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ANTIOXIDANT ACTION OF BENZYLISOQUINOLINE ALKALOIDS

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The antioxidant action of a series of benzylisoquinoline alkaloids has been investigated. Laudanosoline, protopapaverine, anonaine, apomorphine, glaucine, boldine, bulbocapnine, tetrahydroberberine and stepholidine produced a dose-dependent inhibition of microsomal lipid peroxidation induced by Fe^{2+}/ascorbate, CCl_4/NADPH or by Fe^{3+}ADP/NADPH. Apomorphine exerted the highest inhibitory effects in the three systems of induction used, with a potency higher than propyl gallate. Laudanosoline was particularly effective in the first system, while bulbocapnine and anonaine were more potent when CCl_4/NADPH or Fe^{3+}-ADP/NADPH were used as inducers. Laudanosoline, protopapaverine, apomorphine, tetrahydroberberine and stepholidine were also potent inhibitors of nitroblue tetrazolium (NBT) reduction. The presence of a free hydroxyl group or preferably of a catechol group is a feature relevant for inhibition of lipid peroxidation and NBT reduction, nevertheless the antioxidant activity of benzylisoquinoline alkaloids cannot be only ascribed to the formation of phenoxy radicals and other free radical species may be formed during aporphine and tetrahydroprotoberberine oxidation. The influence of this series of compounds on the time course of lipid peroxidation suggests that some of them, like apomorphine and boldine act as chain-breaking antioxidants.

KEY WORDS: Antioxidant, benzylisoquinoline alkaloids, aporphines, protoberberines, microsomal lipid peroxidation.

INTRODUCTION

There is compelling evidence that free radicals are implicated in damage to biomembranes and other cellular components. Free radicals attacking biomembranes lead to the lipid peroxidation process, which in turn has received much attention in connection with its involvement in pathological events.[1,2] There are numerous approaches to the study of molecules able to interact with free radicals in order to control their toxic effects. In this respect, natural products could be an alternative to synthetic antioxidants if they would have the necessary efficacy and potency besides a reduced toxicity to allow *in vivo* application.[3-14]

In previous papers we have reported the antioxidant properties of phenolic compounds such as flavonoids and phenolic acids.[10,12,15] Other natural products, alkaloids, can also be effective antioxidants and/or free radical scavengers.[8,14,16-22] The benzylisoquinoline group include a large number of alkaloids belonging to different structural types. In a recent work[22] we carried out a screening on the antilipoperoxidative activity of phenolic benzylisoquinoline alkaloids belonging to the simple benzylisoquinoline, phtalideisoquinoline, aporphine, protoberberine and

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benzophenanthridine types. The encouraging preliminary results we obtained prompted us to select the active compounds in order to perform a more complete study to validate and extend these findings. Thus, we have compared the effects of simple benzylisoquinolines with those of aporphines and protoberberines, and we have also used a reference antioxidant, propyl gallate, in different experimental models of lipid peroxidation and free radical generation.

MATERIALS AND METHODS

Chemicals

Stepholidine and anonaine were isolated from *Annona cherimolia* stem bark.[23] Tetrahydroberberine was obtained by Clemensen reduction of berberine. Apomorphine, boldine, berberine, bulbocapnine and glaucine were purchased from Sigma Chemical Co. (St. Louis, MO), protopapaverine from Roth GmBH (Karlsruhe, Germany) and laudanosoline from Aldrich Chemie (Steinheim, Germany). Alkaloids were used in salt form and dissolved in distilled water. The rest of the chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Lipid Peroxidation Induced by Fe^{2+}/ascorbate

Rat liver microsomal fractions were prepared as described[24] from livers of male Wistar rats weighing 200–250 g. Reactions mixtures contained 2 mg microsomal protein/ml, 0.1 M Tris-HCl, 1.15% KCl, pH 7.4 and different concentrations of test compounds. Peroxidation was induced by $FeSO_4$ and ascorbate (final concentrations 5 μM and 500 μM, respectively). The samples were incubated in triplicate at 37°C usually for 20 minutes and the extent of lipid peroxidation was determined by the thiobarbituric acid method.[25] Appropriate controls were performed at the end of incubation to assess any possible alkaloid interference with the thiobarbituric acid assay.[26]

Lipid Peroxidation Induced by CCl_4/NADPH

A modification of the method previously described[24] was followed. Final concentrations were: 1.5 mg microsomal protein/ml, the NADPH-generating system (0.2 mM $NADP^+$, 4 mM glucose-6-phosphate, 0.6 units glucose-6-phosphate dehydrogenase), in the same buffer as above. Peroxidation was started by CCl_4 (final concentration 0.02 M). After 15 minutes incubation at 37°C thiobarbituric acid-reactive substances were determined as above.

