Pergamon

Biochemical Pharmacology, Vol. 48, No. 4, pp. 753–759, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0188-Q

SCAVENGING OF REACTIVE OXYGEN SPECIES BY SILIBININ DIHEMISUCCINATE

LURDES MIRA, MANUELA SILVA and C. F. MANSO*

Instituto de Química Fisiológica, Faculdade de Medicina de Lisboa, 1600 Lisboa, Portugal

(*Received* 8 *December* 1993; *accepted* 15 *April* 1994)

Abstract—Silibinin dihemisuccinate (SDH) is a flavonoid of plant origin with hepatoprotective effects which have been partially attributed to its ability to scavenge oxygen free radicals. In the present paper the antioxidant properties of SDH were evaluated by studying the ability of this drug to react with relevant biological oxidants such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) and hypochlorous acid (HOCl). In addition, its effect on lipid peroxidation was investigated. SDH is not a good scavenger of $O_2^{\cdot-}$ and no reaction with H_2O_2 was detected within the sensitivity limit of our assay. However, it reacts rapidly with $HO^{\cdot}$ radicals in free solution at approximately diffusion-controlled rate ($K = (1.0–1.2) \times 10^{10}$/M/sec) and appears to be a weak iron ion chelator. SDH at concentrations in the micromolar range protected α_1-antiproteinase against inactivation by HOCl, showing that it is a potent scavenger of this oxidizing species. Luminol-dependent chemiluminescence induced by HOCl was also inhibited by SDH. The reaction of SDH with HOCl was monitored by the modification of the UV–visible spectrum of SDH. The studies on rat liver microsome lipid peroxidation induced by Fe(III)/ascorbate showed that SDH has an inhibitory effect, which is dependent on its concentration and the magnitude of lipid peroxidation. This work supports the reactive oxygen species scavenger action ascribed to SDH.

Key words: silibinin dihemisuccinate; flavonoids; reactive oxygen species; free radical scavengers; antioxidant properties; lipid peroxidation

Flavonoids are phenolic compounds of plant origin to which antioxidant properties have been attributed. These properties seem to be due to their ability to scavenge free radicals and to chelate metal ions [1–6]. However, their free radical scavenging properties have not yet been fully characterized.

SDH† is a water-soluble form of the main structural isomer constituent of the flavonoid mixture termed silymarin, which also contains the isomers isosilibinin, silichristin and silidianin [7].

Several studies *in vitro* and *in vivo* have shown that silymarin and silibinin possess hepatoprotective effects [8–10]; it has been suggested that one of the mechanisms responsible for this effect is related to their ability to scavenge oxygen free radicals, which may play a major role in liver damage [11].

One approach which may be adopted to elucidate the antioxidant properties of a compound is to study its ability to react with important reactive oxygen species that are generated *in vivo*. One of these is the superoxide anion radical ($O_2^{\cdot-}$), which is formed during normal aerobic metabolism and by activated phagocytic cells in inflammation [12–14]. The superoxide can dismutate into hydrogen peroxide (H_2O_2), which can also be formed directly by some oxidases [15]. However, much of the damage done by $O_2^{\cdot-}$ and H_2O_2 is thought to be due to their metal ion-dependent conversion into a highly reactive species, especially the hydroxyl radical, $HO^{\cdot}$ [16]. Besides $O_2^{\cdot-}$ and H_2O_2, activated neutrophils produce a powerful oxidant, hypochlorous acid (HOCl), through the action of myeloperoxidase [17]. During neutrophil stimulation, myeloperoxidase is also present outside the cell and thus HOCl is generated both intra- and extracellularly [18]. At physiological pH values, hypochlorous acid is present as a mixture of $HOCl/OCl^-$ because of its pK_a of 7.5 [19]. Hypochlorous acid degrades or inactivates a wide range of biomolecules [20, 21], an important extracellular target being the plasma glycoprotein α_1-AP [17]. The inactivation of α_1-AP decreases the protection of tissues against proteolytic attack by proteases such as elastase, which is also released from activated neutrophils [17, 22]. Thus the release of oxidants and proteolytic enzymes by activated phagocytes can cause tissue damage.

