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Relationship between the lipophilicity of gallic acid *n*-alquil esters' derivatives and both myeloperoxidase activity and HOCl scavenging

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Abstract—The gallic acid and several *n*-alkyl gallates, with the same number of hydroxyl substituents, varying only in the side carbonic chain length, with respective lipophilicity defined through the C log *P*, were studied. It evidenced the structure–activity relationship of the myeloperoxidase activity inhibition and the hypochlorous acid scavenger property, as well as its low toxicity in rat hepatic tissue. The gallates with C log *P* below 3.0 (compounds **2**–**7**) were more active against the enzyme activity, what means that the addition of 1–6 carbons (C log *P* between 0.92 and 2.92) at the side chain increased approximately 50% the gallic acid effect. However, a relationship between the HOCl scavenging capability and the lipophilicity was not observed. With these results it is possible to suggest that the gallates protect the HOCl targets through two mechanisms: inhibiting its production by the enzyme and scavenging the reactive specie.

1. Introduction

Neutrophils form a principal front of the cell-mediated immune system in mammals. Their natural function is to overwhelm foreign organisms inactivating them, and to destroy the residues.[1] The myeloperoxidase (MPO), which consists in the azurophilic granules in the phagolysosomes, is the principal enzyme responsible for this action. MPO has a chlorinating activity besides its regular peroxidative activity, unlike other peroxidases. The enzyme contains two heme prosthetic groups and the native form is ferric (MP^{3+}). The resting enzyme (MP^{3+}) reacts with hydrogen peroxide (H_2O_2), which oxidizes chloride (Cl^-) to hypochlorous acid (HOCl) (Eq. 1).[2–4]

$$H_2O_2 + Cl + H^+ \rightarrow HOCl + H_2O \quad (1)$$

In addition to the beneficial effects of the MPO activity, the enzyme can jeopardize the host organism either through the direct effect of its chlorinated products or through the formation of secondary products from the reaction of intermediate enzyme states with phenolic compounds.[1]

HOCl is a powerful oxidant that reacts readily with many biologically important molecules. During pulmonary inflammation an influx of neutrophils is observed. After the activation, they produce prostaglandins, leukotrienes, proteolytic enzymes (e.g., elastase), and reactive oxygen species (ROS). The HOCl formed by MPO may cause rapid inactivation of the α_1-antiproteinase (an elastase inhibitor) by oxidizing the methionine residues at the active site; this will promote an uncontrolled proteinase activity, and the elastase is then able to digest the lung elastin.[5–7] In addition, HOCl can modify human low-density lipoproteins (LDL), and convert them into a form, which is readily taken up by macrophages, suggesting that HOCl generation may be implicated in the pathogenesis of atherosclerosis.[8–10] The MPO activity and HOCl production are implicated in

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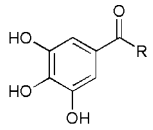
many pathologic processes like some lung damages associated with cystic fibrosis,[11–13] ischemia-reperfusion injury,[14] rheumatoid arthritis, inflammatory bowel diseases[15] as well as other inflammatory processes.[16]

Several reports have suggested that HOCl can halogenate DNA bases in vivo. It has been shown that reaction of HOCl with NH or NH_2 groups of nucleosides resulted in the formation of semistable chloramines.[17] The reaction of HOCl with the double bonds of unsaturated lipids produces α,β-chlorohydrin isomers. Fatty acid acyl and cholesterol chlorohydrins are formed after exposure of red cell membranes to HOCl. The lipid chlorohydrins at the cell membranes can contribute to the cytotoxicity of hypochlorous acid.[18] HOCl is able to penetrate into the cell membrane and oxidize intracellular thiols. Reduced glutathione (GSH) is one of the most preferred biological substrates of myeloperoxidase-derived hypochlorous acid,[19,20] as well as the proteins. Proteins' treatment with HOCl results in alterations in the amino acid side chain, protein fragmentation, and dimerization.[21–23]