Lipid Peroxidation Induced by Fe^{3+}-ADP/NADPH

Reaction mixtures were 1.0 ml, containing 0.25 mg microsomal protein, 10 μl alkaloid solution or 10 μl distilled water for the controls and 10 mM KH_2PO_4-KOH buffer, pH 7.4. ADP (1.7 mM) and Fe^{3+} (100 μM) were pre-mixed before addition to the reaction mixture. Peroxidation was started by adding NADPH (400 μM) followed by incubation at 37°C for 20 min. After this, the extent of peroxidation was assessed using the thiobarbituric acid test.[27] Controls were performed using NADPH consumption to study the influence of alkaloids on microsomal reductase activity.[26]

Nitroblue Tetrazolium Reduction Assay

The incubation mixture contained NADH (166 μM), nitroblue tetrazolium (NBT) (43 μM), test compound and 19 mM phosphate buffer, pH 7.4. The reaction initiated by phenazine methosulfate (2.7 μM) was followed at 560 nm at 20°C for 2 minutes.[28]

Data Analysis

Inhibitory concentration 50% (IC_{50}) was calculated from the concentration/effect regression lines with the results obtained for a range of five appropriate concentrations. Statistical analysis was performed using the Dunnett's *t* test for multiple comparisons.

RESULTS

Lipid Peroxidation

The structures of the benzylisoquinoline alkaloids tested are shown in Figure 1 and Tables 1 and 2. All the alkaloids produced a marked and dose-dependent inhibition of microsomal lipid peroxidation, allowing calculation of IC_{50} values (Table 3). A well-known antioxidant, propyl gallate was used as a positive control. The inhibitory potencies for Fe^{2+}/ascorbate-induced lipid peroxidation were similar to those we reported previously[22] for the system Fe^{2+}/cysteine, with the exception of protopapaverine, anonaine and stepholidine, which showed a higher potency in the last test. When comparing the results obtained in nonenzymic (Fe^{2+}/ascorbate) and enzymic (CCl_4 /NADPH or Fe^{3+}-ADP/NADPH) lipid peroxidation, it can be observed that apomorphine, glaucine and tetrahydroberberine demonstrated similar potencies in the three systems, while the rest of alkaloids showed variable potency depending on the test. Thus laudanosoline was particularly effective in the process induced by Fe^{2+}/ascorbate, while bulbocapnine and anonaine were more potent in suppressing lipid peroxidation when CCl_4 /NADPH or Fe^{3+}-ADP/NADPH were used as inducers and boldine showed a higher potency in the presence of Fe^{3+}-ADP/NADPH. It is interesting to note that apomorphine showed in the three systems IC_{50} values in the μM range and even lower than those showed by the antioxidant propyl gallate. None of the alkaloids inhibited microsomal reduction of Fe^{3+}-ADP (data not shown).

In order to understand better the mechanisms by which the drugs in this study might be capable of acting as antioxidants we evaluated their effects on the time course of nonenzymic lipid peroxidation. Concentrations causing less than 50% inhibition at 20 minutes (final point of incubation used for IC_{50} calculations) were selected in a preliminary study and assayed at different reaction times.

Figures 2, 3 and 4 show that all alkaloids inhibited lipid peroxidation at the first minutes of incubation, although anonaine, glaucine, protopapaverine and bulbocapnine afforded less than 50% of inhibition. As seen for propyl gallate, apomorphine, boldine, tetrahydroberberine and laudanosoline were more effective at 2 minutes than at the end of the incubation period. Nevertheless for stepholidine, protopapaverine, anonaine and bulbocapnine there were slight differences between percentages of inhibition at those two points, while glaucine exerted the same effect throughout the incubation period.

