Biochemical Pharmacology, Vol. 51, pp. 1397–1402, 1996.

ELSEVIER

ISSN 0006-2952/96/$15.00 + 0.00
PII 0006-2952(96)00080-9

Characterization of Novel Indenoindoles. Part I. Structure-Activity Relationships in Different Model Systems of Lipid Peroxidation

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ABSTRACT. Structure-activity relationships are presented for some representative compounds from a novel series of potent inhibitors of lipid peroxidation. The compounds are indenoindole derivatives with oxidation potentials in organic solvents of between 0.2 and 1.5 V. Two of these compounds, cis-5,5a,6,10b-tetrahydro-9-methoxy-7-methylindeno[2,1-b]indole (H 290/51) with an oxidation potential of 0.32 V and cis-4b,5,9b,10-tetrahydro-8-methoxy-6-methylindeno[1,2-b]indole (H 290/30) with an oxidation potential of 0.30 V, have been tested more extensively and compared with reference compounds in several pharmacological models of lipid peroxidation. The inhibitory potencies (pIC_{50}) of the compounds in respect to Fe/Ascorbate-induced production of thiobarbituric acid-reactive substances (TBARS) in a suspension of purified soybean lecithin were calculated. These data are 8.2 for H 290/51; 8.0 for H 290/30; 5.6 for vitamin E; and 6.6 for butylated hydroxytoluene (BHT). In isolated rat renal tissue subjected to hypoxia and reoxygenation, the potency for inhibition of TBARS formation is 6.9 for H 290/51, 6.9 for H 290/30, and <5 for vitamin E. In oxidative modification of low-density lipoproteins (LDL) induced by mouse peritoneal macrophages, the corresponding pIC_{50} values for TBARS inhibition for each compound are: 8.7, 8.3, <5, and 6.9, respectively. It is concluded that the synthetic indenoindoles are potent antioxidants. The results suggest that indenoindoles such as H 290/51 and H 290/30 could be useful as therapeutic agents in pathophysiological situations where lipid peroxidation plays an important role. BIOCHEM PHARMACOL 51;10:1397–1402, 1996.

KEY WORDS. LDL modification; antioxidants; free radicals; hypoxia

Oxygen free radical-induced lipid peroxidation has been suggested to be an important event in many pathophysiological situations, including ischemia/reperfusion, traumatic injuries, and leukocyte-mediated inflammation [1, 2]. Chain-breaking antioxidants, such as vitamin E and its analogs, have been tested and proven effective in protecting biological tissue from oxidative stress [3–5]. Moreover, during the last decade there have been several *in vitro* and *in vivo* studies suggesting that oxidative modification of LDL[‖] by lipid peroxidation increases their atherogenic properties [6, 7]. This hypothesis is further supported by numerous *in vitro* studies showing that antioxidants, including vitamin E and BHT, can protect LDL against oxidation induced by either cells or metal ions [8].

The potential beneficial therapeutic effects of potent inhibitors of lipid peroxidation encouraged us to develop a novel series of compounds which, when tested *in vitro*, would be more potent than vitamin E. In the present study, we describe structure-activity relationships for a series of indenoindoles and protective effects of representative compounds on peroxidation of phospholipid vesicles, macrophage-induced oxidative modification of LDL, and reoxygenation damage to isolated renal tissue. In the accompanying paper, the propensity of redox recycling of the indenoindole H 290/51 with ascorbate is further investigated.

MATERIALS AND METHODS

Chemistry

The indenoindoles were prepared using standard Fischer indole synthesis. Detailed information regarding the syntheses is given elsewhere [9, 10].

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‖ *Abbreviations:* H 290/51, cis-5,5a,6,10b-tetrahydro-9-methoxy-7-methylindeno[2,1-b]indole; H 290/30, cis-4b,5,9b,10-tetrahydro-8-methoxy-6-methylindeno[1,2-b]indole; TBARS, thiobarbituric-acid reactive substances; BHT, butylated hydroxy toluene; pIC_{50}, negative value of the log concentration of the compound needed to reduce TBARS formation by 50%; LDL, low-density lipoproteins; KPi, potassium phosphate buffer; α-MEM, modified Eagle's medium; FCS, fetal calf serum; TBA, Thiobarbituric acid; WHHL, Watanabe heritable hyperlipidemic.