The excess activation of immune system during inflammation causes host cell/tissue damage via harmful MPO activity and HOCl production, among others. The anti-inflammatory activity of many drugs has been attributed to the inhibition of the leukocyte MPO and HOCl scavengers.[24,25] For example, the anti-inflammatory aminopyrine was shown to inhibit the conversion of H_2O_2 to HOCl by trapping MPO[26] and the HOCl scavengers prevent the protein carbonyl formation[27] and α_1-antiproteinase inactivation.[28]

There is a strong relation between the antioxidants' amount in diet and the incidence reduction of many diseases. The carotenoids, for example, are associated with the reduction of pathologic process related to photo-oxidation,[29] a diet rich in phenolic compounds reduces the incidence of atherosclerosis, neurodegenerative diseases, and cancer.[30,31] However, the potent in vitro antioxidant and anti-inflammatory ability of phenolic compounds is less apparent in vivo due in part to a relatively poor bioavailability and low solubility in cell membranes compared with vitamin E.[32,33] The hydrophobic chain of vitamin E is vital for its uptake from gut, systemic transportation, and orientation in cell membranes for optimum biological function. Increasing lipophilicity of water-soluble antioxidants may therefore have therapeutic potential to treat a range of clinical conditions such as stroke, diabetic complications, neurodegenerative conditions, and inflammatory process where macromolecules' oxidation has pathogenic consequences. For example, results from recent studies of lipophilic analogues of gibbilimbols,[34] phaffiaol,[35] chromanols,[36] polyprenylated hydroquinones,[37] dihydrobenzofurans,[38] vitamin E,[39] and esters of ferulic acid[40] emphasize the importance of increased lipophilicity in prevention of lipid oxidation. This property was also observed for gallic acid ester. The hydrophobic group of dodecyl gallate is associated with the antioxidant activity in the mitochondrial lipid peroxidation prevention.[41]



1: Gallic acid - R = H
2: Methyl gallate - R = CH_3
3: Ethyl gallate - R = CH_2CH_3
4: Propyl gallate - R = $(CH_2)_2CH_3$
5: Butyl gallate - R = $(CH_2)_3CH_3$
6: Pentyl gallate - R = $(CH_2)_4CH_3$
7: Hexyl gallate - R = $(CH_2)_5CH_3$
8: Heptyl gallate - R = $(CH_2)_6CH_3$
9: Octyl gallate - R = $(CH_2)_7CH_3$
10: Decyl gallate - R = $(CH_2)_9CH_3$
11: Undecyl gallate - R = $(CH_2)_{10}CH_3$
12: Dodecyl gallate - R = $(CH_2)_{11}CH_3$
13: Tetradecyl gallate - R = $(CH_2)_{13}CH_3$
14: Hexadecyl gallate - R = $(CH_2)_{15}CH_3$
15: Octadecyl gallate - R = $(CH_2)_{17}CH_3$

Figure 1. Chemical structures of gallic acid *n*-alquil esters derivatives.

Thus, in the present study, gallic acid and several *n*-alkyl gallates (see Fig. 1 for structures), with the same number of hydroxyl substituents, varying only in the side carbonic chain length, with respective lipophilicity defined through C log*P*, were studied evidencing the structure–activity relationship in the inhibition of MPO activity and the hypochlorous acid scavenger property, as well its low toxicity in rat hepatic tissue.

2. Results and discussion

Myeloperoxidase is an enzyme present in neutrophils and plays a central role in infection and inflammation. The physiological action of this enzyme is to convert hydrogen peroxide and chloride to HOCl, although it is also able to degrade hydrogen peroxide to oxygen and water,[42] about 5% of the hydrogen peroxide consumed by the enzyme is used to produce tyrosyl radicals.[26,43] The gallates inhibited the enzyme activity strongly, as can be seen in Figure 2, which shows the enzyme inhibition of the more active compound (butyl gallate), the results were re-plotted from the figure inset graphic which shows the kinetics. Through this analysis the $K_{0.5}$ for all compounds were obtained, shown in Table 1.

