by the oxidation of the aromatic thiol, 5-thio-2-nitrobenzoic acid (TNB) [17] or 3,3′,5,5'-tetramethylbenzidine (TMB) at acid pH [18]. These methods are selective to the chlorinating activity of MPO. Particularly, they are largely used for determination of HOCl produced by purified MPO and isolated neutrophil [18]. However, they are not adequate for determination of intracellular production of HOCl, since taurine chloramine could be depleted by reacting with sulfhydryl moieties inside the cells. Moreover, the chromogenic substrates TNB and TMB are susceptible to oxidation by others reactive oxygen species. Therefore, fluorescent probes have been developed for specific intracellular determination of HOCl, which have potential application in flow cytometry-based assays and imaging studies. They are based on xanthene dyes, which became fluorescent upon reacting with HOCl. Among them, 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (HPF) and 2-[6-(4′-amino)phenoxy-3H-xanthen-3-on-9-yl]benzoic acid (APF) have been proposed. The authors showed that both probes can be oxidized by hydroxyl radical, but only HOCl is able to react with APF [19]. More recently, AFP was used to detect the intracellular production of HOCl in neutrophils [20] and HOBr produced by eosinophils using flow cytometry [21]. Another successful method is based on HOCl-promoted cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles. In this technique, the probe shows an emission at 473 nm, which is typical of coumarin moiety. Then, upon addition of HOCl, a cyclization takes place and through resonance energy transfer, the probe starts to emit as a rhodamine at 594 nm [22]. Considering the importance of selective determination of HOCl and HOBr, we had worked in a new analytical approach for determination of halogenating activity of MPO/EPO. Here, the selectivity was based on the electrophilic reactivity of these acids with aromatic compounds. We will show that the fluorescent probe dansylglycine can be used to distinguish the peroxidase and halogenating activities of MPO and EPO.

Materials and methods

Chemicals and reagents

Dansylglycine, taurine, 3,3′,5,5'-tetramethylbenzidine (TMB), 5-fluorotryptamine, 4-hydroxybenzhydrazide, horseradish peroxidase (HRP) and catalase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). MPO and EPO (EC 1.11.1.7) were purchased from Planta Natural Products (Vienna, Austria) and their concentrations were determined from their absorption at 430 nm (ε = 89,000 mol^{-1} L cm^{-1} per heme) and 413 nm (ε = 110,000 mol^{-1} L cm^{-1}) for MPO and EPO, respectively [23,24]. Hydrogen peroxide (H_2O_2) was prepared by diluting a 30% stock solution purchased from Peroxides do Brasil (Sao Paulo, SP, Brazil), and its concentration was calculated using its absorption at 240 nm (ε = 43.6 mol^{-1} L cm^{-1}) [25]. HOCl was prepared by diluting a 5% stock solution and the concentration was determined spectrophotometrically after dilution in 0.01 mol L^{-1} NaOH, pH 12 at 292 nm (ε = 350 mol^{-1} L cm^{-1}) [26]. HOBr was prepared by combining 100 mmol L^{-1} HOCl and 200 mmol L^{-1} NaBr in water [27]. TMB solution (14 mmol L^{-1}) was prepared by dissolving the adequate mass in 1:1 dimethylformamide and 400 mmol L^{-1} acetic acid. To this solution, 100 μmol L^{-1} potassium iodide was added and the reagent immediately used [28]. Stock solution of dansylglycine (2.5 mmol L^{-1}) was prepared by dissolving the adequate mass in 0.01 mol L^{-1} hydrochloric acid. Phosphate buffered saline (PBS) was prepared as follow: phosphate 10 mmol L^{-1}, NaCl 138 mmol L^{-1}, KCl 2.7 mmol L^{-1} and the pH adjusted to 7.4. All solutions were prepared with water purified by a Milli-Q system (Millipore, Bedford, MA, USA). All reagents used for buffer preparation were of analytical grade.

HOCl-promoted halogenation of dansylglycine

The study of HOCl-promoted halogenation of dansylglycine was performed by stopped-flow and conventional spectrofluorimetry. The fast-kinetic experiments were performed using a single-mixing stopped-flow system equipped with a high intensity LED source (360 nm) and cut-off filter (475 nm) (SX20/LED Stopped-Flow System, Applied Photophysics, UK). The relative rate constants were obtained by fitting the fluorescence bleaching to a single exponential decay equation, as follows:

$$F = F_0{*}\, e^{-k{*}\, t}$$

Where F is the fluorescence at any time and F_0 is the initial fluorescence. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} HOCl and increasing concentrations of Br^{-} in PBS, pH 7.4 at 25 °C.

The consumption of dansylglycine was also measured using conventional spectrofluorimetry. In this case, the experiments were adapted to microplate assay using a black 96-well flat bottom microplate. The multi-detector microplate reader (Synergy 2 Multi-Mode, BioTek, USA) was set for spectral scanning analysis (excitation at 340 nm and emission in the range of 400–650 nm) or endpoint reading (excitation at 340 nm and emission at 525 nm). Otherwise stated, the reaction medium was constituted of 50 μmol L^{-1} dansylglycine, 1 mmol L^{-1} Br^{-} and increasing concentrations of HOCl in PBS, pH 7.4 at 25 °C. The reactions were initiated by adding HOCl and incubated for 5 min. The final reaction volume was 250 μL.

