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Evaluation of sulfur, selenium and tellurium catalysts with antioxidant potential

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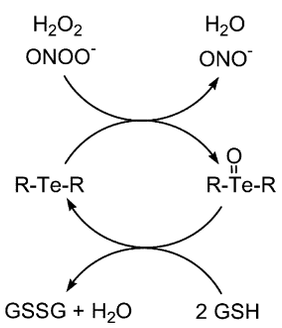
Oxidative stress is implicated, either directly or indirectly, in the pathology of a range of human diseases. As a consequence, the development of efficient antioxidants for medical use has become increasingly important. We have synthesised a range of structurally related organo-sulfur, -selenium and -tellurium agents and demonstrated that a combination of electrochemical methodology, *in vitro* assays and cell culture tests can be used to rationalise the antioxidant activity of these catalytic agents. Based on its exceptionally low anodic oxidation potential (*E*pa) and high activity against the representative oxidative stressors *tert*-butyl hydroperoxide and peroxynitrite, 4,4′-dihydroxydiphenyltelluride is predicted to be a potent antioxidant. This compound exhibits a correspondingly high activity with a remarkably low IC_{50} value of 20 nM, when tested in PC12 cell culture using a bioassay indicative of the early stages of Alzheimer's disease.

Introduction

A wide range of human diseases are associated with a disturbed cellular redox balance known as oxidative stress (OS).[1] OS results from an increase in intracellular concentrations of oxidizing species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to the oxidation of membranes, proteins, DNA and ultimately to cell death. Stoichiometric antioxidant strategies that focus on inhibiting oxidative damage early in the progression of OS related diseases have recently been investigated *in vitro* and *in vivo*.[2–6] These regimes mostly use antioxidants such as the vitamins C and E and cysteine derivatives[7] which reduce oxidative stressors. As stressors consume these antioxidants, high concentrations of antioxidants are frequently required to be effective—a significant barrier in the development of antioxidant therapies.

Theoretically, catalytic antioxidants would have considerable advantages: By catalytically increasing the effects of the naturally occurring antioxidants still present within cells during oxidative stress (such as the cellular tripeptide glutathione, GSH), the catalyst would be able to enhance the rate of reduction of oxidative stressors. Since the catalyst is not consumed during the reaction it also exerts an effect at considerably lower concentrations than stoichiometric antioxidants. High activity at low concentrations—the hallmark of catalysts—also circumvents the considerable pharmacological challenges inherent in trafficking large amounts of a drug to a specific organ.

We are currently investigating the design and application of antioxidant catalysts that mimic the catalytic activity of the human selenoenzyme glutathione peroxidase (GPx).[8–11] This enzyme reduces toxic hydroperoxides and peroxynitrite (PN) in the presence of GSH.[12] Synthetic catalysts that mimic peroxidase activity can be designed by covalently incorporating a selenium or tellurium atom within an organic framework.[9,11,13] In contrast to conventional enzyme mimic design, a broader substrate specificity is actually of advantage, since it enables GPx mimics to detoxify a wider range of ROS and RNS when compared to the parent enzyme (Scheme 1).



Scheme 1 Catalytic cycle for the reduction of peroxynitrite or hydrogen peroxide by organochalcogens. The telluride is initially oxidised to a telluroxide by either peroxynitrite or peroxide, reducing the oxidative stressor in the process. The telluroxide is then reduced by two equivalents of thiol to regenerate the active site *via* a tellurylsulfide intermediate. The overall redox reaction therefore consists of the reduction of an oxidative stressor and the oxidation of two thiols to form a disulfide. Within the cell, the predominance of the GSH thiol results in the formation of GSSG.[10]

One such selenium agent, 2-phenyl-1,2-benzoisoselenazol-3(2*H*)-one (ebselen), has already been clinically evaluated for the treatment of stroke[14] and a set of empirical design criteria for effective GPx mimics have become apparent.[9–11] In order to gain a deeper insight into the biochemical efficiency of organochalcogen catalysts, we have studied a range of structurally related compounds with the following features (for structures see Table 1 and Fig. 1): (i) the presence of a catalytic centre (sulfur, selenium or tellurium); (ii) mono- or dichalcogenides as the catalytic core of the molecule; (iii) different ring substituents to increase activity; (iv) the presence of at least one aryl ring attached to the chalcogen atom to provide chemical stability (telluroxides with alkyl substituents are known to undergo elimination reactions[15]); (v) the incorporation of more than one redox active centre in the same molecule (compound **7**, Fig. 1).