The majority of the compounds inhibited the enzyme except those ones named **14** and **15**. Searching in the literature we found that Kato and collaborators demonstrated a strong MPO inhibition by phenolic antioxidants such as quercetin, curcumin, ferulic, caffeic, and gallic acid.[44] It was also shown that the presence of θ-dihydroxy groups in the gallic acid is the reason of MPO inhibition.[45] The difference between the compounds tested in this work is the width of carbon side chain what gives them a certain grade of lipophilicity, represented as the C log*P*. In Table 1 are listed the C log*P* of each gallic acid derivative. In order to evaluate if the lipophilicity would be related to the enzyme

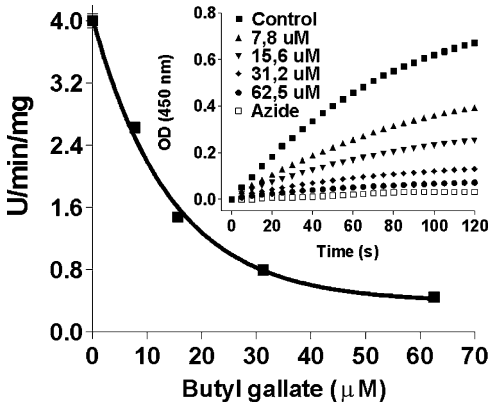


Figure 2. Effect of butyl gallate on the MPO activity. The values of the enzyme activity were calculated from the curves shown in the figure inset. The enzyme activity was measured spectrophotometrically following the oxidation of θ-dianisidine by HOCl produced by the enzyme. More details are described in Section 3. 100% of enzyme activity is 4.0 ± 1.0 U/min/mg.

Table 1. $K_{0.5}$ values for MPO activity inhibition and IC_{50} values for HOCl scavenger of gallic acid and gallates

Gallates	MPO inhibition $K_{0.5}$ (μM)	HOCl scavenger IC_{50} (μM)	Clog*P*
1	28.7 ± 1.1	9.6 ± 1.0	0.89
2	10.0 ± 0.4	12.8 ± 1.6	0.92
3	9.7 ± 0.3	11.2 ± 4.0	1.27
4	8.7 ± 0.2	13.1 ± 1.4	1.73
5	8.5 ± 0.3	9.1 ± 3.7	2.13
6	9.4 ± 0.2	12.5 ± 0.7	2.53
7	11.8 ± 0.6	12.7 ± 1.3	2.92
8	25.7 ± 2.2	8.9 ± 0.8	3.32
9	29.1 ± 1.6	9.1 ± 0.9	3.72
10	38.3 ± 1.8	16.0 ± 0.7	4.51
11	25.2 ± 1.3	10.0 ± 1.3	4.90
12	23.5 ± 0.5	13.7 ± 2.2	5.30
13	22.2 ± 0.9	11.1 ± 0.5	6.09
14	—[a]	10.0 ± 1.2	6.89
15	—[a]	nd	7.68

nd, not determined; the compound promoted a turvation of the medium.
[a] did not inhibit the enzyme.

activity inhibition, the data were analyzed as shown in Figure 3. $1/K_{0.5}$ versus Clog*P* showed that there is a special range of compounds' lipophilicity for the enzyme activity inhibition. The gallates with Clog*P* below 3.0 (compounds **2**–**7**) were more active, what means that the addition of 1–6 carbons (Clog*P* between 0.92 and 2.92) at the side chain increased approximately 50% the gallic acid effect.