MPO and EPO catalyzed bromination of dansylglycine

Unless otherwise stated, the reaction medium was composed of 20 nmol L^{-1} MPO or EPO, 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2, and 10 mmol L^{-1} Br^- in PBS, pH 7.4 at 25 °C. For the endpoint experiments the fluorescence was measured 30 min after the addition of H_2O_2. The final reaction volume was 250 μL and the microplate reader was set at 340/525 nm. For determination of MPO activity, the enzyme concentration was reduced to 4–20 nmol L^{-1} and the time-dependent fluorescence bleaching measured in the linear part of the curve. The microplate reader was set to acquire the fluorescence intensity at 1 min intervals. The calibration curves were raised and the limit of detection (LOD) calculated according to the formula: LOD = 3.3 *Sy/S*. Where *Sy* is the standard deviation of the response (y-intercepts of the regression line) and *S* is the slope of the calibration curve.

MPO chlorination activity by taurine/TMB assay

The chlorination activity of MPO was determined based on the reaction of HOCl with taurine to produce taurine chloramine and subsequent determination of this oxidant using the TMB reagent [28]. The reaction medium was composed of 20 or 80 nmol L^{-1} MPO, 100 μmol L^{-1} H_2O_2, 10 mmol L^{-1} taurine in PBS, pH 7.4 at 25 °C. The final volume was 200 μL. The reactions were initiated by the addition of H_2O_2 and incubated for 30 min. Then, 0.1 mg mL^{-1} catalase was added to stop the reaction, and after 5 min, 50 μL of the TMB reagent was added and the absorbance measured at 650 nm.

HPLC and mass spectrometry

The reaction medium was composed of 1 mmol L^{-1} dansylglycine, 1 mmol L^{-1} HOCl, 10 mmol L^{-1} Br^- in PBS, pH 7.4 at 25 °C. The reactions were conducted for 5 min. The consumption of dansylglycine and formation of products was chromatographically evaluated by HPLC. The analyses were carried out isocratically on a Luna C18 reversed-phase column (250 mm × 4.6 mm, 4 μm), using 0.1% formic acid in water and 0.1% formic acid in acetonitrile (95:5, v:v) as mobile phase and flow rate of 1.0 mL min^{-1} (Jasco, Easton, MD, USA). The high resolution mass spectra (HRMS) analysis were obtained in a micrOTOF-Q II-Bruker spectrometer (Bruker, Massachusetts, EUA).

Electrochemical studies

The H_2O_2 consumption was monitored amperometrically with a H_2O_2-selective electrode coupled to a TBR4100 Free Radical Analyzer (World Precision Instruments, Sarasota, FL, USA). Two reaction system were studied: (1) taurine 10 mmol L^{-1}, H_2O_2 100 μmol L^{-1}, MPO 20 nmol L^{-1} in PBS, pH 7.4 at 25 °C or (2) dansylglycine 50 μmol L^{-1}, Br^- 10 mmol L^{-1}, H_2O_2 100 μmol L^{-1}, MPO 20 nmol L^{-1} in PBS, pH 7.4 at 25 °C. The reactions were triggered by H_2O_2.

Results and discussion

Reaction with HOCl and the effect of Br^-

Dansylglycine is a fluorescent compound used as a site 2 specific ligand of serum albumin [29]. Recently, we demonstrated that dansylglycine reacts promptly with HOBr. In addition, we showed that its apparent second-order rate constant with HOBr (7.3×10^6 mol^{-1} L s^{-1}) was three order of magnitude higher compared to HOCl (5.2×10^2 mol^{-1} L s^{-1}) [30]. Considering that HOCl (E° = 1.28 V) is a stronger oxidant compared to HOBr (E° = 1.13 V) [31]; the higher reactivity of HOBr is an indication that the susceptibility to electrophilic attack, but not to oxidation, must be the major reactional feature of dansylglycine in the context of its reaction with these acids. In agreement, the higher electrophilicity of HOBr compared to HOCl was also demonstrated for others reactions with aromatic compounds as salicylic acid, anisole, acetophenone, uracil and uridine [30]. These finding and the absence free anilinic, enolic or phenolic groups in the molecular structure of dansylglycine leaded us to consider that this fluorescent probe would not be susceptible to direct peroxidase catalytic cycle of MPO. On the other hand, it would be susceptible to the products of the halogenating activity of MPO.

Although the reactivity of dansylglycine with HOBr is significantly higher compared to HOCl, the last is the main halogenating species produced physiologically through the catalytic action of MPO [1,2,5]. Therefore, the first step in this study was to improve the reactivity of dansylglycine with HOCl, which was accomplished by adding the Br^- into the reaction medium. The results in Fig. 1a show the effect of addition of Br^- in the rate of fluorescence bleaching of dansylglycine provoked by HOCl. From these experiments, the relative rate constants were measured and the following values were obtained: 0.148 s^{-1} (Br^- 0 μmol^{-1} L), 0.396 s^{-1} (Br^- 12.5 μmol^{-1} L), 0.604 s^{-1} (Br^- 25 μmol^{-1} L), 1.13 s^{-1} (Br^- 50 μmol^{-1} L) and 2.47 s^{-1} (Br^- 100 μmol^{-1} L). These results confirmed our expectation and can be explained taking into account the formation of HOBr by the reaction between Br^- and HOCl. These results were obtained using fast kinetic experiments, therefore not adequate for conventional microplate-based assays. Thus, we also measured the effect of Br^- in a higher time scale experiment and adapted to microplates (endpoint experiments). The results depicted in Fig. 1b shows the fluorescence bleaching measured after five minutes and the effect of addition of Br^-. A significant increase in the consumption of dansylglycine was obtained using 1.0 mmol L^{-1} of Br^-, a concentration that was used in

