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Antioxidant Activity of Guaiazulene and Protection Against Paracetamol Hepatotoxicity in Rats

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Abstract

The effect of guaiazulene, a lipophilic azulene derivative widely found in nature, on radical-mediated processes is examined. The ability of guaiazulene to inhibit rat hepatic microsomal membrane lipid peroxidation and to scavenge hydroxyl radicals, as well as to interact with 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), was estimated.

It was found that guaiazulene can inhibit lipid peroxidation very significantly, having an IC50 value of 9·8 μM. It can also scavenge hydroxyl radicals and interact with DPPH. The protection afforded by guaiazulene to rats with paracetamol-induced liver injury was also investigated. Paracetamol hepatotoxicity is caused by the reactive metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI), which causes oxidative stress and glutathione (GSH) depletion. Hepatic cytosolic protein, GSH, glutathione transferase and glutathione reductase levels are determined as indices of hepatic injury with or without the administration of guaiazulene. It was found that all parameters affected by paracetamol are restored to normal by guaiazulene treatment, while the administration of guaiazulene alone has no effect on the performed tests compared with the control values.

It was concluded that the significant protection against paracetamol-induced GSH depletion and hepatic damage afforded by guaiazulene is probably connected with its antioxidant activity. A molecular mechanism of action of guaiazulene is suggested.

The oxidation of biomolecules such as lipids, proteins and DNA is considered to be critical in the development of conditions like cancer, cardiovascular and inflammatory diseases, the ageing process and age-related disorders. Oxidative stress can be produced or aggravated by nutritional imbalance, exposure to certain xenobiotics or irradiation, injury or disease states (Chow 1991). Physiological antioxidant defence is offered by preventative enzymes and by chain-breaking small molecules, like ascorbic acid, α-tocopherol and glutathione. When more reactive oxygen species are formed than can be counteracted by the defence mechanism of the organism, the therapeutic use of synthetic or natural antioxidants appears to be a rational approach to the management of oxidative-stress-related conditions (Paya et al 1992).

Guaiazulene (1,4-dimethyl-7-isopropylazulene, Fig. 1) is a component of the oil of *Guajacum officinale* (Zygophyllaceae) and *Matricaria chamomilla* (Asteraceae). Its chemical structure was determined in 1949 by Plattner et al, and it has been reported to possess anti-allergic, anti-inflammatory and anti-ulcer properties (Yanagisawa et al 1990).

Since the above actions are connected with free radical-related conditions, and taking into consideration that guaiazulene is an easily available, nontoxic natural product, we found it interesting to investigate the potential antioxidant properties of guaiazulene. We also examined its effect on liver injury induced by paracetamol, a classical example of a xenobiotic detoxified by glutathione conjugation of its reactive metabolite (James et al 1993).

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Materials and Methods

Materials

Guaiazulene, 5,5′-dithio-bis (2-nitrobenzoic) acid (DTNB) and 2-thiobarbituric acid were purchased from Sigma Chemical Co. (St Louis, MO), paracetamol and 1-chloro-2,4-dinitrobenzene (CDNB) from Merck (Darmstadt, Germany), and 1,1-diphenyl-2-picrylhydrazyl stable radical (DPPH) from Aldrich-Chemie (Steinheim, Germany).

All other chemicals were of the highest purity commercially available.

Animals, animal treatments and preparation of subcellular fractions

Female Fischer-344 rats (180–200 g) were used. Animals were housed in controlled rooms, humidity 50%, temp 23°C, with a 12-h light cycle, and had free access to standard laboratory chow and tap-water.

For the lipid peroxidation experiments, hepatic microsomal fraction from untreated rats was prepared as described earlier (Rekka et al 1989).

For the in-vivo experiment, rats were divided into four groups. Group 1 received paracetamol (600 mg kg^{-1} p.o.) as a micronized aqueous suspension with a few drops of Tween 80. Group 2 received guaiazulene (250 mg kg^{-1} i.p.) as a micronized aqueous suspension with a few drops of Tween 80. Group 3 received paracetamol as for group 1 and then one hour later, guaiazulene was administered as in group 2. Group 4 served as a control group, receiving only the liquid vehicle.