In the present work, we studied the ability of SDH to react with the reactive oxygen species described above. The scavenging of $O_2^{\cdot-}$ was evaluated using the autoxidation of adrenaline to adrenochrome at pH 10.2 [23] and the reaction with H_2O_2 was tested in an assay involving the release of O_2 by catalase [24]. The second-order rate constant of SDH for reaction with $HO^{\cdot}$ was determined using the deoxyribose method [25]. This assay, performed in the absence of EDTA, also allowed us to evaluate the potential ability of the compound to chelate iron ions, interfering with site-specific Fenton chemistry [26]. The scavenging of HOCl by SDH was studied by evaluating the ability of the compound to protect

* Corresponding author: C. F. Manso, Instituto de Química Fisiológica, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1600 Lisboa, Portugal. Tel. (01) 7975128; FAX (01) 7964059.

Abbreviations: SDH, silibinin dihemisuccinate; α_1-AP, α_1-antiproteinase; SOD, superoxide dismutase; TBA, 2-thiobarbituric acid; TCA, trichloroacetic acid; BHT, butylated hydroxy-toluene.

α_1-AP against inactivation [27]. We also examined the ability of SDH to inhibit the luminol-dependent chemiluminescence induced by HOCl/OCl^- [28] and the direct reaction of HOCl with SDH was monitored by measuring the UV–visible spectrum of SDH. In addition, the effect of SDH on the lipid peroxidation of rat liver microsomes induced by Fe(III)/ascorbate was studied.

MATERIALS AND METHODS

Chemicals. All reagents were of the highest quality available and used as supplied either from Sigma Chemical Co. (St Louis, MO, U.S.A.) or from Merck (Darmstadt, Germany). Ferric chloride was from Fluka (Buchs, Switzerland) and sodium hypochlorite from Carlo Erba (Milan, Italy). SDH, disodium salt, was a gift from Madaus AG (Köln, Germany).

Assay for superoxide anion radical. The autoxidation of adrenaline was followed by monitoring the production of adrenochrome, according to the method described by Misra and Fridovich [23]. Each assay contained 100 μM EDTA/50 mM sodium carbonate buffer, pH 10.2, and 300 μM adrenaline in a final volume of 3 mL. All reactions were performed at 30° and started with the addition of adrenaline. Absorbance values were continuously monitored spectrophotometrically at 480 nm in the presence or absence of SDH at the concentrations shown in the figures. Percentage inhibition was evaluated by determining the rate of adrenochrome formation. To compare the inhibitory effect of SDH on adrenochrome formation, a similar study with SOD was performed as a positive control.

Measurement of hydrogen peroxide. H_2O_2 was measured indirectly by the release of O_2 from the decomposition of H_2O_2 by catalase, with a Clark-type oxygen electrode (Gilson 5/6 oxygraph). Using a 50 mM potassium phosphate buffer with 0.15 mM NaCl, pH 7.4, and 0.10–2.25 mM H_2O_2, a standard curve (O_2 production vs H_2O_2 concentration) was constructed. The release of O_2 was started by the addition of 5.8 U/mL of catalase. SDH was incubated at concentrations up to 5 mM with 0.25 and 2 mM H_2O_2 for 30 min at 25°. At the end of this time the remaining H_2O_2 was measured using the catalase-based method.

Detection of hydroxyl radical. The experimental procedure used was essentially as described by Halliwell *et al.* [25] with minor modifications [29]. Each assay contained, in a final volume of 1 mL, 20 mM KH_2PO_4–KOH buffer, pH 7.4, 2.8 mM deoxyribose, 100 μM $FeCl_3$, 104 μM EDTA (when added), 300 μM H_2O_2, SDH at concentrations ranging from 0.05 to 3 mM and 100 μM ascorbic acid.

Ferric chloride and EDTA (when added) were pre-mixed just before addition to the reaction mixture. Solutions of $FeCl_3$ and ascorbate were made up immediately before use. The pH of drug solutions was adjusted to 7.4 before addition to the reaction mixture. Ascorbic acid was added to start the reaction. Reaction mixtures were incubated for 1 hr at 37°. At the end of the incubation period 1 mL of TBA 1% (w/v) in 0.05 M NaOH and 1 mL of TCA 2.8% (w/v) were added. After acidification the SDH precipitated, but this was irrelevant to the assay, where the precipitate was discarded by centrifugation. The supernatant was heated for 15 min at 100° and after cooling, the chromogen was measured at 532 nm.