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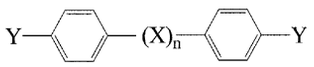
Table 1 Relationship between chemical structure, *E*pa and activity in metallothionein (MT) zinc release and peroxynitrite quenching assays.[11] Experimental details are given in the text. All experiments were repeated at least three times with less than 10% relative error

Oxidation potentials and antioxidant activities of organochalcogens



Compound	X	*n*	Y	*E*pa/mV	MT Zn release (%)	HPA IC_{50}/μM	DHR IC_{50}/μM
Ebselen	NA	NA	NA	+1044	100[b]	741[b]	0.2[b]
1	S	1	H	+1366	0	ND	ND
2	S	2	OCH_3	+1176	0	ND	ND
3	Se	1	OCH_3	+753	20.0	ND	2.38
4	Se	2	OCH_3	+1000	20.1	381	0.5
5	Te	1	OCH_3	+368	53.5	109[c]	0.24[c]
6	Te	2	OCH_3	+578	53.8	75	0.3
7	Te	1	[a]	+527	47.9	174	0.22
8	Te	1	$O(O)CCH_3$	+448	32.9	135	0.17
9	Te	1	[a]	+694	32.6	138	8.41
10	Te	1	OH	+299	74.2	128[c]	0.29[c]

[a] Formula shown in Fig. 1. [b] The amount of zinc released from MT by ebselen was virtually identical to the theoretical zinc content based on the MT concentration; ebselen has previously been evaluated in the PN assays, where it gave IC_{50} values of 60 μM (using 10-fold less PN) in the 4-hydroxyphenylacetate (HPA) assay and 0.2 μM in the dihydrorhodamine-123 (DHR) assay.[27] [c] The activities of ompounds **5** and **10** have previously been evaluated in the HPA assay (at 20 μM) and the DHR assay (at 3 μM), where they inhibited the nitration of HPA to 67% and 30% of the maximum and the oxidation of DHR to 59% and 17% of the maximum, respectively.[10] NA, not applicable; ND, not determined.

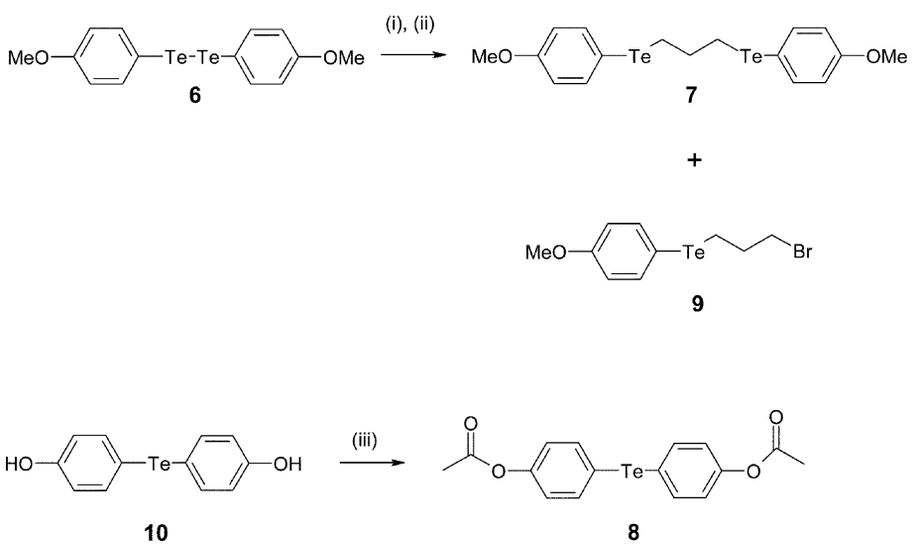


Fig. 1 Synthetic pathways leading to tellurides **7**, **8** and **9**. *Reagents*: (i) $NaBH_4$, Ar, EtOH; (ii) 1,3-dibromopropane; (iii) acetic anhydride.

This paper describes the synthesis and evaluation of sulfur, selenium and tellurium agents with antioxidant potential, particularly emphasising the importance of a multidisciplinary approach towards antioxidant characterisation that includes electrochemistry, *in vitro* and PC12 cell culture methods.[11] We discuss a correlation between the "ease of oxidation" given by the anodic oxidation potential (*E*pa) in aqueous media and the activity in *in vitro* assays that exists for a range of structurally related organochalcogens. Selenium and tellurium agents are shown to act as suitable antioxidants against diverse oxidative stressors with different underlying redox mechanisms,[9,10,16] hence supporting the notion that catalysts might provide the most effective antioxidant defence. Based on the encouraging results obtained for 4,4′-dihydroxydiphenyltelluride in PC12 cells, we propose that catalytic redox agents are promising antioxidant candidates with possible medical use in the therapy of Alzheimer's disease (AD).