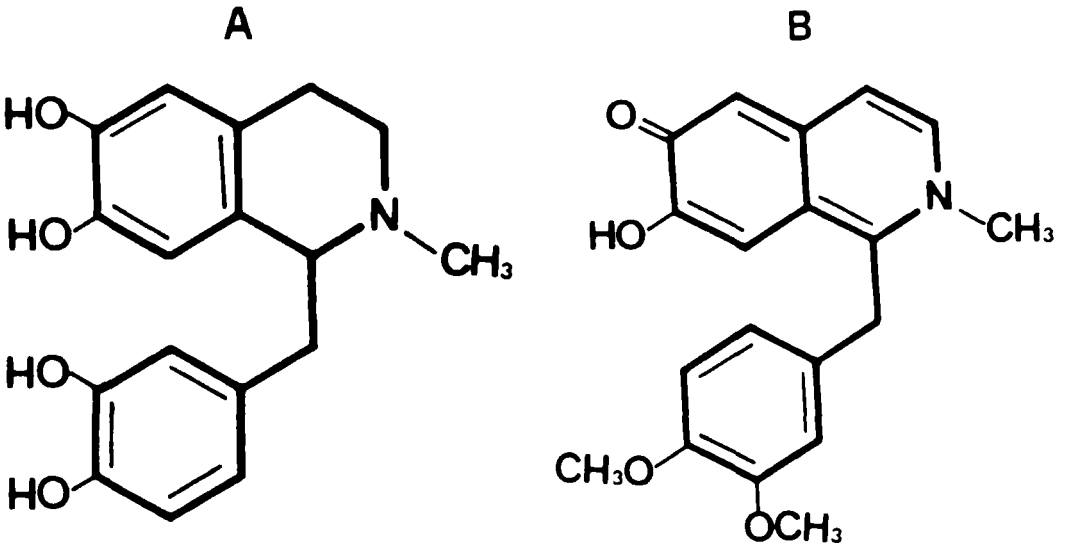
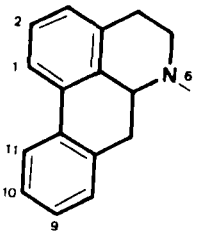


FIGURE 1 Structures of the simple benzylisoquinolines tested. A = laudanosoline. B = protopapaverine.

TABLE 1
Structure of the aporphines tested

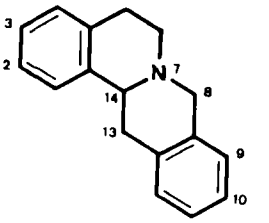


Name	1	2	6	9	10	11
Apomorphine	H	H	CH_3	H	OH	OH
Boldine	OCH_3	OH	CH_3	OH	OCH_3	H
Glaucine	OCH_3	OCH_3	CH_3	OCH_3	OCH_3	H
Anonaine	$-O-CH_2-O-$		H	H	H	H
Bulbocapnine	$-O-CH_2-O-$		CH_3	H	OCH_3	H

NBT Reduction

Since some of these benzylisoquinoline alkaloids themselves reduced cytochrome *c* (unpublished results) and they could not be studied using the rate of cytochrome *c* reduction as a direct parameter of its superoxide scavenging ability, the NBT reduction test was performed. Table 4 shows that laudanosoline, protopapaverine, apomorphine, tetrahydroberberine and stepholidine were the best inhibitors.

TABLE 2
Structure of the protoberberines tested



Name	2	3	9	10
Stepholidine	OCH_3	OH	OH	OCH_3
Tetrahydroberberine	$-O-CH_2-O-$		OCH_3	OCH_3

TABLE 3
Effect of alkaloids on microsomal lipid peroxidation

Compound	Fe^{2+}/ascorbate $IC_{50}(\mu M)$	CCl_4/NADPH $IC_{50}(\mu M)$	Fe^{3+}-ADP/NADPH $IC_{50}(\mu M)$
Laudanosoline	6.9 ± 0.2	52.1 ± 8.3	17.7 ± 0.4
Protopapaverine	16.1 ± 0.5	82.7 ± 5.2	22.7 ± 0.3
Apomorphine	2.3 ± 0.1	2.3 ± 0.4	3.7 ± 0.2
Bulbocapnine	23.0 ± 0.1	8.8 ± 1.8	9.5 ● 0.2
Boldine	23.3 ± 0.5	15.0 ± 1.4	5.6 ± 0.2
Glaucine	21.6 ± 1.0	14.1 ± 2.5	17.5 ± 0.2
Anonaine	86.0 ± 4.6	23.0 ± 2.8	16.3 ± 0.3
Tetrahydroberberine	13.7 ± 0.3	10.6 ± 1.2	7.3 ± 0.2
Stepholidine	72.8 ± 0.6	39.1 ± 0.2	12.7 ± 0.2

Results show mean ± S.E.M. (n = 6–12). The antioxidant propyl gallate showed IC_{50} = 4.5 ± 0.6 μM (CCl_4/NADPH), IC_{50} = 4.9 ± 0.1 μM (Fe^{2+}/ascorbate) and IC_{50} = 8.0 ± 0.1 μM (Fe^{3+}-ADP/NADPH).