The rate constants for the reaction of SDH with HO$^\bullet$ (K_{SDH}) were calculated from the slopes of the straight lines obtained representing $1/A$ against [SDH] (slope = $K_{SDH}/K_{DR}[DR]\,A°$) [25], where the absorbance in the presence of SDH was $A = K_{SDH}$ [SDH] [HO$^\bullet$] and the absorbance in the absence of SDH was $A° = K_{SDH}$ [SDH] [HO$^\bullet$] + K_{DR} [DR] [HO$^\bullet$]. The deoxyribose concentration [DR] was 2.8 mM and the rate constant for the reaction of deoxyribose with HO$^\bullet$ was $K_{DR} = 3.1 \times 10^9$/M/sec).

For all concentrations of SDH, controls in which deoxyribose was omitted from the reaction mixture were performed and revealed that SDH did not release TBA-reactive material when attacked by HO$^\bullet$. It was also found that SDH did not interfere with subsequent measurement of deoxyribose degradation products since it had no effect when added to the reaction mixture at the end of incubation, just before addition of TCA and TBA.

Assay for hypochlorous acid. The inactivation of α_1-AP by HOCl and the resulting decrease in inhibition of elastase was assayed essentially as described by Aruoma *et al.* [27] albeit with a few modifications. Full details are given in the legend of Table 1. HOCl was produced immediately before use by adjusting NaOCl to pH 6.2 with diluted H_2SO_4. The concentration of HOCl was determined using an extinction coefficient of 100/M/cm at 235 nm [30].

Chemiluminescence was measured in a LKB Wallac luminometer (model 1251) using luminol as luminophore. Experimental solutions containing 50 mM potassium phosphate buffer with 0.15 mM NaCl, pH 7.4, 20 μM luminol and SDH were pre-mixed in polyethylene tubes placed in the luminometer counting chamber at 25°. The HOCl/OCl^- was then added and the luminescence, expressed as light intensity (LI) in mV, measured every second. The effect of SDH was determined from the maximal light intensity values obtained in each assay. For each assay, five replicates were done.

Spectrum modifications of SDH by 100 μM HOCl were monitored in a Pye Unicam SP 8-100 UV spectrophotometer.

Microsomal peroxidation. Rat liver microsomes from male Sprague–Dawley rats (250–300 g) were prepared by differential centrifugation [31]. Each microsomal preparation was obtained from three different livers. The protein content was determined by the method of Lowry *et al.* [32].

The microsomal lipid peroxidation induced in the presence of $FeCl_3$ and ascorbate was measured by the TBA test [27]. The reaction mixtures contained, in a final volume of 1 mL, phosphate saline buffer (3.4 mM Na_2HPO_4/NaH_2PO_4, 0.15 M NaCl, pH 7.4), 0.25 mg microsomal protein, (10, 25 or 50 μM) $FeCl_3$ and ascorbate in equimolar concentrations with iron. SDH was added to the reaction mixtures to give the final concentrations shown in the figures. The pH of SDH solutions was adjusted to 7.4. Peroxidation

Table 1. Action of HOCl on elastase-inhibitory capacity of α_1-AP: effect of SDH

Composition of the first 1.0 mL reaction mixture	Elastase activity in final reaction mixture $\Delta A_{410} \times 10^3$/sec	α_1-AP activity (% inhibition of elastase)
Buffer, only	50	—
Buffer, α_1-AP	0	100
Buffer, α_1-AP, HOCl	50	0
+SDH (15 μM)	43	14
+SDH (30 μM)	15	70
+SDH (45 μM)	5	90
+SDH (60 μM)	0	100
+SDH (100 μM)	0	100
+SDH (150 μM)	0	100

α_1-AP (1.0 mg/mL), HOCl (100 μM) and SDH (if present) were incubated in a final volume of 1.0 mL in buffer (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4, pH 7.4) at 26° for 30 min. Then 1.6 mL of the same buffer and 0.03 mL of 0.7% (w/v) porcine pancreatic elastase solution in buffer were added, followed by incubation at 26° for a further 30 min to allow any α_1-AP still active to inhibit elastase. Any HOCl remaining in solution is sufficiently diluted so that it does not affect elastase itself. The remaining elastase activity is then measured by adding 375 μL of 12 mM elastase substrate (*N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide). The hydrolyse of the substrate is followed by a linear increase of absorbance at 410 nm. SDH was mixed with α_1-AP and buffer immediately before adding HOCl. Values shown are from a representative experiment.