Received 3 July 1995; accepted 19 January 1996.

The oxidation potentials of the compounds were determined by cyclic voltammetry, a standard electrochemical method where the oxidation/reduction potential of a given compound is determined by a cyclic variation of the potential of an electrode and measurement of the resultant current. One mg of compound was dissolved in 3.4 mL of 99.5% ethanol, and 0.60 mL ammonium acetate (0.2 M) was added. The voltammograms were produced using a Voltamograph CV-27, Cellstand C1B, a glassy carbon-working electrode, an Ag/AgCl-reference electrode, and a Pt-auxiliary electrode (Bioanalytical Systems, BAS, Kent Ave, West Lafayette, IN, U.S.A.).

Fe-Ascorbate-Mediated Lipid Peroxidation

Lecithin (1 g) was partially purified from soybean lecithin concentrates (soya lecithin granulate, Lucas Meyer, Hamburg, Germany). The procedure involved 3 washes with 10 mL of chloroform/acetone (2:8 v/v) containing 10 mM Na_2-EDTA and one with 10 mL acetone. After each wash, the tubes were centrifuged at 2000 rpm and the supernatant decanted. The last residue of partially purified lecithin was dissolved at approximately 250 mg/mL in chloroform and stored under N_2 at −20°C.

On the day of the experiment, 50 μL of the stored lecithin solution was extracted with 10 mL acetone. Following centrifugation, the supernatant was discarded and the lecithin dried under N_2. To each 2.5 mg of dried lecithin was added 6.25 mL of 50 mM potassium phosphate buffer (KPi), pH 7.4. This suspension was sonicated until it became translucent, and was used within 12 hr. Inspection of the suspension by dark-field microscopy confirmed that no vesicles were present.

The antioxidants to be tested were dissolved in 99.5% ethanol as stock solutions. Dilution series from 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, and 10^{-11} M were freshly prepared, also in ethanol. First, a 250-μL lecithin suspension with 730 μL KPi was preincubated with 10 μL antioxidant for 60 min at room temperature in BRAND disposable tubes. Then, the peroxidation reaction was initiated by addition of 20 μL of 10 mM ascorbic acid plus 10 μL 1 mM $Fe(NH_4)(SO_4)_2$. The tubes were further incubated at 37°C in a shaking water bath for 60 min. The reaction was stopped by addition of 50 μL of 20 mM BHT in ethanol. For each compound, the experiments were performed at least twice, and the measurements were done in duplicate or triplicate.

TBARS formed during oxidation were evaluated by using the fluorometric procedure of Yagi [11], and 1,1,3,3-tetrahydroxy propane in the concentration range of 5–200 μM was used as a standard.

The inhibitory potency of the tested compounds (pIC_{50}) was calculated as the negative value of the log concentration (M) of the compound needed to reduce TBARS formation by 50%.

A standard set of BHT dilutions was used to correct for variability in the preparation of the lecithin suspension. The pIC_{50} values reported are corrected values assuming a constant value for BHT in the system of 6.6, which was measured repeatedly (n = 10) with a single preparation.

Lipid Peroxidation Inhibition in Isolated Renal Tissue

Kidneys obtained from pentobarbital-anesthetized Sprague-Dawley rats (300–370 g) were cut into 2-mm slices and put into 50 mL Erlenmeyer flasks containing 40 mM HEPES buffer (pH 7.40) 37°C (10–100 mg tissue per 4 mL buffer). The renal tissue was made hypoxic for 20 min by bubbling argon gas through the buffer. Then, either an indenoindole, vitamin E, or BHT in final concentrations ranging from 10^{-8} to 10^{-5} M, or the corresponding vehicle, was added and the tissue kept under argon for an additional period of 20 min. The sample was then reoxygenated by bubbling the buffer with 95.5% O_2/CO_2. After 30 min of reoxygenation, the iron chelator desferoxamine (final concentration 45 μM) was added and the sample homogenized on ice, quickly frozen in dry ice and alcohol, and stored at −70°C. The formation of TBARS was quantified using an automated continuous-flow technique [12]. The lipid peroxidation inhibitory potency of the compounds is presented as pIC_{50} values. Each pIC_{50} value was calculated from dose-response curves obtained by testing 7 concentrations of each compound. The number of dose-response curves obtained for each compound varied between 2–6.