Results and discussion

Cyclic voltammetry

The cyclic voltammograms of most chalcogen compounds displayed only one irreversible oxidation peak (*E*pa) between 0 and +1200 mV *vs.* standard silver electrode (SSE) (Table 1) with the exception of telluride **10** (Fig. 2) that exhibited two reversible redox couples, one at +277 mV (Epa_1 = +299 mV, Epc_1 = +255 mV, Δ*E*p = 44 mV, 1.3 electron transfer) and the second at +792 mV (Epa_2 = +820 mV, Epc_2 = +763 mV, Δ*E*p = 57 mV, 1.0 electron transfer, with a very small reduction current ic_2; this special feature of **10** is discussed below in more detail). For the other organochalcogens, the value for the oxidation potential ranged from +300 to +1400 mV *vs.* SSE with a clearly observable trend, the tellurides having the lowest potential averaging around +400 mV, increasing to approximately +900 mV for the selenides and to +1200 mV for the sulfides. Interestingly, both mono- and dichalcogenides displayed an oxidation peak, allowing a basic comparison of the two species.

In aqueous solution anodic oxidation of these chalcogens (apart from **10**, see below) is associated with an irreversible process[11] that involves the formation of an intermediate radical cation species rapidly followed by reaction with water to form the chalcogen- or dichalcogen-oxide.[17,18] The formation of such species (*e.g.* disulfide *S*-oxides, selenoxides) is also observed in biochemical studies[19,20] and has already been implicated in catalytic antioxidation (*e.g.* oxidative activation of ditellurides, selenylsulfides).[17] On the assumption that the ease of electro-

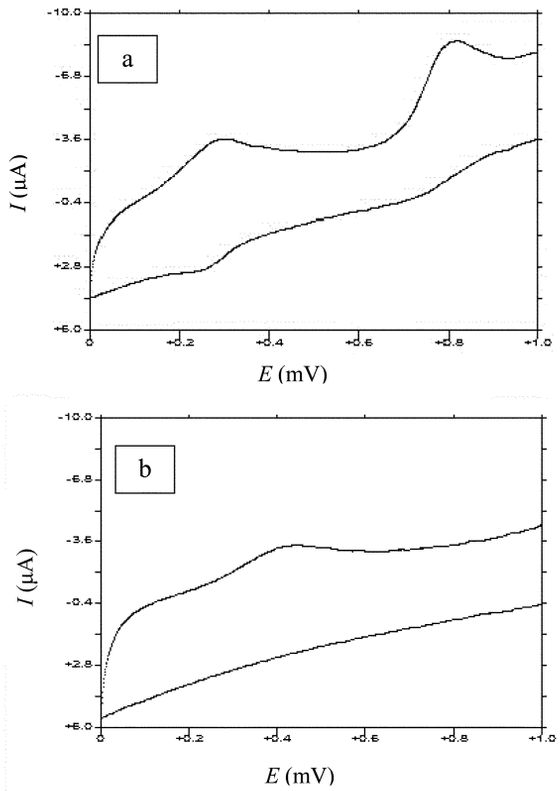


Fig. 2 Cyclic voltammogram of (a) telluride **10** and (b) its methoxyphenyl-analogue **5**. Organochalcogens (50 μM) were studied in potassium phosphate buffer (50 mM, pH 7.4) containing 30% methanol at 25 °C using a SSE reference electrode, a Pt counter electrode and a glassy carbon working electrode. The scan rate was 500 mV s^{-1}. Since the presence of methanol affects the pH value of solutions, pH was measured with the inclusion of 30% MeOH in the buffer.

chemical oxidation is correlated with the ease of oxidation of the chalcogen *in vitro* and *in vivo*, *E*pa may be useful as a predictor of biological activity.

In vitro characterisation of antioxidant activity

In order to evaluate this relationship between *E*pa and biochemical redox activity, the efficacy of compounds was examined *in vitro* by monitoring their effect on the interaction of the representative oxidative stressors *tert*-butyl hydroperoxide and PN with biological targets.