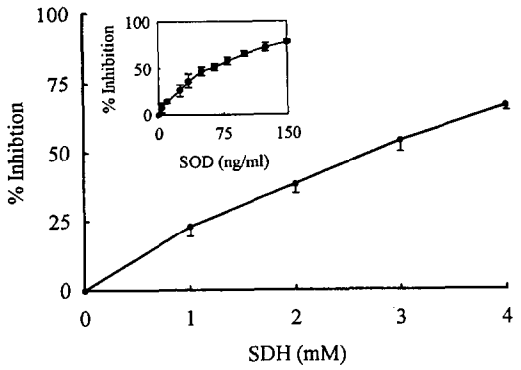

Fig. 1. Inhibition of the autoxidation of epinephrine, at pH 10.2, by SDH or SOD. Each assay contained, in a final volume of 3 mL, 100 μM EDTA/50 mM sodium carbonate buffer (pH 10.2), SDH or SOD and 300 μM epinephrine. The reactions were performed at 30°. Data are given as means ± SD, N = 4.

was initiated by adding ascorbate and the reaction mixtures incubated for 1 hr at 37°. TBA reactivity was assayed by adding 20 μL of BHT 1% (w/v) and 0.5 mL of TBA 1% (w/v) in 0.05 M NaOH followed by 1 mL of TCA 2.8% (w/v). The reaction mixtures were then heated for 15 min at 100° and chromogen extracted with 1-butanol. The absorbance of the upper (organic) layer was measured at 532 nm. BHT was added to suppress peroxidation during the TBA assay itself.

RESULTS

Scavenging of O_2^-

The autoxidation of adrenaline to adrenochrome at pH 10.2 proceeds by a free radical chain reaction involving O_2^- [21]. Any compound itself able to react with O_2^- will decrease the rate of adrenochrome formation. Under our reaction conditions, SOD inhibits adrenochrome formation with an IC_{50} value (the concentration required for 50% inhibition of O_2^-) of approximately 59 ng/mL. SDH also inhibits the reaction but with a higher IC_{50} (2.8 mM ≈2.0 mg/mL) indicating that it is not an effective O_2^- scavenger (Fig. 1).

Scavenging of H_2O_2

The rates of O_2 production, calculated from the slopes of the linear portion of the curves, are proportional to the concentration of H_2O_2 added between 0.10 and 2.25 mM (data not shown).

If a compound reacts with H_2O_2, it will decrease the rate of O_2 production. There was no change in the rates of O_2 production in reaction mixtures containing both SDH (0.1–5.0 mM) and H_2O_2 (0.25 and 2 mM).

Scavenging of HO·

In the deoxyribose method HO· radicals are generated in free solution by a mixture of ascorbic acid, H_2O_2 and Fe(III)–EDTA. They can be detected by attack on deoxyribose which originates fragments that, when heated with TBA at low pH, generate a pink chromogen [33]. If another compound is present in the solution it will compete with deoxyribose for HO· and therefore inhibit deoxyribose degradation. This depends on the concentration of the compound in the reaction mixture and on its rate constant for reaction with HO· [25].

Unlike other flavonoids such as quercetin and myricetin [34], SDH did not stimulate HO· generation in reaction mixtures containing Fe(III)–

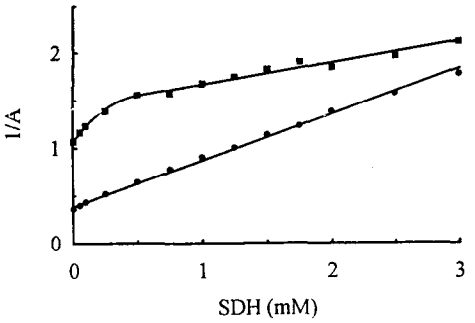

Fig. 2. Inhibition of deoxyribose degradation by SDH. Each assay contained, in a final volume of 1 mL, 20 mM KH_2PO_4–KOH buffer (pH 7.4), 2.8 mM deoxyribose, 100 μM $FeCl_3$ and 104 μM EDTA (when added), 300 μM H_2O_2, SDH and 100 μM ascorbic acid. The reaction mixtures were incubated for 1 hr at 37° and the colour was developed by the TBA method. (● with EDTA; ■ without EDTA). Data represent the means of at least four experiments done in triplicate that differed by no more than 10%. The rate constants were determined from the slopes of the lines obtained in the presence of EDTA (K_{SDH} = slope × K_{DR} × [DR] × $A°$, where K_{DR} = 3.1×10^9/M/sec, [DR] = 2.8 mM and $A°$ is the absorbance obtained in the absence of SDH) [25].