Oxidative Modification of Low-Density Lipoprotein

LDL was isolated at a density of 1.020 to 1.060 from EDTA plasma from fasting healthy volunteers by two-step ultracentrifugation in NaCl/KBr solutions. To remove the high salt concentration, the LDL fraction was then dialysed against PBS buffer (9 g/L NaCl, 10 mM KH_2PO_4, pH 7.4, without Ca^{2+} and Mg^{2+}, containing 0.11 g/L EDTA). The dialysis was performed for 72 hr at 4°C with 4 changes of the PBS. The LDL fraction was then stored for a maximum of 2 weeks under nitrogen until used in cell-culture experiments.

Before the cell-culture experiments the EDTA-containing buffer of the LDL fraction was changed to PBS containing Ca^{2+} (36 mg/L) and Mg^{2+} (12 mg/L) by dialysis or by gel chromatography on PD-10 columns.

The LDL fraction was analysed for total cholesterol by using an enzymatic colorimetric assay (Boehringer Enzymatics kits, Boehringer, Mannheim, cat no 237-574), and the LDL protein was determined according to the modified method of Lowry *et al.* [13], using bovine serum albumin as standard.

Resident mouse peritoneal macrophages were harvested from female NMRI mice using PBS solution, as previously described [14]. The macrophages were collected by centrifugation (300 × *g* for 5 min). Cells were resuspended in modified Eagle's medium (α-MEM, ICN Biomedicals Ltd.,

U.K.) containing gentamicin sulfate (50 μg/mL) and fungizone (0.25 μg/mL) (medium A), to which was added 15% (v/v) heat-inactivated FCS. The cells (~10^6) were plated on 35-mm cell-culture dishes and incubated for 24 hr at 37°C in air enriched by CO_2 (5%). After this incubation, the cells were washed 3 times with 0.5 mL of HAM's F10 medium (ICN Biomedicals, Ltd., U.K.). Prior to the experiment, the cells were left to rest for 1 hr in the third wash.

Cell Culture Experiment

HAM's F10 medium, without Phenol red because of the antioxidant property of the dye, was supplemented with L-glutamine (2 mM), gentamicin sulfate (50 μg/mL) and LDL (25 μg protein/mL) (medium B). The compounds to be tested were dissolved in ethanol and added to cell medium B, giving a final concentration of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M. The concentration of ethanol in the medium was 0.1%. One mL of medium B, containing the test substances, was added to the cells, which were then incubated at 37°C in air enriched by CO_2 (5%) for 24 hr. The experiments were performed in triplicate. Medium from each dish was then collected and separated from discharged cells by centrifugation (1000 × *g*) for 5 min.

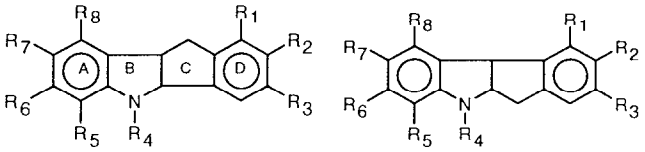
To determine the lipid peroxidation of the LDL, 250 μL of the cell media was mixed with 1.5 mL of 0.67% TBA and 1.5 mL of 20% trichloroacetic acid in glass tubes. The tubes were heated for 30 min at 95°C and then chilled in an ice bath. After addition of 200 μL 1% sodium dodecyl sulphate to eliminate turbidity, and after centrifugation (1000 × *g*) for 5 min, the formation of TBARS was measured [11].

RESULTS AND DISCUSSION

In the present study, we describe structure-activity relationships for a novel series of antioxidants, based on indenoindoles that inhibit chain-mediated lipid peroxidation. This type of compound is very potent, and the relationship between ionization potential and antioxidant effect *in vitro* is established in the present study, confirming earlier results by Brown *et al.* [15].