The experiment was terminated 18 h after paracetamol administration, livers were excised, weighed, the cytosolic fraction was prepared individually (Rekka et al 1994) and stored at − 80°C until use.

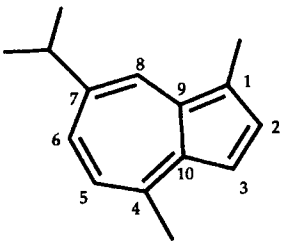

FIG. 1. The chemical structure of guaiazulen.

In-vitro lipid peroxidation

For the in-vitro lipid peroxidation experiments, the incubation mixture contained heat-inactivated (90°C, 90 s) hepatic microsomal fraction, corresponding to 0·125 g liver mL^{-1} (final concn), ascorbic acid (0·2 mM) in Tris-HCl/KCl buffer (50 mM/150 mM, pH 7·4) and various concentrations (5–20 μM) of guaiazulene dissolved in dimethylsulphoxide (DMSO). The reaction was started by the addition of a freshly prepared $FeSO_4$ solution (10 μM) and the mixture was incubated for 45 min. Samples (0·3 mL) from the incubation mixture were taken at various time intervals. Lipid peroxidation was assayed spectrophotometrically (535 against 600 nm) by the determination of the 2-thiobarbituric acid reactive material (Tani et al 1994). Under the above experimental conditions, guaiazulene and DMSO were tested and found not to interfere with the assay. Each experiment was performed at least in triplicate.

Assays in the cytosolic fractions

Cytosolic-reduced GSH was determined by a spectrophotometric method based on the reaction of GSH with DTNB, according to the literature (Akerboom et al 1982).

Cytosolic glutathione reductase activity was estimated spectroscopically from the consumption of NADPH by glutathione disulphide (GSSG) (Carlberg & Mannervik 1975), and the spectroscopic determination of cytosolic glutathione *S*-transferases was performed using CDNB as a substrate (Habig et al 1974).

Cytosolic protein was determined using bovine serum albumin as a standard (Lowry et al 1951).

Competition of guaiazulene with DMSO for hydroxyl radicals

Hydroxyl radicals (HO•) were generated by the Fe^{3+}/ascorbate system and detected by the determination of formaldehyde produced during the HO•-mediated oxidation of DMSO (Klein et al 1981). The reaction mixture contained EDTA 0·1 mM, Fe^{3+} (as a 1 : 2 mixture with EDTA) 167 μM, DMSO 33 mM in phosphate buffer (50 mM, pH 7·4). Guaiazulene was dissolved in a 2% (v/v) dimethylformamide (DMF)/buffer mixture and added at various concentrations (5–20 mM). The final DMF concentration (0·5%, v/v) was tested and found to have negligible effect on the assay. The reaction was started by the addition of 2 mM ascorbic acid and the mixture (final vol 750 μL) was incubated at 37°C for 30 min (Rekka & Kourounakis 1991). The reaction was stopped by the addition of 250 μL trichloroacetic acid (17·5%, w/v), and the formaldehyde produced was assayed spectrophotometrically by the method of Nash (1953). Each experiment was performed at least in triplicate.

Interaction of guaiazulene with the stable radical DPPH

Guaiazulene was dissolved in absolute ethanol (analytical grade, iron content less than 10^{-5}% (w/v)), added to an equal volume of an ethanolic solution of DPPH (final concn 0·2 mM) at various concentrations (0·1–0·4 mM) and kept at room temperature (22±2°C). Absorbance (517 nm) was recorded at different time intervals for 6 h (Alexidis et al 1995). Each experiment was performed at least in triplicate.

Results

The effect of guaiazulene on the in-vitro peroxidation of rat hepatic microsomal membrane lipids is shown in Fig. 2. The observed inhibition was time- and concentration-dependent and was still detectable at 5 μM. D-α-Tocopherol acetate, used as a reference compound, was found to inhibit lipid peroxidation by 100 and 10% at 1 mM and 0·5 mM, respectively, under the same experimental conditions. The concentration of guaiazulene that inhibited lipid peroxidation by 50% after 45 min of incubation (IC50) was calculated to be 9·8 μM.