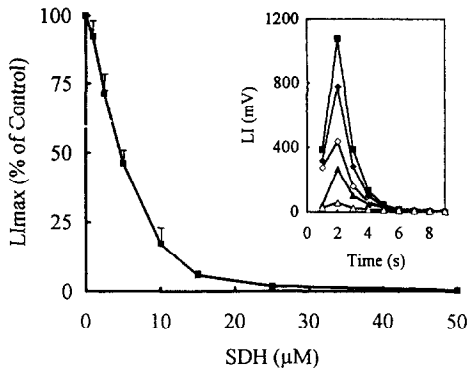

Fig. 3. Effect of SDH concentration on luminol-chemiluminescence induced by hypochlorous acid. The luminescence, expressed as maximal light intensity (LI_{max}) in mV, was measured every second after the addition of 100 μM HOCl/OCl^- to the experimental solutions containing saline phosphate buffer (pH 7.4), 20 μM luminol, and SDH. The reactions were performed at 25°. Data are given as means ± SD, from four experiments done in quintuplicate. Inset: chemiluminescence profiles in the presence of increasing amounts of SDH of a representative experiment (■ control; ◆ 2.5 μM; ◇ 5 μM; ▲ 10 μM and Δ 15 μM SDH).

EDTA and H_2O_2. Figure 2 shows the ability of SDH to inhibit deoxyribose degradation in reaction mixtures, with or without EDTA. From the slope of the competition plot obtained in the presence of EDTA, a second-order rate constant of 1.0–1.2×10^{10}/M/sec was calculated. SDH also inhibited deoxyribose degradation in reaction mixtures not containing EDTA, suggesting that it has some degree of metal binding capability, but this inhibition was proportionally less at higher concentrations of SDH. In this assay without EDTA, the only substances that inhibit deoxyribose degradation are those that bind iron ions strongly enough to remove them from deoxyribose and form complexes less reactive in generating HO• [26, 35].

Scavenging of HOCl

The effect of SDH on inactivation of α_1-AP by HOCl and the resulting elastase activity are shown in Table 1. We selected a concentration of α_1-AP just sufficient to inhibit elastase activity completely. The addition of 100 μM HOCl abolished its elastase-inhibitory effect. However, the additional presence of SDH in the reaction mixture at concentrations above 15 μM protected α_1-AP against inactivation by HOCl and a concentration of 60 μM protected it completely.

Control experiments showed that SDH, HOCl and the product resulting from its reaction did not interfere with elastase activity. Nor did SDH interfere with the ability of α_1-AP to inhibit elastase.

In the chemiluminescence assays an extremely rapid response was induced by the addition of HOCl/OCl^- to the buffer solution containing luminol (within 2 sec). SDH inhibited the luminol chemiluminescence induced by HOCl/OCl^- in a concentration-dependent manner (Fig. 3), with an IC_{50} of about 5 μM.

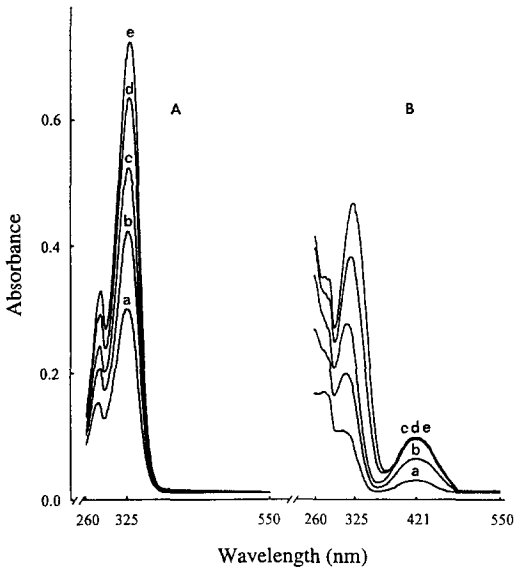

Fig. 4. Modification of the absorption spectra of SDH by HOCl. (**A**) Absorption spectra of SDH in saline phosphate buffer (pH 7.4). Curve (a) 15 μM; curve (b) 20 μM; curve (c) 25 μM; curve (d) 30 μM; curve (e) 35 μM. (**B**) Absorption spectra of SDH with 100 μM HOCl in saline phosphate buffer (pH 7.4).