The inherent ease of oxidation of a compound is reflected in its electrochemical oxidation potential. The peak oxidation potentials for some selected indenoindoles are given in Table 1. The center of the antioxidant activity probably resides on the anilinic nitrogen atom with its lone pair of electrons. Delocalisation of this electron pair over the aromatic system seems to be of great importance, because removal of this possibility *via* N-acylation (e.g. compound 2) effectively decreased the ease of oxidation (>0.9 V). N-Alkylation (i.e. compound 3) also reduced the ease of oxidation somewhat. Steric strain, resulting in decreased overlap between the nitrogen-centered pair orbital and the aromatic π-orbitals, could be one of the causes of this effect. As expected, electron-donating alkyl groups on the A-ring (compounds 4 and 5) increased the ease of oxidation. In compound 6, the positive effect of A-ring methylation and the negative effect of N-methylation seem to balance one another effectively. Increasing the electron donation into the A-ring even further by the addition of a methoxy group (compounds 7 and 10) attenuated the oxidizability even further. Compound 8, incorporating a hydroxy group in direct conjugation with the anilinic nitro-

TABLE 1. Structure and corresponding oxidation potentials of the indenoindole compounds



a b

Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	Oxidpot.
1 (a)	H	H	H	H	H	H	H	H	0.52
2 (a)	H	H	H	Ac	H	H	H	H	>0.9
3 (a)	H	H	H	Et	H	H	H	H	0.62
4 (a)	H	H	H	H	H	H	Me	H	0.42
5 (a)	H	H	H	H	Me	H	Me	H	0.38
6 (a)	H	H	H	Me	H	H	Me	H	0.57
7 (a)	H	H	H	H	Me	Me	OMe	Me	0.29
8 (a)	H	H	H	H	H	Me	OH	Me	-0.15
9 (a)	H	H	H	H	H	H	NO2	H	0.76
10 (a)	H	H	H	H	Me	H	OMe	H	0.30
11 (a)	Me	OMe	Me	H	H	H	H	H	0.50
12 (b)	H	H	H	H	H	H	H	H	0.53
13 (b)	H	H	H	H	Me	H	OMe	H	0.32
BHT									0.80
Vitamin E									0.38

gen, extended this effect and made this compound very unstable. Again, as expected, incorporation of the electron-withdrawing nitro group into the A-ring (compound 9) effectively decreased the ease of oxidation. Substitution in the D-ring, on the other hand, as in compound 11, did not influence the oxidation potential to any greater extent. This is, again, as expected because the D-ring is isolated from the indoline unit. Finally, as is evident from compounds 12 and 13, reversal of the C-ring geometry, giving the iso-indenoindole series (b in Table 1), had little effect on the ease of oxidation.

Three different *in vitro* models were used to test the effectiveness of these indenoindole compounds in inhibiting lipid peroxidation. The most basic one involved Fe/ascorbate-induced lipid peroxidation of sonicated soybean phospholipid suspensions. Representative dose-response curves for BHT and compound 10 (H 290/30) are shown in Fig. 1. In the second model, the inhibitory potency of the compounds in relation to reoxygenation-induced lipid peroxidation of kidney tissue was determined. In the third model, the inhibitory effect on cell-mediated oxidative modification of LDL was measured. The inhibitory potencies of the indenoindole compounds tested in the 3 models are given in Table 2. In general, as is evident from Tables 1 and 2 and Fig. 2, there exists a good correlation between the pIC_{50} values in the 3 test models, as well as for the measured oxidation potential of the compounds. However, compound 8 was relatively ineffective in the models in which it was tested, despite its very high oxidizability. This might be attributed to its low chemical stability, which would result in rapid deterioration under the experimental conditions used in these investigations. Compound 2 is the only one from Table 2 that is not included in Fig. 2. As expected, it did not have any inhibitory effect at 10 μM in any of the experimental models