TABLE 4
Effect of alkaloids on NBT reduction

Compound	%I (100 μM)	$IC_{50}(\mu M)$
Laudanosoline	73.0 ± 1.5**	7.5 ± 0.5
Protopapaverine	71.3 ± 0.9**	9.6 ● 1.2
Apomorphine	80.2 ± 1.2**	11.2 ± 1.6
Bulbocapnine	15.9 ± 2.5**	> 100
Boldine	41.5 ± 2.1**	> 100
Glaucine	8.5 ± 2.6**	> 100
Anonaine	21.1 ● 1.0**	> 100
Tetrahydroberberine	96.7 ± 1.2**	20.2 ± 1.3
Stepholidine	55.8 ± 0.5**	85.1 ± 3.7

Results show mean ± S.E.M. (n = 6–12) of percentages of inhibition at the highest concentration tested (100 μM) and IC_{50} μM. **P < 0.01. The antioxidant propyl gallate showed an IC_{50} = 8.4 ± 1.3 μM.

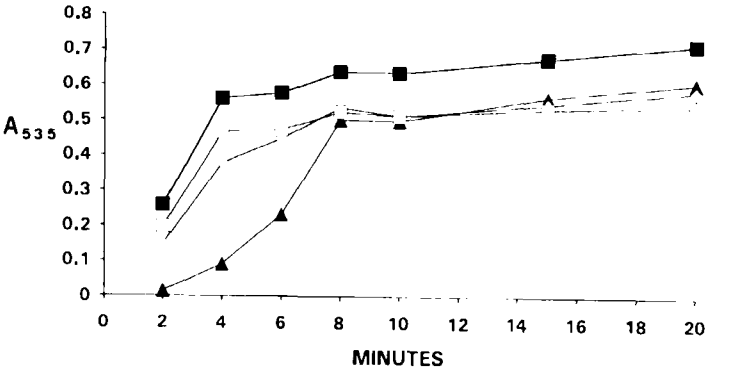


FIGURE 2 Effect of alkaloids on the time course of nonenzymic lipid peroxidation. Results show mean values of absorbance units from 6 determinations. S.E. have been omitted for simplicity. Control (■), 0.1 μM apomorphine (▲), 30 μM anonaine (◇), 10 μM glaucine (□).

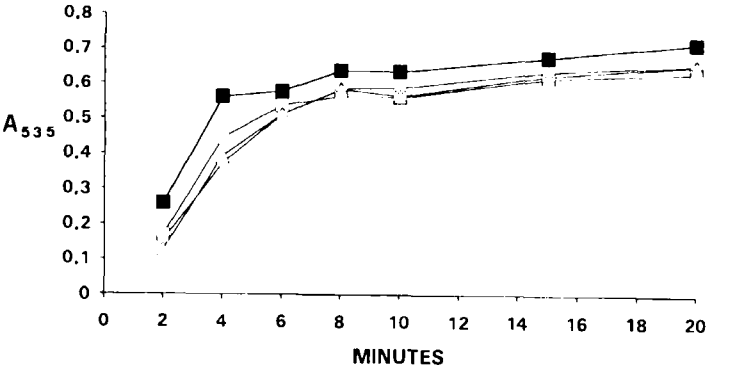


FIGURE 3 Effect of alkaloids on the time course of nonenzymic lipid peroxidation. Results show mean values of absorbance units from 6 determinations. S.E. have been omitted for simplicity. Control (■), 10 μM bulbocapnine (□), 10 μM protopapaverine (◇), 20 μM stepholidine (△).