A concentration of 100 μM HOCl modified the spectra recorded for SDH (10–40 μM). A reduction of absorption at 325 nm and an absorption band

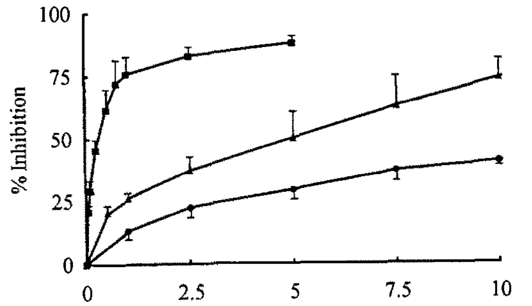

Fig. 5. Effect of SDH on the peroxidation of rat liver microsomes induced by different equimolar Fe(III)/ascorbate concentrations. Each assay was done in duplicate and contained, in a final volume of 1 mL, 3.4 mM NaH_2PO_4–Na_2HPO_4, 0.15 M NaCl buffer (pH 7.4), 0.25 mg of microsomal protein, SDH and $FeCl_3$/ascorbate in equimolar concentrations (■ 10 μM; ▲ 25 μM; ● 50 μM). The reaction mixtures were incubated for 1 hr at 37° and peroxidation measured by the formation of TBA-reactive products in the presence of BHT. Data are given as means ± SD of at least five experiments (N = 5–8).

appeared at 421 nm (Fig. 4). The reaction was complete within the time interval between mixing and recording of spectra (1–2 min). The absorption band at 421 nm increased with increasing concentrations of SDH up to 25 μM. Higher concentrations of SDH (30, 35 and 40 μM) caused no further increase of absorption at 421 nm.

Studies on lipid peroxidation

Peroxidation of mouse liver microsomes was studied using three different equimolar concentrations of Fe(III)/ascorbate to stimulate lipid peroxidation. SDH tested at concentrations up to 10 mM inhibited peroxidation to an extent that depended on Fe(III)/ascorbate concentration (Fig. 5). As expected, lower concentrations of SDH were needed to inhibit lipid peroxidation in the system with 10 μM Fe(III)/ascorbate. In control experiments, we verified that SDH did not interfere with the TBA test; when SDH was added just before TBA, the absorbance values obtained were the same as those when it was omitted.

DISCUSSION

To know if a compound has relevant antioxidant properties it is necessary to evaluate its ability to react with those biological oxidants thought to be generated *in vivo* and also to establish if it is or is not able to chelate transition metal ions.

In this work, we studied the reaction of SDH with some reactive oxygen species, such as $O_2^{\cdot-}$, H_2O_2, $HO^{\cdot}$ and HOCl. The effect of SDH upon lipid peroxidation stimulated by the iron/ascorbate system was also investigated.

All the methods used for the detection of reactive oxygen species were indirect. The adrenochrome assay is a convenient and sensitive method for

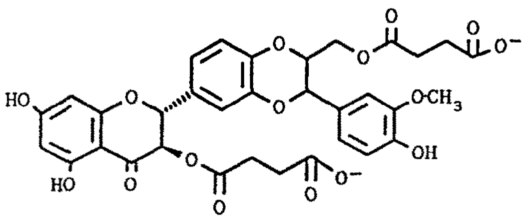

Fig. 6. Chemical structure of SDH.

assaying the ability of a compound to react with $O_2^{\cdot-}$, an unstable free radical which cannot be detected directly by conventional analytic tools [36]. The deoxyribose method is a simple "test-tube" assay for determination of the rate constants for the reaction of a compound with $HO^{\cdot}$, broadly comparable to those obtained by pulse radiolysis [25]. The advantage of sensitivity, simplicity and quickness makes the luminol-amplified chemiluminescence induced by HOCl a very useful way to study the scavenging of HOCl by compounds. The inactivation of α_1-AP by HOCl was also used because it is a more physiological way to test whether a compound might be capable of protecting biological targets from oxidative HOCl attack. A standard test of antioxidant ability is compound's effectiveness in inhibiting peroxidation of membranes such as microsomes. Although the TBA is affected by multiple factors it works well in microsomal systems provided the necessary controls are performed [16].