In the metallothionein (MT) assay, peroxidase activity was quantified by measuring the effect of the compounds on the rate of reduction of *tert*-butyl hydroperoxide by MT. MT is a small protein (MW ~ 6 kDa) whose 20 cysteines tightly bind seven zinc atoms in a Zn_4Cys_{11} and Zn_3Cys_9 cluster. Its thiol ligands are redox-sensitive but their oxidation is generally considerably slower than the oxidation of "free thiols". MT, in combination with the chromophoric dye 4-(2-pyridylazo)-resorcinol (PAR), can therefore be used in a highly reliable assay for monitoring the catalytic effect of organochalcogens on the interaction of peroxides with thiols.[9,21–23]

Table 1 and Fig. 3 show that *E*pa correlates well with the activity in the MT assay, with the lowest potentials associated with the highest zinc release. A reasonably linear relationship was obtained when the *E*pa values were plotted against percent zinc release (Fig. 3), supporting the notion of *E*pa as a predictor of activity. As expected, the sulfur analogues showed no activity in the assay, in accord with their high *E*pa values while compound **10** was by far the most active, with both the lowest *E*pa of +299 mV and the highest zinc release of 74.2%.

It is important, however, to emphasise that this comparison only works for compounds with the same (or similar) underlying redox mechanisms. The monoselenide **3** and the diselenide

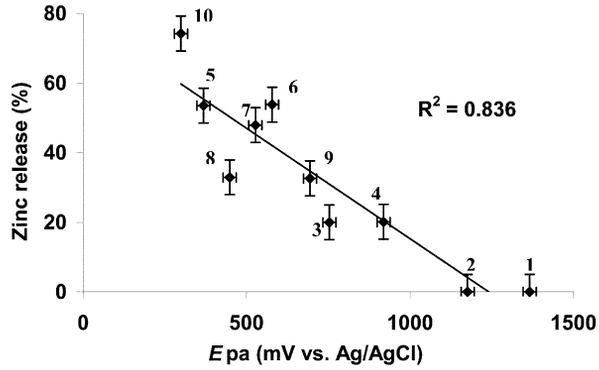


Fig. 3 Correlation between the lowest anodic potential (*E*pa) and zinc release from metallothionein for **1**–**10**. The maximum zinc transferred from MT (0.5 μM) was established by incubation with ebselen (10 μM) and PAR (100 μM). The values for zinc release by the compounds (200 nM for **1**–**6**, **8**–**10** and 100 nM for **7** to allow for two catalytic sites) were established by continuously monitoring the formation of the $Zn(PAR)_2$ complex and using the reading after 60 min. Values are expressed as a percentage of the maximum, data points are labelled according to the assignments in Table 1.[11]

4 showed a 200 mV difference in *E*pa but displayed similar *in vitro* activities, a result also obtained for the mono- and ditellurides **5** and **6**. Dichalcogenides are known to react with thiols *via* formation of mixed selenylsulfides (Se–S) and tellurylsulfides (Te–S), while monochalcogenides react *via* selenoxides (Se=O) and telluroxides (Te=O).[9,19] The oxidation of dichalcogenides is further complicated by the ability of the oxidised species to undergo dissociation and recombination reactions.[24,25] These significant differences in the redox mechanism of structurally related compounds must be taken into consideration when discussing oxidation and reduction potentials.[26]

The second assay evaluated activity against PN, a stressor that can undergo electrophilic as well as radical reactions. In contrast to the peroxides, PN can induce both oxidation and nitration reactions in biological targets, resulting in damage to DNA and a wide range of proteins and lipids. These two reactions can be assayed independently by examining the PN-mediated oxidation of dihydrorhodamine-123 (DHR) and the nitration of 4-hydroxyphenylacetate (HPA).[10,27] These oxidation and nitration reactions can be counteracted by the addition of organochalcogens, the efficacy indicating the antioxidant effect of the compound.

Organochalcogens are known to interfere with both the oxidation and nitration reactions of PN. The HPA assay is not catalytic, rather it monitors a stoichiometric interaction between PN and catalyst that competes with the PN–HPA nitration reaction. Compounds with *E*pa below +700 mV showed roughly similar ability to quench the nitrating effects of PN with a mean IC_{50} of around 125 μM (Table 1), indicating a stoichiometric, *i.e.* non-catalytic activity of these agents. In contrast, compounds with more positive *E*pa values were considerably less active.