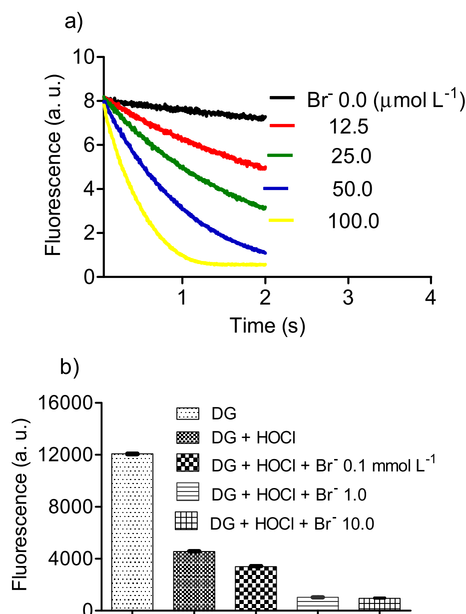

Fig. 1. Reactivity of dansylglycine (DG) with HOCl and the effect of Br^-. (a) Kinetic profile of dansylglycine consumption. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} HOCl and increasing concentrations of Br^- in PBS, pH 7.4 at 25 °C. The stopped-flow system was set as follows: excitation 340 nm LED and emission 475 nm cut-off filter. **(b)** Effect of Br^-. In this case the remaining fluorescence was measured after 5 min of the addition of HOCl (endpoint experiment). The results are the mean and SD of three experiments.

the subsequent experiments with HOCl.

In the sequence, we evaluated how sensitive was the fluorescence bleaching of dansylglycine regarding the concentration of HOCl. In these studies, HOCl was varied from 0 to 100 μmol L^{-1}, a range typically found in studies using MPO and HOCl [32–34]. The results depicted in Fig. 2 show the excellent correlation between the HOCl concentration and fluorescence bleaching of dansylglycine. The data were plotted as the variation of fluorescence ($FLuor_{Initial}$ − $Fluor_{final}$) as a function of concentration of HOCl. The detection limit was 2.3 μM (measured as LOD = 3.3 *Sy/S*).

Identification of the reaction products

As we have stated above, the chemical principle for the application of dansylglycine as a potential substrate for measurement of halogenating activity of MPO and EPO is based on its susceptibility to electrophilic attack of HOBr/HOCl (Fig. 3). This hypothesis was confirmed by measuring the exact mass of the reaction products. The mass spectrum of the reaction product confirmed the formation of monobrominated derivative of dansylglycine, as concluded by comparison with the reactant mass spectrum, examination of the exact mass of the molecular ion and by the expected isotopic distribution due to the presence of bromine atom ([M-H]: 384.9933 (51%) and 386.9895 (49%) (Supplementary Material).

Reaction of dansylglycine with $MPO/H_2O_2/Cl^-/Br^-$

So far, we have demonstrated the susceptibility of dansylglycine to electrophilic attack of $HOCl/Br^-$. The next step was to study the efficiency of the reaction using MPO as the source of HOCl. Initially, the role of Br^- in the reaction efficiency was evaluated. In these experiments, the concentrations of the components were MPO (20 nmol L^{-1}), H_2O_2 (100 μmol L^{-1}), Cl^- (PBS, 140 mmol L^{-1}) and Br^- varied. The results depicted in Fig. 4a show that dansylglycine also reacted with the HOCl generated by the enzymatic system in the presence of Br^-. Compared to the experiments with pure $HOCl/Br^-$, the reaction rate was lower; therefore, the consumption of

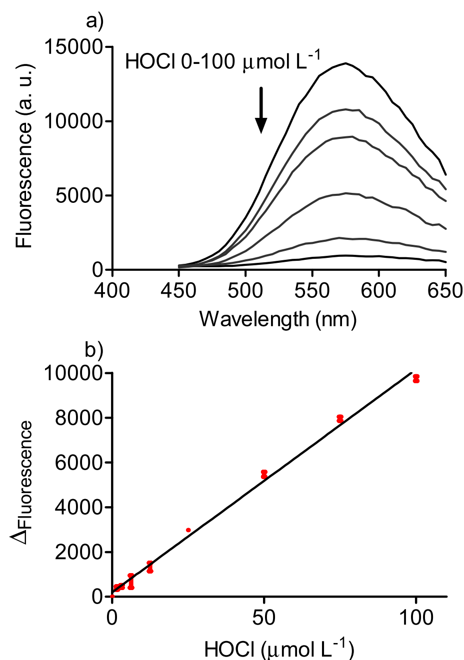

Fig. 2. Determination of HOCl by its reactivity with dansylglycine. **(a)** Fluorescence spectra of dansylglycine measured 5 min after addition of HOCl (0, 12.5, 25.0, 50.0, 75.0 and 100 μmol L^{-1}). **(b)** Analytical curve $\Delta_{fluorescence}$ versus HOCl concentration. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 1 mmol L^{-1} Br^- and increasing concentrations of HOCl in PBS, pH 7.4 at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments, r^2 = 0.9944, *LOD* = 2.3 μmol L^{-1}.

