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THE CORRELATION OF THE OXIDATION POTENTIALS OF STRUCTURALLY RELATED DIBENZO[1,4]DICHALCOGENINES TO THEIR ANTIOXIDANCE CAPACITY IN BIOLOGICAL SYSTEMS UNDERGOING FREE RADICAL-INDUCED LIPID PEROXIDATION

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Abstract—A series of structurally related dibenzo[1,4]dichalcogenines possessing similar lipid solubilities but greatly differing oxidation potentials were tested for their ability to inhibit stimulated lipid peroxidation in ADP/Fe^{2+}/ascorbate-treated liver microsomes and in hepatocytes treated with either *t*-butylhydroperoxide or diquat. In general, there was a close correlation between the half-wave oxidation potential of a particular compound and its antioxidant activity in the microsome and cell systems, with compounds possessing the lowest potential being the most potent antioxidants and vice versa. The Te/O and Te/S and S/O-substituted compounds, with oxidation potentials between 0.65 and 0.87 V, demonstrated most potent activity. Above this potential the antioxidant activity of the structure declined rapidly. The Te/O and Te/S compounds are among the most potent synthetic antioxidants described possessing IC_{50} values in the microsome system lower than 0.5 μM. This study clarifies the critical role of redox potential of an antioxidant site on a particular molecule without the complication of variable lipid solubility and may allow the definition of an optimal potential for antioxidant activity in biological systems.

Interest in organo-selenium compounds as medicinal agents is increasing. We have been working to develop selenoorganic anti-inflammatory agents, particular for use in the lung. Some time ago Ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one; **1**; Fig. 1) was shown to inhibit lipid peroxidation in biological systems [1–4] and catalyse thiol-dependent glutathione peroxidase-like breakdown of hydroperoxides [1–3]. These "antioxidant" activities may potentially interfere with events occurring in the process of acute and chronic inflammation in a number of ways, especially by affecting the consequences of activation of the oxidative burst of polymorphonuclear leukocytes [5, 6]. Ebselen has been demonstrated to be an effective anti-inflammatory agent in the treatment of a variety of animal models of inflammation including monoarthritis and alveolitis/bronchiolitis in the rat [7–9].

In an effort to define which biological "antioxidant" property of Ebselen contributes to the observed anti-inflammatory effects of the structure we began the search for other structures which might possess one or the other activity. The oxidation potential is probably an important controlling factor in the biological activity of an antioxidant. However, the systematic variation of the oxidizability of a series of compounds (e.g. by substitution) can often not be achieved without changing other relevant parameters (like lipophilicity, conformation etc.). In order to determine the role of the oxidation potential we designed a series of compounds similar in shape and lipophilicity. It became apparent that dibenzo[1,4]-dichalcogenines should be suitable as model compounds since: (a) a wide range of oxidation potentials can be expected by variation of the nature and combination of chalcogen atoms; (b) benzannelation results in rigid, similar conformations of the studied compounds; and (c) substances with similar lipophilicity can be expected permitting accurate comparisons. Here we report the investigation of a series of very closely related dibenzo[1,4]-dichalcogenines (**2–8**) possessing similar lipid solubility but displaying vastly different redox potentials. A striking dependence on the redox potential of the dichalcogenine heteroatoms for the biological activity of the carrier molecule as an antioxidant in microsomal and cellular systems undergoing lipid peroxidation was revealed.

MATERIALS AND METHODS

All the dibenzo[1,4]dichalcogenines used in these studies were synthesized and characterized as reported previously [10]. Tertiary butyl hydroperoxide (*t*-BH; 70% (v/v) aqueous solution) and

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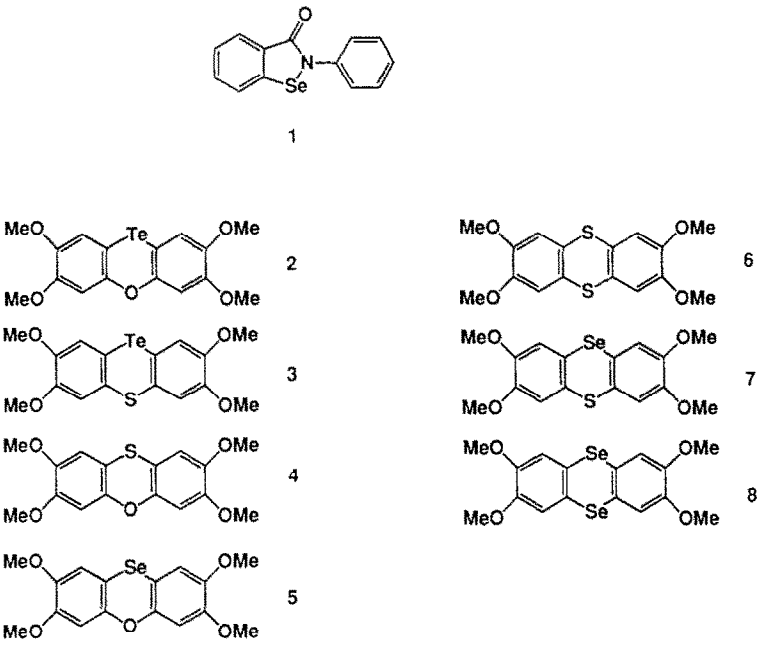