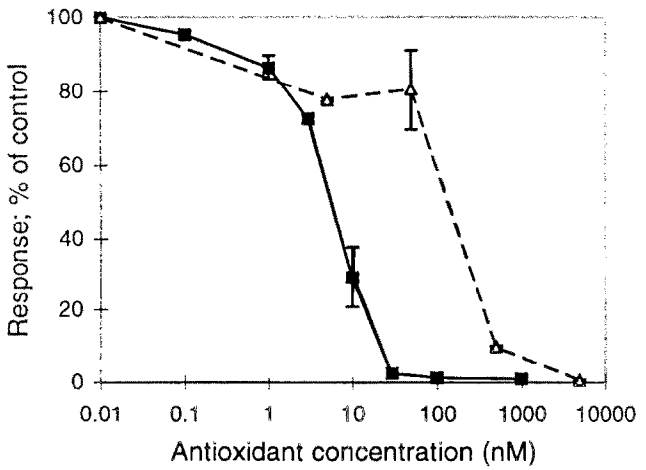


FIG. 1. Representative dose-response curves for inhibition of Fe/ascorbate-induced lipid peroxidation in phospholipid vesicles from soybean lecithin. The dotted line represents BHT and the solid one H 290/30. Values are presented as mean ± SD. The experiments were performed twice, each time in triplicate.

TABLE 2. Potency (pIC_{50} values) for inhibition of *in vitro* lipid peroxidation by a series of novel indenoindoles

Compound no.	LDL	Fe/Asc	Kidney
1	7	7.2	5.7
2	<5	<5	<5
3	6.4	6.1	<5
4	6.9	7.1	6.4
5	7.4	7.3	6.5
6	6.6	6.4	5.3
7	7.9	8.0	*nd*
8	6.6	6.4	*nd*
9	*nd*	6.1	<5
10*	8.3	8.0	6.8
11	7.1	7.2	6.0
12	6.7	6.9	5.8
13†	8.7	8.2	6.8
Reference compounds:			
BHT	6.9	6.6	5.3
Vitamin E	*nd*	5.6	<5
Trolox	7.1	*nd*	4.7

* Compound no. 10 and †compound no. 13 are referred to in the text using the official nomenclature H 290/30 and H 290/51, respectively. *nd*, not determined; pIC_{50}, -log (M compound) to reduce TBARS formation by 50%; LDL, oxidative modification of LDL by mouse peritoneal macrophages; Fe/Asc, Fe/ascorbate-induced lipid peroxidation in phospholipid vesicles from soybean lecithin; Kidney, isolated rat renal tissue subjected to hypoxia and reoxygenation.

tested. Compared to the two reference compounds, vitamin E and BHT, the most potent indenoindole compounds were 10- to 100-fold more potent.

The exact mechanism of action of these new antioxidants has not been studied in detail, although the available data and their high lipophilicity indicate that they mainly act as chain-breaking antioxidants. As is evident from the accompanying paper, compound 13 (H 290/51) at least, is also capable of recycling with ascorbic acid. Furthermore, this compound also seems to be able to quench vitamin E radicals; thus, acting as an inhibitor of tocopherol-mediated lipid peroxidation.

The concentration of compound 13 (H 290/51) that gives half-maximal inhibition (IC_{50}) of lipid peroxidation is approximately 0.01 μM in the Fe/ascorbate assay. This is a negligible concentration compared to the 10 μM iron anions used to induce lipid peroxidation. We can, therefore, exclude the possibility that the inhibition of peroxidation in this assay, caused by the indenoindoles, depends on a metal-chelating property of these compounds. Moreover, H 290/51 is also a powerful antioxidant in the metal-free system described in the accompanying paper.

In the *in vitro* model using isolated slices from rat kidneys, the tissue was subjected to hypoxia and reoxygenation with either the test compound or the vehicle present in the medium. In Table 2, the potency values for the different compounds tested are presented. The most potent indenoindoles, compound 10 and 13 (H 290/30 and H 290/51, respectively) were equipotent and 10-fold more effective lipid peroxidation inhibitors than the reference compound BHT and approximately 100-fold more potent than vitamin E and its water-soluble analog Trolox.