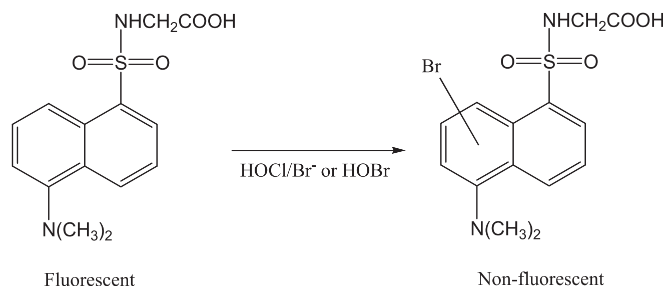

Fig. 3. Chemical equation for the monobromination of dansylglycine.

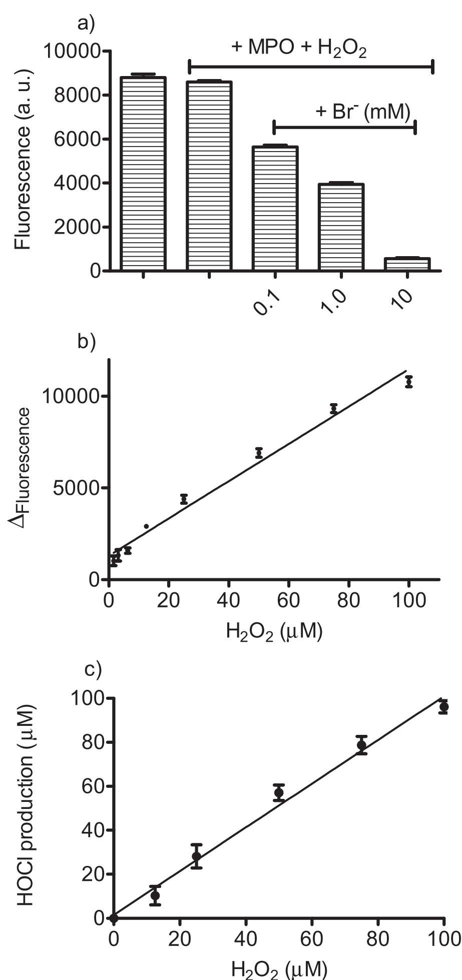

Fig. 4. Determination of HOCl produced by MPO/H_2O_2 using the dansylglycine method. **(a)** Dansylglycine fluorescence measured 30 min after addition of H_2O_2 in the presence or absence of Br^-. The reaction mixture was composed of 20 nmol L^{-1} MPO, 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2 and the indicated concentration of Br^- in PBS, pH 7.4 at 25 °C **(b)** Analytical curve $\Delta_{fluorescence}$ versus H_2O_2 concentration. The reaction mixture was composed of 20 nmol L^{-1} MPO, 50 μmol L^{-1} dansylglycine, 10 mmol L^{-1} Br^- in PBS and increasing H_2O_2, pH 7.4 at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments, r^2 = 0.9782, *LOD* = 5.5 μM. C **(c)** Relationship between HOCl produced versus H_2O_2 added in the enzymatic reaction medium.

dansylglycine was measured after 30 min of incubation at 37 °C. Obviously, the lower reaction rate can be explained considering the necessity of initial formation of HOCl by the MPO/H_2O_2 system. Our results also show that 10 mmol L^{-1} of Br^- was enough to provoke the complete consumption of dansylglycine after 30 min. From these results, it is reasonable to suppose that a relationship between the concentration of H_2O_2 and the fluorescence bleaching of dansylglycine should be also obtained by keeping constant the other components of the reaction. The results show in Fig. 4b confirmed our expectation, since a linear correlation was obtained for H_2O_2 versus Δfluorescence. The detection limit for H_2O_2 was 5.5 mmol L^{-1}. Finally, a linear correlation between the concentration of H_2O_2 and HOCl was also obtained (Fig. 4c). In these experiments, the equation obtained from the linear regression between the concentration of pure HOCl and Δfluorescence of dansylglycine was used to calculate the production of HOCl by MPO as a function of H_2O_2. In short, these results show that in the presence of dansylglycine and Br^-, the conversion of H_2O_2 to HOCl through catalytic action of MPO was quantitative and a stoichiometry of 1:1 (H_2O_2:HOCl) was obtained.