As every compound has θ-dihydroxy group, the lipophilicity in fact has influence on this activity. One can suggest that increasing the number of carbons in the lipophilic side chain can promote a spatial inhibition of the enzyme active site. Mekapati and collaborators [46] have demonstrated that allosteric reaction has been found in a variety of instances in biological activity of many substances. An inverted parabolic relationship between biological activity and hydrophobicity is apparent, that is the activity

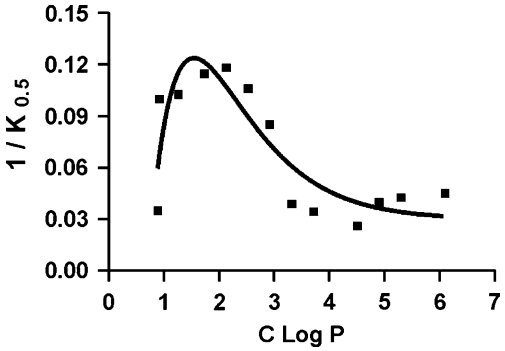


Figure 3. Relationship between the MPO activity inhibition and the compounds lipophilicity. $1/K_{0.5}$ versus Clog*P*, higher the rate higher the enzyme inhibitory effect. The line is only an eye guide. The corresponding Clog*P* for each compound is shown in Table 1.

first decreases as the hydrophobicity increases and after a certain point, the activity begins to increase again. This could be attributed to the ligands that cause a change in the receptor structure. Our results partially agree with this report. The MPO inhibition shows the inverted parabolic relationship, with the inhibitory activity first increasing as the hydrophobicity increased and after a certain point, the effect begins to reduce and does not increase again. From these results we assume that the phenolic and dihydroxy groups are the principal components responsible for the MPO inhibitory effect of these compounds and the lipophilicity influences this activity, however the inhibition mechanism is unknown and more detailed studies are necessary.

Searching in the literature we found some results to support this hypothesis. It was shown that alkyl (*n*-butyl, *n*-octyl and *n*-dodecyl) gallates modify the PgP function in PgP overexpressing KB-C2 cells, and that this modification is exerted by both: the gallic acid moiety and a long alkyl chain.[47] The same interpretation was given to the inhibition of a protein kinase (PTK) by lauryl gallate.[48]

The inhibition of θ-dianisidine oxidation by the compounds could be indirectly by the scavenging of HOCl. As mentioned in the introduction it has been reported that such compounds can act as reactive oxygen species scavengers, also of HOCl. The assay for HOCl scavenging was carried out for all compounds according to the method described in Section 3. The IC_{50}s obtained are listed in Table 1. The results show that all compounds were able to scavenge the reactive specie demonstrated by the inhibition of TNB oxidation, in a low μM range. However, differently from the results obtained for MPO activity, a relationship between the HOCl scavenging capability and the lipophilicity was not observed (Fig. 4), as can be seen in Figure 3 (plot $1/IC_{50} \times$ Clog*P*). Additionally, the compound named **14** (hexadecyl gallate) was not able to inhibit the enzyme although, it was able to scavenge HOCl. Through these observations, it is possible to suggest that the gallates protect the HOCl targets through two mechanisms: inhibiting its production by the enzyme and scavenging the reactive specie although, only the first way is dependent on the gallate lipophilicity.

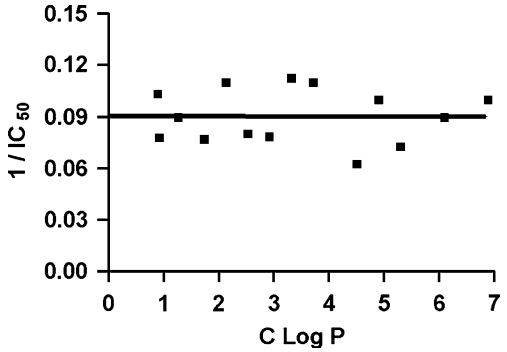


Figure 4. Relationship between the HOCl scavenging and the compounds' lipophilicity. $1/IC_{50}$ versus C log *P*, higher the rate higher the antioxidant effect. The line is only an eye guide. The corresponding C log *P* for each compound is shown in Table 1.