Activity in the DHR oxidation assay indicative of PN-induced oxidation was similar to the reported ebselen activity[27] where the compounds protected DHR from PN-induced oxidation at concentrations of around 0.25 μM (Table 1). As is the case for the HPA assay, the DHR assay is not a catalytic assay and the oxidation potential of the chalcogen atom is not sufficient in itself to account for the different quenching effects.

Redox potential versus redox mechanism: implications for *in vitro* activity

These *in vitro* findings support the notion that selenium and tellurium agents have a broad-range specificity and mechanistically can act in different ways—and the MT, HPA and DHR

assays exemplify this broad activity. While a general correlation exists for peroxidation (*i.e.* between *E*pa and activity in the catalytic MT assay), specific mechanistic details of oxidation of individual agents have to be considered in each case.

This is particularly true for compound **10**, which contains a hydroxy group in each *para* position. This compound was therefore subjected to a more rigorous electrochemical analysis. A correlation of the variation in Epa_1 with pH showed a considerable pH dependence (Fig. 4) as was expected from the presence of the ionisable OH groups in the chemical structure. A change of almost 150 mV was observed between pH 3 and 10. From a mechanistic point of view, the pH dependency, together with the reversibility of a redox couple at rather low electrochemical potentials, might hint at a specific, phenol-based redox pathway (Fig. 5) that is not possible for organochalcogens with methoxy or acetylated OH groups—although electronic effects of the phenolate on the tellurium atom must also be considered. In line with the notion that the phenol group plays an important role in compound **10**, the methylated and acetylated forms of **10** (compounds **5** and **8**, respectively) were found to exhibit distinctively different electrochemical behaviour (Fig. 2b). Interestingly, the sulfur analogue of compound **10**, *i.e.* 4,4′-dihydroxydiphenylsulfide, also showed an exceptionally low *E*pa value for a sulfide (551 mV), underlining the importance of the hydroxy groups for the redox behaviour of these compounds.

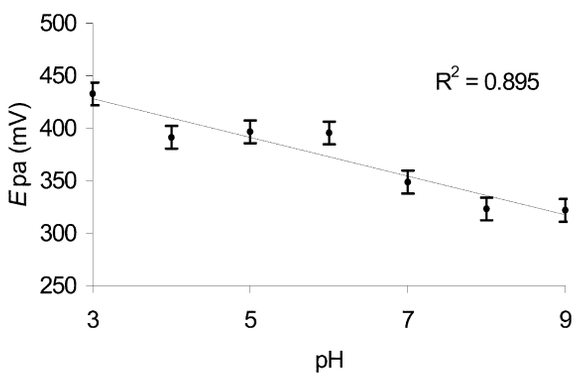


Fig. 4 Correlation between pH and Epa_1 for **10**. *E*pa values for **10** (50 μM) were obtained by cyclic voltammetry in potassium phosphate buffer (50 mM containing 30% MeOH to ensure solubility) and correlated with the pH of the buffer.

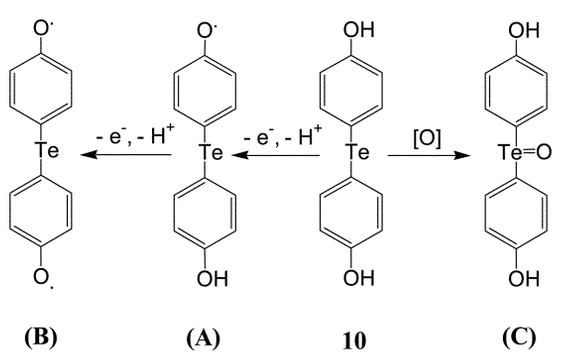


Fig. 5 Proposed oxidation pathways for **10**. The organotelluride can be oxidised by two successive one electron transfers resulting in phenolic oxidation products (A) and (B) or by oxygen atom transfer resulting in a telluroxide (C). This phenol-based oxidation behaviour is specific to compound **10** and not possible for the other agents studied.

From an antioxidant point of view, hydroxyphenyl compounds are particularly interesting since the pH dependence of their redox potential could provide a possible pH switch for antioxidant activity. The observed linear correlation between *E*pa and pH also suggests that it might be possible to fine-tune the compound's activity in the future by adding groups with different pK_a values onto the aromatic ring adjacent to the hydroxy group. Furthermore, phenolic compounds are already used as antioxidants.[28]

PC12 neuronal cell culture experiments indicative of AD

Since **10** exhibited the lowest *E*pa, showed the highest activity in the MT assay and acted as an efficient quencher of PN, it was further evaluated in a previously established PC12 cell culture model indicative of AD.[29,30] For comparison, **4**, the established GPx-mimic ebselen and selenocystamine (SeCA) were also examined.