The dansylglycine assay was compared with the well-stablished and widely used assay based in the capture of HOCl by taurine

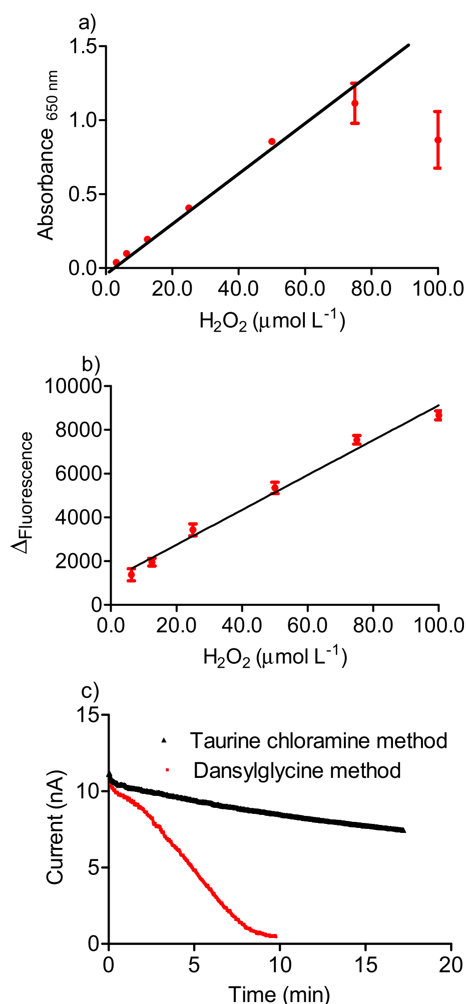

Fig. 5. Comparison of methodologies: Taurine/TMB versus dansylglycine. The reaction mixtures were composed of 5 nmol L^{-1} MPO, H_2O_2 in the range 0–100 μmol L^{-1} and (**a**) 10 mmol L^{-1} taurine or (**b**) 50 μmol L^{-1} dansylglycine in PBS, pH 7.4 at 25 °C. The results are the mean and SD of three experiments. (**c**) Amperometric monitoring the consumption of H_2O_2 by selective electrode.

leading to accumulation of taurine chloramine and its measurement by oxidation of the chromogenic substrate TMB [18]. The results depicted in Fig. 5a confirmed the efficacy of this methodology, but also showed that using only 5 nmol L^{-1} MPO, the enzyme seems to be partially inactivated, what could explain the lost of curve linearity above 70 μmol L^{-1} H_2O_2. These results are not unexpected, since the denaturation of MPO by H_2O_2 is a well-documented subject [35]. By comparing the results of the taurine/TMB assay with those obtained using dansylglycine (Fig. 5a and b), we can conclude that the methodology in development was less susceptible to MPO degradation. An explanation for these findings is the high reactivity of HOBr with dansylglycine, which promotes a fast depletion of this oxidant avoiding the degradation of the enzyme. It addition, the second-order rate constant for the reaction between MPO-I and Br^- leading to HOBr (1.1×10^6 mol^{-1} L s^{-1}) is significantly higher compared to Cl^- leading to HOCl (2.5×10^4 mol^{-1} L s^{-1}) [36]. Therefore, in the presence of Br^- the depletion of H_2O_2 must be also faster causing less degradation of the enzyme. In opposition of that, taurine chloramine and H_2O_2 remain longer in contact with the enzyme before the revelation by adding TMB. Hence, it is reasonable to consider that it could be partially inactivated. To support this proposal, the efficient of the catalytic performance of MPO using dansylglycine was also compared to the taurine method by amperometric measurement of the consumption of H_2O_2 using a selective electrode. The results depicted in Fig. 5c show the efficiency of H_2O_2 consumption in both cases. Corroborant with the previous results, the consumption of H_2O_2 was significantly faster using dansylglycine/Br^-.

Determination of MPO

The above results led us to consider that the dansylglycine methodology could be also useful for determination of MPO activity. In this case, the concentration of H_2O_2, Br^- and dansylglycine were fixed and the enzyme varied. The reaction rate was measured in the linear phase of the fluorescence versus time curve (Fig. 6a). The results in Fig. 6b confirmed our expectation and show that this methodology can be used for directly measurement of the halogenating activity of MPO. The detection limit of MPO was 6 nmol L^{-1}. The excellent linearity obtained is an indication that the enzyme did not lose its activity, or at least, any loss that occurs is consistent under each enzyme concentration. Once more, the efficiency of the reaction was demonstrated.

Selectivity for halogenation activity of MPO

How stated above, we argued that this methodology could be selective regarding the discrimination between peroxidase and chlorinating activity of MPO. This hypothesis was tested by removing, one by one, the components of the reaction system. As expected, the consumption of dansylglycine was dependent of the presence of MPO and H_2O_2 (Table 1). In the same direction, the removal of Br^- impaired the reaction. These results are in agreement with the higher electrophilicity of the HOBr compared to HOCl, which is essential for reacting with dansylglycine. Finally, we replaced MPO by HRP and found that dansylglycine was not consumed, even though Cl^- and Br^- were present in the reaction medium. As well-known HRP is able to catalyze the oxidation of many substrates through its peroxidase cycle involving the correspondent active forms HRP-I and HRP-II. However, due to its low oxidation potential it is not able to oxidize halides [37]. Altogether, these results confirm the specificity of dansylglycine as a substrate for determination of the halogenating activity of MPO.