In conclusion, these compounds are good candidates to act as anti-inflammatory, since they inhibit a key enzyme of the inflammatory processes, and are capable to scavenge one of the most important deleterious products of the enzyme. Moreover, all compounds did not present cytotoxicity in rat hepatic tissue (results not shown), even at 200 μM. In another work of our group, the antioxidant activity of these gallates as well as their low toxicity were demonstrated, they presented CC_{50} (cytotoxic concentration) in Vero cells above 1000 μM, while the effective concentration was found below 100 μM.[49]

3. Materials and methods

3.1. Materials

5,5′-Dithio-bis(2-nitrobenzoic acid) (DTNB), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), hexadecyl trimethyl-ammonium bromide (HTMAB), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (HEPES), MPO, and θ-dianisidine were purchased from Sigma® (St. Louis, MO, USA). All other reagents were of analytical grade.

3.2. Animals

The lungs and the liver were obtained from male Wistar rats weighing 160–200 g and 42–54 days old that were maintained in the animal facilities of the Universidade Federal de Santa Catarina and housed in 12 h light–dark cycle at an ambient temperature of approximately 24 °C. The animals were maintained with pelleted food and tap water available ad libitum. The animals were maintained in accordance with ethical recommendations of Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation.

3.3. Compounds

The compounds tested in this work were synthesized by MSc. Paulo C. Leal from the group of Dr. Rosendo A. Yunes and Ricardo J. Nunes as described previously.[49]

3.4. Assay for myeloperoxidase activity

The lungs were homogenized in an ice-cold phosphate buffer (50 mM, pH 6.0), containing 0.5% HTMAB and three times freeze-thawed.[50,51] The samples were centrifuged at 12,000*g* at 4 °C for 20 min. The supernatant was assayed in a reaction medium containing NaH_2PO_4 (50 mM) at 25 °C, θ-dianisidine (0.167 mg/mL), and H_2O_2 (0.0005%). The enzyme activity was determined by the slope of the absorption curve set at 450 nm. A standard curve of myeloperoxidase activity was previously obtained with a commercial enzyme batch (Sigma®). A control with sodium azide (250 μM), inhibiting completely the MPO activity, run in parallel.[52]

3.5. Hypochlorous acid scavenging

3.5.1. Hypochlorous acid synthesis. For the assay, HOCl (70 μM) was immediately prepared before adjusting a solution of NaOCl to pH 6.6 with KH_2PO_4 (50 mM). The concentration of HOCl was further spectrophotometrically determined at 290 nm using the molar absorption coefficient of 350 M^{-1} cm^{-1}.[53]

3.5.2. 5-Thio-2-nitrobenzoic acid (TNB) synthesis. TNB was prepared according to a described procedure.[53] Briefly, sodium borohydride (20 mM) was added to a solution of DTNB (1 mM) in KH_2PO_4 buffer (50 mM, pH 6.6), containing EDTA (5 mM). The solution was incubated at 37 °C for 30 min. The TNB concentration was monitored at 412 nm using the molar absorption coefficient of 13.600 M^{-1} cm^{-1}.

3.5.3. Hypochlorous acid scavenging assay. The assay was performed at room temperature in a cuvette containing a TNB (70 μM) solution, with or without gallates (0–40 μM). The absorbance was measured at 412 nm before and 5 min after hypochlorous acid (25 μM) addition.

3.6. C log *P* determination

The C log *P* value was determined by TSAR 3D program.[54]

3.7. Cell viability

Liver slices (thickness 400 μm) were incubated for 1 h at 37 °C in a medium containing (mM): HEPES (25), glucose (12), $CaCl_2$ (1), NaCl (124), KCl (4), and $MgSO_4$ (1.2), in the presence or absence of the compounds (up to 200 μM). After that the medium was discarded and the slices were further incubated for 45 min with MTT (0.5 mg/mL). The cell viability was monitored spectrophotometrically at 550 nm, after the reaction medium substitution by dimethylsulfoxide. Cell respiration was assessed by reduction of MTT to formazan.[55,56]

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