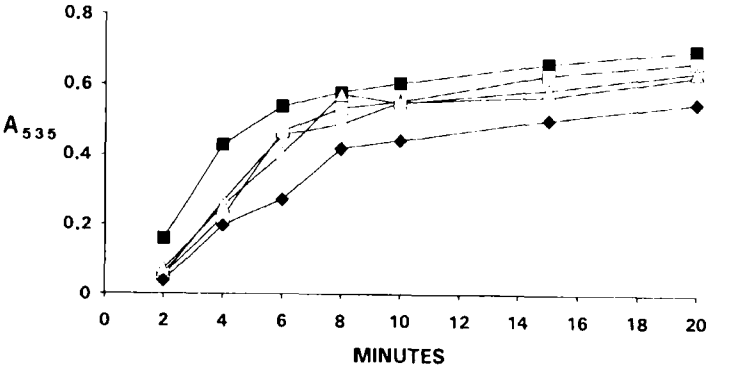


FIGURE 4 Effect of alkaloids on the time course of nonenzymic lipid peroxidation. Results show mean values of absorbance units from 6 determinations. S.E. have been omitted for simplicity. Control (■), 1μM propylgallate (□), 5 μM tetrahydroberberine (◇), 5 μM laudanosoline (△), 10 μM boldine (♦).

DISCUSSION

Our results confirm and extend previous data[14,22] on the antioxidant properties of benzylisoquinoline alkaloids, which can be related at least in part to their free radical scavenging activity. Simple benzylisoquinolines could act like phenolic antioxidants with formation of phenoxy radicals stabilized by resonance to the semiquinone form. The results on NBT reduction suggest a smaller reactivity of the aporphine derivatives with oxygen radicals in comparison with simple isoquinolines and protoberberines, since the only potent inhibitor in the aporphine group was apomorphine, which possesses the reactive catechol group. The participation of free hydroxyl groups in the scavenging activity of these compounds is indicated by the fact that their blockade by methylation is detrimental (glaucine versus boldine), nevertheless the hydroxyl at 9 position does not seem to have a high influence on the superoxide scavenging activity. The catechol group is present in the most potent inhibitors of NBT reduction and of lipid peroxidation, laudanosoline and apomorphine which could form the ortho-semiquinone and ortho-quinone radicals.

On the other hand, the antioxidant activity of benzylisoquinoline alkaloids cannot be only ascribed to the formation of phenoxy radicals, since compounds without free hydroxyls, such as glaucine and tetrahydroberberine were also active. In this respect, it should be taken into account when studying the antioxidant properties of this group of alkaloids, the chemical characteristics of aporphines and tetrahydroprotoberberines, which can be oxidized to dehydroaporphines or oxoaporphines, and protoberberines, respectively.[29] Thus, free radical species may be formed during aporphine and tetrahydroprotoberberine oxidation and the presence of some hydroxyl group would increase the antioxidative potency in the aporphines (bulbocapnine-anonaine), while in the tetrahydroprotoberberines the methylenedioxy group is more effective (tetrahydroberberine-stepholidine).

The alkaloids apomorphine and boldine at low concentrations were able to introduce a lag period into the peroxidative process, while a reduced effect was exerted by tetrahydroberberine and laudanosoline. Since chain-breaking antioxidants are consumed by reaction with peroxyl radicals,[26] it is likely that the antioxidant function of such alkaloids includes the removal of peroxyl radicals in microsomal membranes.

The results obtained with boldine are in agreement with those reported recently in rat brain homogenate auto-oxidation, 2,2′-azobis(2-amidinopropane)-induced lipid peroxidation of red cell plasma membranes and inactivation of lysozyme[14] and its ability to interact with free radical-mediated reactions has been confirmed.

CCl_4 is activated by the NADPH-cytochrome P-450 system, with formation of the $CCl_3\cdot$ radical and in aerobic conditions, of the $CCl_3O_2\cdot$ radical which initiates lipid peroxidation of polyunsaturated fatty acids.[30] The higher potency of bulbocapnine and anonaine on enzymic lipid peroxidation, in relation with that obtained in nonenzymic lipid peroxidation, suggests that their activity is partly due to an inhibitory influence on the activation of CCl_4 or Fe^{3+}-ADP by the NADPH-cytochrome P-450 system. Nevertheless, we have observed that benzylisoquinoline alkaloids do not influence microsomal reductase activity and thus other possibilities should be considered to explain such a behaviour.

Benzylisoquinoline alkaloids can interfere with the lipid peroxidation process at different stages. They can scavenge free radicals participating in the initiation stage of lipid peroxidation, like activated oxygen species which induce polyunsaturated fatty acid peroxidation and radical propagation.[26,31] Our results indicate that

apomorphine and boldine could act as chain-breaking antioxidants, by interaction with peroxyl radicals during the propagation stage of lipid peroxidation. Finally, some alkaloids may also interact with other reactive species, as well as influence iron-mediated reactions. Notwithstanding preliminary observations indicate that such interactions are very complex and they are currently under study.

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