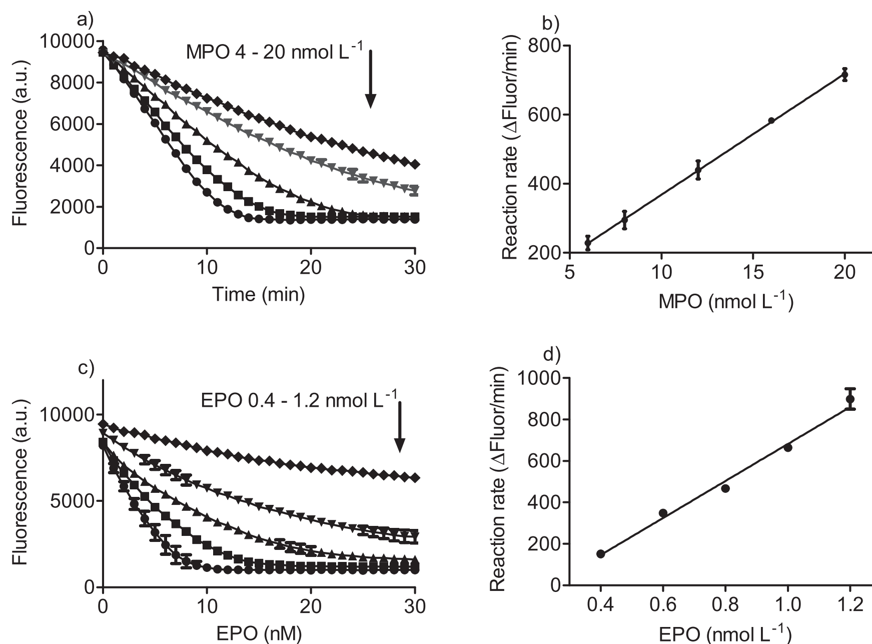

Fig. 6. Kinetic determination of MPO and EPO using the dansylglycine assay. (**a,c**) Kinetic profile of bleaching of dansylglycine fluorescence and (**b,d**) analytical curve $\Delta_{Fluorescence}$/min versus MPO and EPO, respectively. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2, 10 mmol L^{-1} Br^- and the indicated concentration of MPO and EPO in PBS, pH 7.4 at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments. MPO (r^2 = 0.9692), EPO (r^2 = 0.9692).

Table 1
Dansylglycine assay: Specificity for halogenating activity of MPO.

	Dansylglycine consumption (μmol L^{-1})	Dansylglycine consumption (%)
Control[a]	45,9 ± 0,7	91,8 ± 1,4
- MPO	3.0 ± 2.6	6.0 ± 5.2
- H_2O_2	3.8 ± 2.6	7.6 ± 5.2
- Br^-	2.9 ± 2.2	5.8 ± 4.4
HRP[b]	1.3 ± 1.2	2.6 ± 2.4

Note. The results are presented as mean and standard deviation.
[a] Dansylglycine 50 μmol L^{-1}, MPO 20 nmol L^{-1}, Br^- 10 mmol L^{-1} and H_2O_2 100 μmol L^{-1} in PBS, pH 7.4 at 25 °C.
[b] MPO was replaced by HRP.

Selectivity for halogenating activity of eosinophil peroxidase

While MPO can oxidize both Cl^- and Br^-, EPO has much more affinity to Br^- [38]. Therefore, as Br^- is an important component of the dansylglycine assay, we also tested the methodology to measure HOBr produced by EPO. The results depicted in Fig. 6c show the excellent correlation between the EPO concentration and the fluorescence decay of dansylglycine. Moreover, the detection limit for EPO was higher compared to MPO, highlighting the efficiency by which HOBr is produced by the halogenating cycle of EPO and consumed by dansylglycine (Fig. 6d). These results can be explained taking into account that the reaction rate between EPO-I and Br^- (1.0×10^7 mol^{-1} L s^{-1}) is 10-fold higher compared to MPO-I and Br^- (1.1×10^6 mol^{-1} L s^{-1}) at pH 7.0 [36,39]. The selectivity for halogenating activity using EPO was also verified (Table 2).

Application of the dansylglycine assay for evaluation of inhibitors

As stated in the introduction section, the numerous evidence of the involvement of MPO and EPO in the physiopathology of chronic and inflammatory diseases have led for a constant search for new inhibitors of these enzymes and evaluation of their potency [14,15,40,41]. Regarding the evaluation of the inhibitory potency of new compounds, the most used methods are the taurine chloramine assay and the use of H_2O_2-specific electrode [18,42]. The first one is highly specific for chlorination activity, however, it has the

Table 2
Dansylglycine assay: Specificity for halogenating activity of EPO.

	Dansylglycine consumption (μmol L^{-1})	Dansylglycine consumption (%)
Control[a]	47,3 ± 0,1	94.6 ± 0.2
- EPO	1.0 ± 0.3	2.0 ± 0.6
- H_2O_2	1.4 ± 1.0	2.8 ± 2.0
- Br^-	1.1 ± 0.4	2.2 ± 0.8

Note. The results are presented as mean and standard deviation.
[a] Dansylglycine 50 μmol L^{-1}, EPO 20 nmol L^{-1}, Br^- 10 mmol L^{-1} and H_2O_2 100 μmol L^{-1} in PBS, pH 7.4 at 25 °C.