Fig. 1. The structural formulae of the compounds (**1–8**) detailed in this study.

thiobarbituric acid (TBA: 99%) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Diquat was the kind gift of Dr Lewis Smith, ICI (Macclesfield, U.K.). Bis-chloronitrosourea (BCNU: >99%) was obtained from Bristol-Meyers (Stockholm, Sweden). Other reagents and chemicals were of the highest grade obtained from local suppliers.

Lipid peroxidation in microsomes. The livers of male Sprague–Dawley rats were exsanguinated, excised and homogenized in an ice-cold sucrose (250 mM)/phosphate (50 mM) buffer, pH 7.4, using a polytron. The homogenate was centrifuged once at 12,000 *g*, at 4° for 30 min and the supernatant recentrifuged at 105,000 *g*, at 4° for 60 min. The pellet was resuspended and washed twice with 150 mM KCl before being used in the experiments. Microsomes were prepared fresh before each batch of experiments.

Microsomal lipid peroxidation was performed in incubations constructed as follows: incubations (1 mL) in phosphate (50 mM) buffer, pH 7.4, contained microsomal protein (1 mg), ADP (200 μM), $FeSO_4$ (1 μM) and vehicle/test substance, were preincubated for 5 min at 37° before addition of the initiation stimulus ascorbate (50 μM). For screening experiments the accumulation of MDA over 30 min of incubation in antioxidant-treated samples was compared to control levels in microsomes treated with dimethyl sulphoxide (DMSO) compared to control levels in microsomes treated with DMSO vehicle only. Individual 50% inhibition concentrations (IC_{50} values) were calculated from the best-fit curve of the effect of a range of concentrations of the compounds. Controls demonstrated that the compounds did not react with TBA-reactive substances in the system. In addition, effects of selected compounds on the time course of peroxidation up to 30 min was studied more closely. In all cases the DMSO concentration of the incubations was less than 0.5% (v/v). This concentration of the vehicle did not affect the time course of peroxidation or the extent of peroxidation after 30 min. The extent of lipid peroxidation was assessed by the assay of TBA-reactive substances as described below.

Lipid peroxidation in hepatocytes. Hepatocytes were prepared by collagenase perfusion of livers as described previously [11]. Freshly isolated hepatocytes (>95% viable by tryphan blue exclusion) were incubated in round-bottomed flasks at a concentration of 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, supplemented with 12.5 mM HEPES. In some cases cells were treated with BCNU (50 μM) for 30 min prior to use. Two distinct peroxidation stimuli were employed, one induced by a *t*-BH (1 mM) driven organic fenton reaction in the cells to yield the hydroxyl radical ($^{\cdot}OH$) [12] and the other involving the intracellular redox cycling of diquat (1 mM) with molecular oxygen to yield $^{\cdot}OH$ in cells whose glutathione reductase was inhibited with BCNU [4]. Compounds were introduced into the incubation in DMSO [which was never in excess of 0.5% (v/v)] and allowed to pre-equilibrate for 5 min before addition of the peroxidation stimuli. Aliquots (0.5 mL) were taken at intervals for determination of TBA-reactive substances [13]. Inhibition parameters for the compounds were calculated as for microsomes.

Chemical and biochemical analysis. Lipid peroxidation was assessed by assay of the accumulation of TBA-reactive substances in supernatants of

trichloracetic acid-precipitated samples of cells and microsomes as described previously [13].

Briefly, aliquots (0.5 mL) of incubations were mixed with equal volumes of TCA (10% v/v) containing 10 mM butylated hydroxytoluene and then reacted with TBA at 95° for 15 min. The samples were then centrifuged (1000 *g*, 5 min) and the absorbance determined at 535 nM. The concentration of MDA was determined using $e = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$. We cannot exclude that the compounds affect the cellular metabolism of MDA in the hepatocyte system studied. However, from the data obtained it is deemed unlikely that inhibition/activation of MDA metabolism would be so closely related to the redox potentials of the compounds.