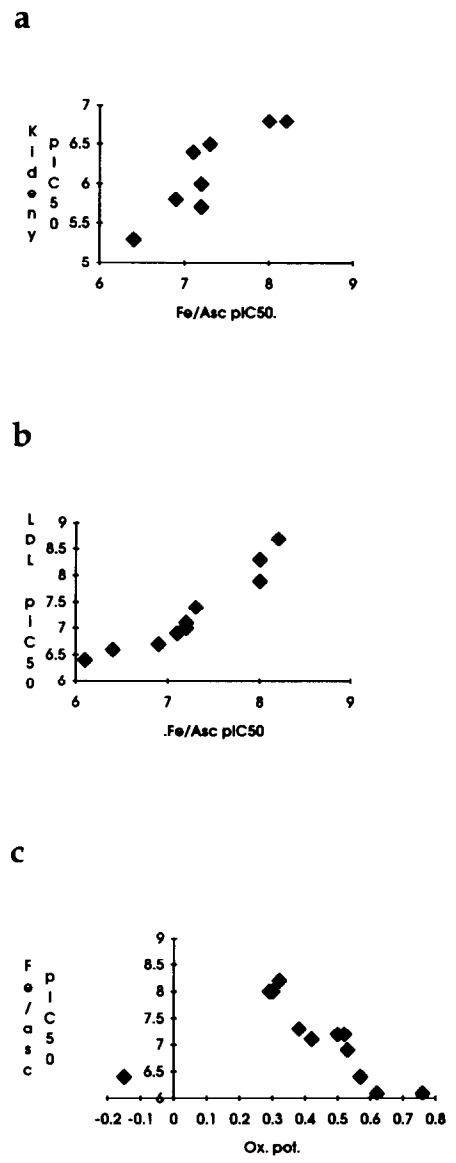


FIG. 2. Correlation for different indenoindoles in 3 experimental models on lipid peroxidation. pIC_{50}, -log (M compound) to reduce TBARS formation by 50%; LDL, oxidative modification of LDL by mouse peritoneal macrophages; Fe/Asc, Fe/ascorbate-induced lipid peroxidation in phospholipid vesicles form soybean lecithin; kidney, rat isolated renal tissue subjected to hypoxia and reoxygenation.

To evaluate whether or not indenoindole compounds, such as the ones tested in this study, also have broader pharmacological effectiveness, additional *in vivo* pathophysiology studies are needed. One such study was recently conducted in rabbits [16]. In these experiments, the animals were subjected to left renal artery occlusion for 60 min. Before reperfusion, H 290/51 (20 μmol/kg) was given i.v. All 8 rabbits that had received H 290/51 survived the 7-day observation period and, at the end of the follow-up period, normalized plasma creatinine values were observed. In the control group, on the other hand, 6 of 8 rabbits died during the observation period, and plasma creatinine was drastically increased.

The results from the *in vivo* studies on kidneys indicate that indenoindole compounds such as H 290/51, which reduces reoxygenation-induced TBAR-formation *in vitro*, could also drastically improve kidney function and survival *in vivo* after severe renal ischemia and reperfusion. In this respect, the screening procedure used in this study has been a relevant method for selecting potent lipid peroxidation inhibitors that have proven beneficial in an *in vivo* pathology model.

The indenoindole compounds presented in this study are very potent inhibitors of macrophage-induced oxidative modification of LDL (Table 2). The most potent compound, 13 (H 290/51), has a pIC_{50} value of 8.7. The effects of the indenoindoles on oxidative modification of LDL suggest that these compounds could have antiatherogenic properties. It has been proposed that atherosclerosis is initiated by the presence of oxidatively modified LDL in the intima of the arterial wall. The prevention of LDL modification could be expected to attenuate foam cell formation and the initiation of plaques. Probucol, a hypolipemic drug with antioxidant properties, has been shown to have an antiatherogenic effect in WHHL rabbits [17, 18]. Carew *et al.* [17] demonstrated that a considerable part of the antiatherogenic effect of probucol was not due to its hypolipemic properties. Moreover, BHT, the reference antioxidant in this paper, which does not cause any hypolipemic effects in rabbits, has been demonstrated to have an antiatherogenic effect in cholesterol-fed rabbits [19]. This further supports the hypothesis that antioxidants might have antiatherogenic properties.

The protective effects of indenoindoles on oxidative modification of LDL presented in this paper merit further *in vivo* studies on possible antiatherogenic effects on these lipid peroxidation inhibitors.

The authors gratefully acknowledge Dr. Johanna Deinum, Astra Hässle Preclinical Research Laboratories, for valuable advice and helpful discussions.

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