AD is a mental dementia that has been associated with OS due to the detection of lipid peroxidation, DNA oxidation, protein nitration and advanced Maillard reaction products in AD brain cells.[31] Neuropathologically, AD is characterised by the formation of two lesions in the brain known as the amyloid plaque and the neurofibrillary tangle. The primary components of the plaque are aggregated amyloid beta (Aβ) peptides, derived from cleavage of the amyloid precursor protein (APP) by the β- and γ-secretases.[32] This abnormal processing of APP results in Aβ peptides consisting of the first 40–43 residues of the protein.[33] When neurons are exposed to Aβ they undergo an influx of extracellular Ca^{2+} *via* plasma membrane channels due to an abnormal generation of oxidative stressors[34] damaging the ion-motive ATPases in the plasma membrane.[35] We have recently utilised this model to develop a clinically relevant bioassay for testing the efficacy of antioxidants in AD.[11,29,30] Exposure of cells to amyloid beta peptide residues 1 to 40 ($A\beta P_{(1-40)}$) causes a dramatic enhancement of Ca^{2+} channel current in the clonal cell line PC12.[29,30] We have confirmed that this event is linked to the generation of oxidative stressors as it is fully prevented by antioxidants. Since the degree of Ca^{2+} channel enhancement caused by $A\beta P_{(1-40)}$ and the concentration-dependent degree of inhibition caused by antioxidants can be monitored with high accuracy, the assay provides a highly sensitive means of evaluating potential antioxidant compounds at the low concentrations expected to be present in brain tissue.

Although all the agents tested reversed the current augmentation induced by $A\beta P_{(1-40)}$ without any detectable loss of cell viability (Fig. 6), there were considerable differences in activity. Ebselen (*E*pa determined as +1044 mV) was efficient with an EC_{50} of ~1 μM. In line with a variation in *E*pa of approximately 700 mV and significantly higher activity in the catalytic MT-based assay, **10** was active in nanomolar concentrations (EC_{50} ~ 20 nM). The diselenide **4** was also found to be highly active in cell culture (EC_{50} ~ 160 nM), albeit considerably less active than **10** in line with its higher oxidation potential (+1000 mV) and lower bioassay activity. In accordance with a high oxidation potential (*E*pa = +1155 mV) the commonly studied selenide SeCA proved rather inactive (EC_{50} ~ 10 μM).

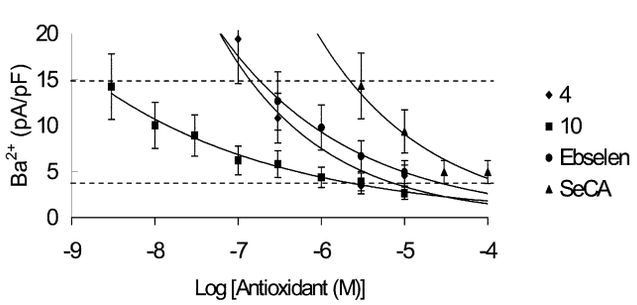


Fig. 6 Activity of catalytic antioxidants in cultured PC12 cells. $A\beta P_{(1-40)}$ induces current augmentation of PC12 cells. The lower dashed line indicates current density in the control cells while the upper dashed line indicates current density 24 h after being challenged with $A\beta P_{(1-40)}$ (100 nM). The ability of **4**, **10**, ebselen and SeCA to reverse the effects of $A\beta P_{(1-40)}$ was established by adding different concentrations of these compounds to the incubation and re-establishing the current density.[11]

Overall, the cell culture experiments confirmed the biochemical activity of the redox catalysts. The low toxicity of the catalysts, due to the minute quantities required for activity, was paired with a high activity against the effects of the Aβ peptide. Although the precise mechanistic details of this biological activity are not yet known, the effects observed *in vitro* and in cell culture support the idea that redox catalysts can provide efficient protection from oxidative stress.