Furthermore, guaiazulene could inhibit the HO•-mediated autoxidation of DMSO (33 mM) by 40, 30 and 25% at 20 mM, 10 mM and 5mM, respectively. As a reference, mannitol, a known HO• scavenger (Goldstein & Czapski 1984), was tested and found to inhibit the oxidation of DMSO (33 mM) by 22% at 25 mM.

The time-dependent interaction of guaiazulene with the N-centred stable radical DPPH is shown in Fig. 3. At equimolar concentrations, the extent of this interaction was 25% after 6 h, while guaiazulene interacted by about 50% within 3h and about 80% after 6 h at double the concentration of DPPH.

The administration of guaiazulene to rats did not cause any macroscopic toxic symptom and it did not affect liver weight, hepatic protein content or the levels of the examined cytosolic glutathione and glutathione-related enzymes in liver (Table 1). Paracetamol, under our experimental protocol, caused a ten-fold decrease in hepatic GSH, compared with controls, but it did not influence any other of the examined parameters.

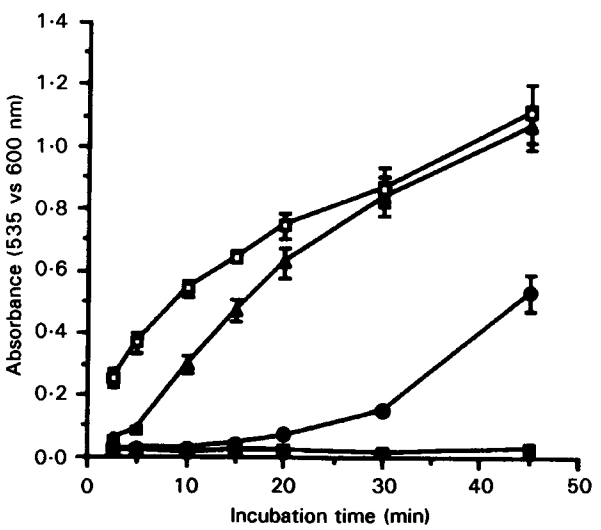

FIG. 2. Time course of lipid peroxidation, as affected by various concentrations of guaiazulene. □ Control; ▲ guaiazulene (5 μM); ● guaiazulene (10 M); ■ guaiazulene (20 μM). Bars represent standard deviation in absorbance values (n = 3–5).

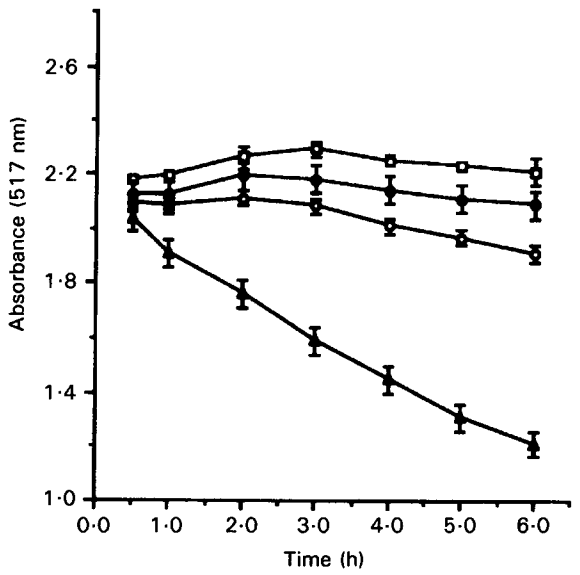

FIG. 3. Time-dependent interaction of guaiazulene (0·1–0·4 mM) with DPPH (0·2 mM). □ Control; ● guaiazulene (0·1 mM); ○ guaiazulene (0·2 mM); ▲ guaiazulene (0·4 mM). Bars represent standard deviation in absorbance values (n = 3–5).