The autoxidation of adrenaline to adrenochrome at pH 10.2, which is mediated by $O_2^{\cdot-}$, was inhibited by SOD and by SDH with IC_{50} values which indicate that SDH is not a good scavenger of $O_2^{\cdot-}$. The IC_{50} values for effective $O_2^{\cdot-}$ scavengers in this assay are in the micromolar range [37].

The generation of $O_2^{\cdot-}$ by the hypoxanthine–xanthine oxidase system could not be used because we had verified that SDH is a strong inhibitor of the enzyme [38].

Under our reaction conditions, SDH did not seem to react with H_2O_2 because the rate of O_2 production was the same when catalase was added to reaction systems containing either H_2O_2 or H_2O_2 plus SDH. Thus SDH cannot be an effective scavenger of H_2O_2 which is present at very low physiological concentrations.

On the contrary, SDH proved to be a powerful scavenger of $HO^{\cdot}$ radical. In fact, SDH (Fig. 6) can react with $HO^{\cdot}$ through different mechanisms: addition reactions on the aromatic rings, abstraction of phenolic hydrogens and decarboxylation reactions of the carboxyl groups [39, 40]. The calculated second order rate constant for the reaction of SDH with $HO^{\cdot}$ indicates that SDH reacts with $HO^{\cdot}$ generated in free solution at an approximately diffusion-controlled rate. SDH is a good scavenger of $HO^{\cdot}$, slightly more effective than compounds such as glucose and mannitol but comparable to some anti-inflammatory drugs [41]. However, this fact will only have physiological relevance if SDH is present *in vivo* in concentrations high enough to compete with biological molecules for $HO^{\cdot}$. Low concentrations of SDH also inhibited deoxyribose degradation in

reaction mixtures not containing EDTA. This means that it has some degree of metal binding capability. This property might be of great importance as the binding of iron ions can prevent the damage done by reactive species generated from O_2^- and H_2O_2 in the presence of transition metal ions.

The neutrophil-derived enzyme myeloperoxidase catalyses the conversion of H_2O_2 and Cl^- to a complex mixture of chlorinated oxidants which includes $HOCl/OCl^-$. A primary role was found for HOCl in neutrophil-mediated inactivation of α_1-AP, a plasma protein capable of inhibiting a variety of serine proteases and possessing a very high avidity for neutrophil elastase [21]. The inactivation of α_1-AP by HOCl results in the loss of its elastase-inhibitory effect. Therefore, if a compound is a good scavenger of HOCl it will protect α_1-AP against inactivation by HOCl over the concentration range present *in vivo*. Micromolar concentrations of SDH were able to protect α_1-AP against inactivation by HOCl in reaction mixtures when added simultaneously with HOCl. The results of the chemiluminescence assays confirm that SDH is a very strong $HOCl/OCl^-$ scavenger. Several chemical groups are susceptible to being attacked by HOCl [14, 17]. Halogenation reactions involving the formation of carbon–chlorine derivatives can result from the attack of HOCl on the aromatic rings of SDH. The spectrophotometric analysis suggests that under our assay conditions 25 μM SDH is sufficient to react with 100 μM HOCl. It is known that when SDH is administered in a dose of 5 mg/kg, a concentration of 50 μg/mL (68.8 μM) is reached at the end of a 2-hr infusion (Madaus A.G., unpublished data). Thus, SDH may scavenge HOCl at a rate fast enough to protect important targets *in vivo*, such as α_1-AP.

The effect of a compound on lipid peroxidation, a free radical mediated process, can provide some information as to its antioxidant capability. The studies on rat liver microsome lipid peroxidation showed that SDH had an inhibitory effect, the magnitude of which was dependent on the concentration of Fe(III)/ascorbate used to stimulate lipid peroxidation. In the system wherein a lesser degree of peroxidation was induced (10 μM Fe(III)/ascorbate) probably simulating more closely what happens *in vivo*, very low concentrations of SDH are needed to inhibit lipid peroxidation. The antiperoxidative activity of SDH can be influenced through two different mechanisms: divalent metal chelation and free radical scavenging [3, 6, 42]. SDH possesses a hydroxyl group at C-5 in addition to the carbonyl group at 4 and two carboxylate groups which may form chelates with divalent cations [6, 43]. The free hydroxyl groups at C-5 and C-7 on SDH structure may also favour the inhibition of lipid peroxidation by reacting with peroxy radicals [42].

The results of this study, when combined with the knowledge that silibinin has low toxicity, support a potential role for silibinin as an antioxidant drug.

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