Conclusion

The design of effective antioxidants that can function in the complex redox environment of human cells and organs is far from trivial. Oxidative stress is the result of a complicated interaction of a wide range of oxidative stressors and antioxidant defence systems involving numerous cellular redox cascades.[20,36–38] The redox behaviour of antioxidant molecules can be rationalised by employing an array of different tools ranging from electrochemistry to cell culture experiments. Cyclic voltammetry is helpful in evaluating the redox pathways and reactivities of different antioxidants. There is at present considerable interest in fully understanding the electrochemistry of organochalcogens.[11,39] In conjunction with bioassays, it is possible to select promising agents that might provide a lead for innovative antioxidant research into prototype drugs for AD and related diseases. Compound **10** is an example, exhibiting the lowest *E*pa and the most promising activity in the *in vitro* bioassays.

Experimental

Materials

UV/VIS spectra were recorded on a Cary 50 Bio UV/VIS spectrophotometer (Varian) and fluorescence spectra were recorded on an LS-5 luminescence spectrometer (Perkin-Elmer). ^{1}H-NMR and ^{13}C-NMR spectra were obtained at 300 MHz on a Bruker spectrometer and cyclic voltammograms were recorded on a BAS 100B/W workstation. Mass spectra were obtained by the Mass Spectrometry Service of the School of Chemistry, University of Exeter, UK. Samples were analysed using a Micromass GCT instrument running in chemical ionisation mode with methane as the reagent gas.

Hydrogen peroxide and metallothionein (MT, rabbit liver Zn,Cd-MT-2 form), were obtained from Sigma (Poole, UK). 4-(2-Pyridylazo)resorcinol (PAR) was purchased from Fluka (Gillingham, UK). All chemicals were analytical grade and used without further purification. Metal free nitrogen-purged buffers were used for the MT oxidation assays.[9,21,22] PN was synthesised by mixing sodium nitrite and acidified H_2O_2 followed instantly by stabilisation in NaOH and the subsequent addition of MnO_2 to remove excess H_2O_2 as previously described.[40] The concentration of PN was determined spectrophotometrically [log (ε_{302}/M^{-1} cm^{-1}) = 3.2]. Compounds **1** and **2** were purchased from Aldrich (Poole, UK).

Synthesis

Unless otherwise indicated, monochalcogens were synthesised by the route of Suzuki and Nakamura[41] and dichalcogens according to the general procedure of Engman and Persson.[42] 4,4′-Dihydroxydiphenyltelluride (**10**) was synthesised by the reaction of tellurium tetrachloride with phenol to form hydroxyphenyl trichloride followed by reduction with ascorbate.[43] Synthesised compounds were stored at −20 °C in the dark.

1-Methoxy-4-[3-(4-methoxyphenyltelluryl)propyltelluryl]-benzene (7)

Diaryl ditelluride **6** (100 mg, 0.2 mmol) was dissolved in absolute ethanol (40 ml) under an argon atmosphere. Sodium borohydride (23 mg, 0.6 mmol) was rapidly added and the solution stirred under argon for 1 h. 1,3-Dibromopropane (34 μl, 0.33 mmol) was added *via* a syringe and the solution stirred for a further 3 h. The solvent was removed *in vacuo* and the residue dissolved in ethyl acetate (50 ml) and washed with water (3 × 30 ml). The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. The product was then dissolved in $CHCl_3$ (1 ml) and further purified by silica gel column chromatography (20% ether–petrol) then dried over silica gel to yield compound **7** as a yellow oil (57 mg, 0.11 mmol, 57% yield). R_f = 0.35 (20% ether–petrol). ^{1}H NMR ($CDCl_3$) δ 7.66 (d, J = 8.8 Hz, 4H), 6.77 (d, J = 8.7 Hz, 4H), 3.82 (s, 6H), 2.86 (t, J = 7.4 Hz, 4H), 2.20–2.10 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 160.12, 141.53, 115.51, 100.67, 55.52, 33.38, 11.09; *m*/*z* (M + H)$^+$ calcd. 516.9666, found 516.9667.

4,4′-Diacetoxydiphenyltelluride (8)

Telluride **10** (100 mg, 0.32 mmol) was dissolved in acetic anhydride (40 ml) and triethylamine (220 μl, 1.6 mmol) was added. The solution was stirred overnight and then extracted into ethyl acetate (60 ml). The mixture was washed with saturated aqueous $NaHCO_3$ (3 × 30 ml) and H_2O (3 × 30 ml) and then dried over $MgSO_4$. The crude material was purified by column chromatography ($CHCl_3$) then dried over silica gel to yield compound **8** as a cream coloured solid (83 mg, 0.21 mmol, 66% yield). R_f = 0.42 ($CHCl_3$). Melting point 155.6–156.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.72 (d, J = 8.8 Hz, 4H), 6.99 (d, J = 8.7 Hz, 4H), 2.32 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 169.41, 150.94, 139.45, 123.11, 111.07, 21.32; *m*/*z* (M + H)$^+$ calcd. 401.0032, found 401.0008.