Guaiazulene, combined with paracetamol treatment of rats, restored the values affected by paracetamol towards normal levels (Table 1).

Discussion

Radical-mediated impairment of cellular functions has been associated with a number of disorders. Since biological membranes contain highly oxidizable polyunsaturated fatty acids, they are particularly vulnerable to radical attack. Thus, treatment with exogenous antioxidants appears to be helpful in conditions related to oxidative stress. It is anticipated that an antioxidant would be useful as a therapeutic agent if it is easily available (e.g. with the diet), nontoxic and possesses suitable pharmacokinetic properties such as the ability to be present in the immediate environment of the radical attack and to be conserved by the kidneys (Rose & Bode 1993).

Guaiazulene is a nontoxic compound, widely distributed in nature, reported to exert anti-inflammatory and anti-ulcer activities. In this investigation, the effect of guaiazulene against various reactive oxygen species is examined.

Guaiazulene proved to inhibit very significantly the in-vitro peroxidation of microsomal membrane lipids. Its IC50 value is comparable with that of known potent antioxidants such as quercetin (IC50 17 μM) (Rekka & Kourounakis 1991) or propyl gallate (IC50 10 μM) (Poli et al 1978). Furthermore, the lag period observed in the time course of lipid peroxidation is considered to characterize chain-breaking antioxidants, compounds able to scavenge intermediate radicals such as peroxyl or alkoxyl radicals (Halliwell 1990) thus preventing continued allylic hydrogen abstraction from lipids. We attribute the protection against the peroxidation of membrane lipids afforded by guaiazulene to its allylic hydrogen atom of the isopropyl group at position 7 (Fig. 1). This hydrogen can be abstracted by the attacking radical, while the resulting radical of the guaiazulene molecule could be stabilized by resonance with the aromatic moiety, thus becoming less reactive. Moreover, the high lipophilicity of guaiazulene (its CLOGP value theoretically calculated according to the method of Hansch & Leo (1979) was found to be 5·74), is expected to contribute to its effective access, retention and interaction with biological membranes, the site of lipid peroxidation.

It is generally accepted that an effective inhibitor of lipid peroxidation should combine two major properties: the ability to deactivate oxygen-derived radicals without propagating chain reactions itself; and possession of a lipophilicity usually corresponding to a log P value of about 3, which would permit its access to the site of the peroxidation process (Fraisse et al 1993). We could thus conclude that guaiazulene, fulfilling most of the generally accepted criteria, may prove a useful inhibitor of oxidative damage due to lipid peroxidation.

In order to obtain further insight into the mechanism of the antioxidant activity of guaiazulene, its ability to scavenge HO• was also investigated. It was found that guaiazulene could effectively compete with DMSO for HO• generated by the Fe^{3+}/ascorbate system, being more potent than mannitol, a known HO• scavenger. However, we could not attribute the strong inhibitory action against lipid peroxidation entirely to the moderate HO•-scavenging properties of guaiazulene. In an analogous case, the hydroxyl radical scavenger mannitol, tested at 1 mM, was found to have no effect on lipid peroxidation under the same experimental conditions. It has been reported (Patterson 1981) that, although the generation of hydroxyl radicals in biological systems seems to be certain, their major sites of attack are not the bis-allylic hydrogen atoms of lipids.

Table 1. Effect of paracetamol (600 mg kg^{-1}) and guaiazulene (250 mg kg^{-1}) treatment on hepatic weight, cytosolic protein, glutathione and glutathione-related enzymes in rats.

Treatment	Hepatic weight (g/100 g body)	Cytosolic protein (mg (g liver)$^{-1}$)	Glutathione (mg (g liver)$^{-1}$)	Glutathione-transferase activity (int. units (g liver)$^{-1}$)	Glutathione-reductase activity (μmol NADPH min^{-1} (g liver)$^{-1}$)
Control	3·65±0·10	116·0±9·2	4·55±0·24	88·54±0·10	150·42±12·44
Paracetamol	3·65±0·16	104·8±14·8	0·52±0·04*	85·22±3·78	161·63±7·46
Guaiazulene	3·66±0·15	105·0±10·0	3·72±0·19	96·90±2·70	156·47±12·98
Paracetamol + guaiazulene	3·79±0·25	121·3±24·5	4·97±0·27	92·24±3·45	145·76±4·23

Results are presented as means ± s.d. from at least four rats (n = 4–6); *P<0·001 (Student's t-test, compared with all other groups).