drawback of being a two-step method, and therefore, it is not adequate kinetic monitoring the effect of the inhibitors in the chlorination rate. The use of H_2O_2-specific electrode allows the kinetic monitoring of the reaction, but is not selective for halogenating activity. Furthermore, the electrode is sensitive to interferences of the medium [18]. Here, we found that the dansylglycine assay, besides selective, is adequate for real-time measurement of the MPO/EPO activity. The results depicted in Fig. 7a and c shows the effect of 5-fluorotryptamine, a well-known and efficient reversible inhibitor of the halogenating activity of MPO [43], on the rate of dansylglycine fluorescence bleaching catalyzed by MPO and EPO, respectively. In this experiments a biphasic behavior was obtained, which can be explained considering the well-known effect of 5-fluorotryptamine on MPO, as follow: 5-Fluorotryptamine competes with Cl^- by the redox active MPO-I leading to the inhibiting of HOCl production [43]. Then, once 5-fluorotryptamine is consumed, the production of HOCl can be reestablished. This property of 5-fluorotryptamine could explain the strong inhibition observed in the first minutes (lag phase) and, with the progress of the reaction and its depletion, the rate of consumption of dansylglycine almost returned to the initial condition. This kinetic behavior was still better observed using EPO (Fig. 7c). The lag phase was almost proportional to the concentration of 5-fluorotryptamine.

To reinforce the applicability of the dansylglycine method for elucidation of the mechanism of action of MPO and EPO inhibitors, we also tested the effect of 4-hydroxybenzhydrazide. This compound belongs to the benzoic acid hydrazide derivatives, which are potent and irreversible inhibitors of MPO [44]. The oxidation of hydrazides leads to free radical intermediates that destroy the heme group of MPO leading to its irreversible inactivation [44]. The results depicted in Fig. 8 show that, differently of 5-fluorotryptamine, the inhibition provoked by 4-hydroxybenzhydrazide did not present a biphasic behavior, i.e., without an initial lag phase. These results are consistent with the irreversible inhibitory character of this class of compounds. In short, dansylglycine method could be useful to differentiate the mechanism of action of MPO/EPO inhibitors, i.e., reversible or irreversible. Finally, it is interesting to note that due to the higher stability of EPO compared to MPO, only 1.2 mmol L^{-1} was necessary to perform the kinetic studies. This is an additional advantage of this method, since purified EPO is the most expensive reagent for the screening of inhibitor for this enzyme.

Discrimination between MPO and EPO

The reactivity of MPO-I with Cl^- leading to HOCl is pH dependent, being favored at acidic pH as indicated by its apparent second-order rate constants 3.9×10^6 mol^{-1} L s^{-1} and 2.5×10^4 mol^{-1} L s^{-1}, at pH 5.0 and 7.0, respectively [36]. In addition, the reactivity of HOCl is increased in its protonated form [45,46], the predominate species at pH 5.0 (pKa 7.44). For this reason, we hypothesized that by conducting the reaction at pH 5.0, the dependence of Br^- could be diminished or totally unnecessary. The results depicted in Fig. 9 show a comparison of the dansylglycine fluorescence bleaching at pH 5.0 and 7.4 in the absence of Br^- and confirmed our expectation. EPO-I also follows the same pH-dependence pattern, but its reactivity with Cl^- is significantly lower compared to MPO-I, as can be noted by its apparent second-order rate constants 2.6×10^4 mol^{-1} L s^{-1} and 3.1×10^3 mol^{-1} L s^{-1} at pH 5.0 and 7,0, respectively [39]. In short, at pH 5.0 and in the absence of Br^- the difference between MPO and EPO reaches two-order of magnitude. Therefore, we used these properties to discriminate MPO and EPO using the dansylglycine assay. Corroborant with our expectation, the conduction of the reaction without Br^- at pH 5.0 results in a clear difference between MPO and EPO (Fig. 9), and could be used as a kinetic assay for discrimination of the enzymes.

Conclusions

In summary, we have developed a non-expensive, one-step and

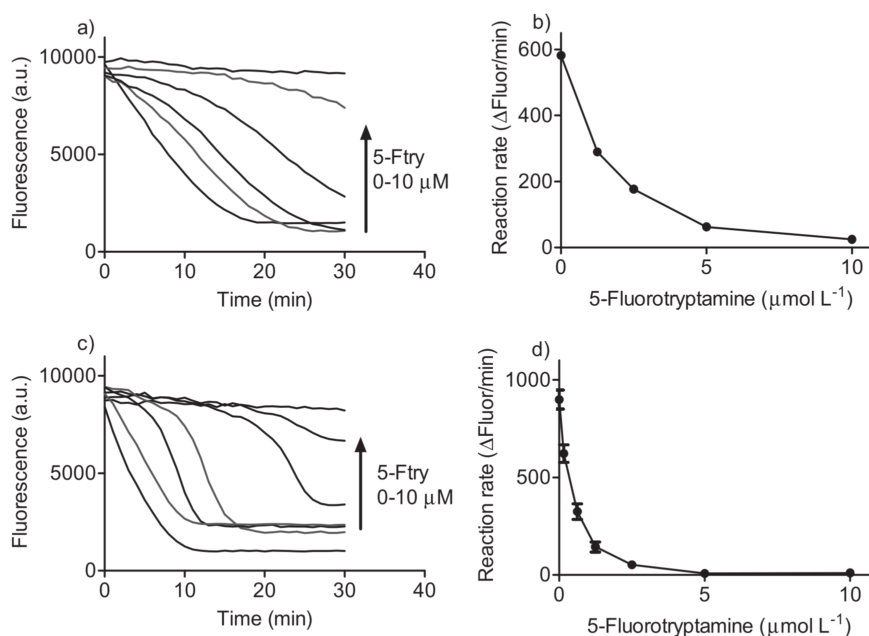

Fig. 7. Application of dansylglycine assay for studying MPO and EPO inhibition by 5-fluorotryptamine. Effect of 5-fluorotryptamine (5-Ftry) on kinetic profile of dansylglycine bleaching mediated by (**a**) MPO and (**c**) EPO. Concentration-dependent curve ($\Delta_{Fluorescence}$/min) and the inhibitory effect of 5-fluorotryptamine on (**b**) MPO and (**d**) EPO. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2, 10 mmol L^{-1} Br^-, 16 nmol L^{-1} MPO or 1.2 nmol L^{-1} EPO in PBS, pH 7.4 at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments.