Half-wave oxidation potentials were determined by cyclic voltammetry under nitrogen in a thermostatted, undivided IBM cell with a platinum-button working electrode and a platinum-wire counter electrode with an s.c.e. reference electrode. The cell was controlled with a PAR 173 galvanostat equipped with a 175 programmer and a 179 coulometer. Scan rate = 100 mV/sec. Substrate concentration was 2 mM in a 0.15 M electrolyte solution (Bu_4NClO_4) in dry dichloromethane, with *ca.* 1% trifluoroacetic anhydride added [10].

Lipophilicity measurements were performed according to the method described by Valkó [14].

RESULTS

Table 1 reports the results of the determination of the half wave oxidation potentials for a series of O-, S-, Se- and Te-containing 2,3,7,8-tetramethoxy dibenzo[1,4]dichalcogenines (A) their relative lipophilicities log $k'(0)$ (B) and their relative efficacy of inhibition of ADP/Fe^{2+}/ascorbate-induced lipid peroxidation in liver microsomes (C) and *t*-BH- (D) and diquat- (E) induced lipid peroxidation in isolated hepatocytes.

It can be seen that the compound with the lowest oxidation potential, and thus the most easily oxidizable substance, contained Te and O in the central ring ($E_{1/2} = 0.65$ V) (compound **2**). The oxidation potential of the compounds were shown to increase steadily with [1,4] substitution in the order Te/O (**2**) < Te/S (**3**) < S/O (**4**) ~ Se/O (**5**) < S/S (**6**) < Se/S (**7**). The Se/Se compound (compound **8**) was shown to possess the highest potential of 1.06 V.

In the biological systems used to generate lipid peroxidation, control peroxidation rates amounted to 16 μM MDA/mg protein/30 min in microsomes and 6.6 μM/10^6 cells/hr in *t*-BH-treated hepatocytes and 6.5 μM/10^6 cells/2 hr in those BCNU-pretreated cells treated with diquat. Examination of the kinetics of microsomal peroxidation confirmed the linearity of peroxidation over 30 min of incubation but a lag-phase was not evident after 5 min of incubation (Fig. 2). This figure also shows that compound **2** did not yield a lag-phase in the induction of peroxidation at concentrations <100 nM but that this lag in microsomal peroxidation was clearly evident at 500 nM under the condition of the assay. It can be

Table 1. A comparison of the half-wave oxidation potentials and relative lipophilicities of structurally-related dibenzo[1,4]dichalcogenines with their antioxidance capacity in a variety of biological systems undergoing lipid peroxidation

Compound No.	A Half-wave oxidation potentials E_i in CH_2Cl_2 volts*	B Relative lipophilicity log k' (0)	C ADP/Fe^{2+}/ascorbate-induced lipid peroxidation in liver microsomes		D *t*-BH-induced lipid peroxidation in isolated hepatocytes		E Diquat-induced peroxidation in BCNU-treated hepatocytes
			% Inhibition 50 μM†	IC_{50} (μM)‡	% Inhibition 50 μM†	IC_{50} (μM)‡	% Inhibition 50 μM†
2	0.65	2.41	100	0.05	100	<1	78
3	0.79	2.75	100	0.25	93	1	100
4	0.87	2.59	100	25	74	12	88
5	0.89	2.51	69	30	51	48	88
6	0.98	2.78	19	>50	36	>50	70
7	1.02	2.75	14	>50	22	>50	47
8	1.06	2.77	25	>50	20	>50	42

* Potentials recorded in CH_2Cl_2 at scan rate of 100 mV/sec.
† % Inhibition of the accumulation rate of TBA-reactive substances as compared to controls performed in parallel.
‡ The concentration of substance required to inhibit the control peroxidation rate by 50% calculated from best-line fit of concentration vs peroxidation data for at least 10 concentrations of the test compound.