It has been confirmed that the primary reaction of HO• with lipids is a reversible addition which affects the stereochemistry of the lipid (Dix & Aikens 1993). Thus, we could suggest that the HO•-scavenging ability of guaiazulene constitutes part of the overall mechanism of its antioxidant activity.

The interaction of guaiazulene with the stable, N-centred free radical DPPH indicates its radical scavenging ability in an iron-free system. Guaiazulene was found to possess a time- and concentration-dependent capacity to interact with DPPH. Since DPPH is a hydrophobic, electron-accepting radical (Ratty et al 1988), we suggest that the observed interaction is again due to the allylic hydrogen abstraction from guaiazulene by DPPH, although obviously at a lower rate, compared with the lipid peroxidation process, supporting our proposed mechanism of the lipid peroxidation inhibition offered by guaiazulene.

These in-vitro results prompted us to investigate the effect of guaiazulene on paracetamol-induced hepatic injury. The analgesic drug paracetamol, at high doses, is used as a model hepatotoxic agent for many animal species, including Fischer-344 rats (Kostrubsky et al 1995). Paracetamol hepatotoxicity is associated with its biotransformation to the reactive metabolite NAPQI, although the mechanism of NAPQI toxicity is not completely elucidated. Covalent binding of NAPQI to essential hepatocellular proteins is the predominant theory, however, the hypothesis that the oxidizing ability of this reactive metabolite is also responsible for toxicity is equally supported (Hinson et al 1995). Although lipid peroxidation has been reported to occur after exposure to paracetamol both in-vivo and in-vitro, the exact mechanism is not fully verified. It is not clear, therefore, whether lipid peroxidation is an early event related to radicals generated during the hepatic metabolism of paracetamol, for example after a hydrogen abstraction to form an amide radical intermediate to the formation of NAPQI (Nelson 1995), or a late event related to extrahepatocellular processes. GSH, the predominant intracellular nonprotein sulphhydryl present in the cytosol, is a strong nucleophile, able to react with electrophilic, potentially toxic species such as NAPQI, and a reducing agent that contributes to the protection of cells against oxidative damage (Meister 1992).

Prevention of GSH depletion and induction of glutathione transferase and glutathione reductase are the most efficient ways of direct protection against paracetamol hepatotoxicity. Moreover, antioxidants like promethazine and α-tocopherol have been found to offer protection against paracetamol-induced liver damage (Vermeulen et al 1992).

The administration of paracetamol to rats caused a severe depletion of hepatic GSH, which can be attributed to GSH consumption by glutathione transferase for metabolite conjugation, and to GSH oxidation for the defence against the produced oxidative stress. Guaiazulene alone had no effect on GSH or the GSH-related enzymes, however, GSH depletion was completely prevented when paracetamol intoxication was followed by guaiazulene administration. These results suggest that the mechanism of protection may involve a direct interaction of the antioxidant guaiazulene with the pro-oxidant metabolite of paracetamol, and not an effect on enzyme activity. By this interaction, the conjugation of NAPQI with GSH and the oxidation of GSH could be prevented, and GSH depletion avoided.

In conclusion, our results demonstrate that guaiazulene, a nontoxic, easily available, lipophilic compound can act as a chain-breaking antioxidant, inhibiting very significantly the microsomal membrane lipid peroxidation in-vitro, and preventing paracetamol-induced liver injury in-vivo. These properties suggest possible therapeutic applications of guaiazulene. Finally, a molecular mechanism which could explain the antioxidant and hepatoprotective action of guaiazulene is proposed. Further in-vivo lipid-peroxidation experiments, using liver tissue from paracetamol-treated rats, are being performed, for verification of the suggested mechanism of action.

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