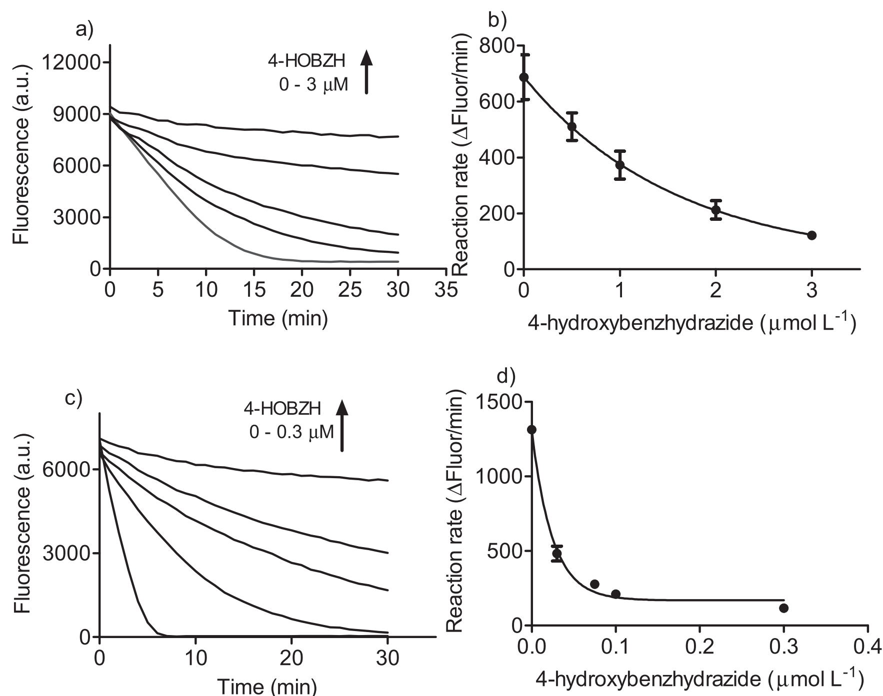

Fig. 8. Application of dansylglycine assay for studying MPO and EPO inhibition by 4-hydroxybenzhydrazide. Effect of 4-hydroxybenzhydrazide (4-HOBZH) on kinetic profile of dansylglycine bleaching catalyzed by (**a**) MPO and (**c**) EPO. Concentration-dependent curve ($\Delta_{Fluorescence}$/min) and the inhibitory effect of 5-fluorotryptamine on (**b**) MPO and (**d**) EPO. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2, 10 mmol L^{-1} Br^-, 16 nmol L^{-1} MPO or 1.2 nmol L^{-1} EPO in PBS, pH 7.4 at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments.

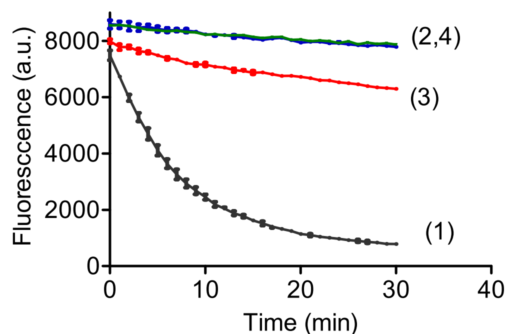

Fig. 9. Effect of pH on dansylglycine fluorescence bleaching in the absence of Br^-. Discrimination between MPO and EPO. (1) MPO pH 5.0, (2) MPO pH 7.4, (3) EPO pH 5.0 and (4) EPO pH 7.4. The reaction mixture was composed of 50 μmol L^{-1} dansylglycine, 100 μmol L^{-1} H_2O_2, MPO or EPO (5 nmol L^{-1}) in PBS (pH 7.4) or 10 mmol L^{-1} phosphate buffer (pH 5.0) at 25 °C. The final reaction volume was 250 μL and the microplate reader was set at 340/550. The results are the mean and SD of three experiments.

selective procedure for analysis of the halogenating activity of MPO and EPO. We demonstrated that dansylglycine is a fluorescent probe susceptible to halogenating, but not to the peroxidase activity of MPO/EPO. The fluorescence bleaching of dansylglycine can be real-time monitored and correlated with the concentration of the enzyme. Therefore, the technique can be useful to study the efficiency and mechanism of action of potential inhibitors of these enzymes. The discrimination between MPO and EPO can be performed by altering the pH of the reaction mixture. Finally, the method may be of limited use in cases where endogenous compounds compete with dansylglycine for reaction with HOBr. This, presumably, may be the case when measuring MPO and EPO activities in crude enzyme/tissue preparations. Hence, further studies must be performed to address these issues.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2017.05.029.

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