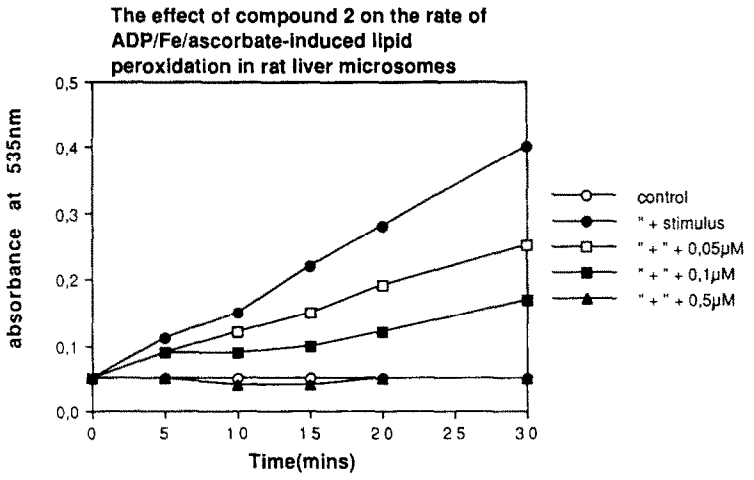

Fig. 2. Incubations were performed as in Materials and Methods with the addition of compound **2** at various concentrations. Peroxidation was assessed as TBA-reactive substances and quantitated spectrophotometrically. Stimulus = ADP/Fe^{2+}/ascorbate plus DMSO vehicle.

seen that in the microsomal system and the *t*-BH-treated hepatocytes, the gross rank-order of antioxidance, as judged by comparative inhibitory effects at 50 μM compound and their respective IC_{50} values, were very similar to the rank order of redox potentials. In these two systems compounds **2** and **3** were clearly most active possessing IC_{50} values equal to, or well below 1 μM. Indeed, in the *t*-BH hepatocyte system compound **2** still produced 80% inhibition at 500 nM (data not shown). Although the rank ordering of inhibitory capacity of the seven potent antioxidants in the microsome system was somewhat different from the redox potential order, it is clear from the IC_{50} data that compounds **6**, **7** and **8** represent a subclass of compounds with weak antioxidance activity in both the microsome and the *t*-BH-treated cell systems.

In the case of the diquat-treated cell system the rank order of the efficacy of the antioxidance of the compounds agreed well with the redox potentials except for compound **2** which was not the most effective agent, inhibiting only 78% of the control peroxidation at 50 μM. However, compound **3** was 100% effective followed by compounds **4**, **5** and **6**, respectively. Again compounds **7** and **8** were clearly in a subclass of their own inhibiting only 47% and 42% of the control peroxidation at 50 μM, respectively.

DISCUSSION

It is clear from the data presented in Table 1 that having standardized the lipid solubility of a potential antioxidance backbone there is indeed a great degree of homology between the oxidation potential of a potential electron donor site and the biological effect of the structure as an antioxidant. However, a note of caution in interpreting the data from the cellular experiments must be conceded as the compounds may have effects at other levels of the respective mechanisms of toxicity of *t*-BH and diquat such as on Ca^{2+} movements and reduced nucleotide balance.

Chemically, increased chalcogen substitution in the central ring normally leads to a lowering of the oxidation potential of the donor [15]. However, in the series of tetramethoxy-substituted tricyclic systems studied here the lowest oxidation potential was found for an electron donor with oxygen substitution, i.e. $E_{1/2}$O/Te < O/S ~ O/Se. This may be explained by the increased planarity of the oxygen-containing heterocycles [16], allowing more efficient delocalization of the cation radical over the aromatic π electron system. It has been shown [16] that the cation radical of the non-planar compound **6** is in fact planar in the tri-iodide salt. In addition, it is interesting to note that similar structures such as the phenothiazines possess antioxidance capacity [17].

In addition to demonstrating a clear dependence on oxidation potential for biological activity the most active compounds (**2** and **3**) represent two of the most potent synthetic, biologically relevant antioxidants thus-far described. In the systems tested, effective phenolic antioxidants such as butylated hydroxyanisole (BHA) had IC_{50} values of 6 μM in the microsome system and 10 μM in the cell system treated with *t*-BH [12]. It is also of interest to note that Ebselen was relatively ineffective by comparison to the present structures in the microsome and *t*-BH systems (IC_{50} values of 3 and >50 μM, respectively) and actually caused *increased* peroxidation in the diquat system (unpublished observations).

It is hoped that these and other studies will help establish rational grounds for the use of selenium and tellurium-containing antioxidants as therapeutic agents. We are at present exploring their efficacy in a small animal model of pulmonary inflammation [9]. These and other model mechanistic studies may allow us to predict structures with the appropriate

redox potentials, predict other structural details facilitating optimal lipid solubility and define other aspects of systemic disposition to facilitate antioxidant drug design.

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