1-(3-Bromopropyltelluryl)-4-methoxybenzene (9)

The same reaction procedure was followed as for the synthesis of **7**, but a large excess of 1,3-dibromopropane (2 ml, 19.6 mmol) was added. The product was purified by column chromatography (10% ether–petrol) and dried over silica gel to yield compound **9** as a yellow oil (32 mg, 0.09 mmol, 45% yield). R_f = 0.42 (10% ether–petrol). ^{1}H NMR ($CDCl_3$) δ 7.71 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 8.8 Hz, 2H), 3.82 (s, 3H), 3.47 (t, J = 6.5 Hz, 2H), 2.91 (t, J = 7.2 Hz, 2H), 2.29–2.20 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 159.99, 141.33, 115.35, 99.99, 55.29, 35.36, 34.31, 6.27; *m*/*z* (M$^+$) calcd. 357.9212, found 357.9207.

Cyclic voltammetry

Cyclic voltammograms of organochalcogens (50 μM) were recorded in potassium phosphate buffer (50 mM, pH 7.4) containing 30% methanol (due to the limited solubility of the organochalcogens in aqueous media) using a standard Ag/AgCl reference electrode, a platinum wire counter electrode and a glassy carbon working electrode that was thoroughly cleaned and polished (with Al_2O_3) after each scan. Cyclic voltammograms were recorded at a scan rate of 500 mV s^{-1} at 25 °C, unless otherwise stated.

In vitro assays: metallothionein zinc release and peroxynitrite quenching assays

Zn_7MT-2 was prepared from the commercially obtained Zn,Cd-form according to the established procedure.[44] The rate of oxidation of MT was established by monitoring the formation of the Zn(PAR)$_2$ complex [log (ε_{500}/M^{-1} cm^{-1}) = 4.8] in HEPES–Na$^+$ (20 mM, pH 7.5) at 25 °C. Catalytic activity was established by incubating each compound (200 nM) with MT (0.5 μM), PAR (100 μM) and *tert*-butyl hydroperoxide (500 μM) and continuously monitoring the zinc release by UV/VIS spectroscopy at 500 nm for 60 min. The maximum zinc that can be released from MT was established by incubating MT with ebselen (10 μM) and the amount of zinc released by different antioxidants was expressed as a percentage of this maxi-

mum.[9,21] As previously described, the use of HEPES buffer was essential in these assays since, unlike phosphate buffer, it does not significantly interact with MT or zinc ions.[45] Nitrogen purged buffers were used throughout.

The DHR assay was performed as previously described[13] with minor modifications. The compound to be tested and DHR (500 nM) were dissolved in potassium phosphate buffer (100 mM, pH 7.3) containing diethylenetriaminepentaacetic acid (DTPA, 0.1 mM). PN (100 nM) was added as a bolus and the concentration of the resulting rhodamine-123 determined by fluorescence spectroscopy (λ_{ex} = 500 nm, λ_{em} = 536 nm). The assay was repeated with varying concentrations of compounds and IC_{50} values were derived by interpolation.

Iron-catalysed nitration of HPA by PN was evaluated by dissolving the selected compounds and HPA (10 mM) in potassium phosphate buffer (100 mM, pH 7.3) containing an Fe(III)–EDTA complex (0.5 mM). PN (0.1 mM) was added with stirring and the assay mixture incubated at ambient temperature for 30 min. NaOH (1 M) was then added to adjust the pH to 11 and the concentration of nitrated HPA determined spectrophotometrically [log (ε_{430}/M^{-1} cm^{-1}) = 3.6].[13] Both PN assays were conducted in phosphate buffer since HEPES reacts with PN to liberate H_2O_2.[46]

Cell culture assays and electrophysiology

PC12 cells were maintained in culture as previously described.[29,30] 24 h prior to study, aliquots were exposed to $A\beta P_{(1–40)}$ (100 nM) in the absence or presence of varying concentrations of selected organochalcogens. Ca^{2+} channel currents were recorded using the whole-cell patch clamp technique, with Ba^{2+} as the charge carrier. Pipettes were filled with a solution (pH 7.4) of the following composition: NaCl (110 mM), CsCl (5 mM), $MgCl_2$ (0.6 mM), $BaCl_2$ (20 mM), HEPES (5 mM), glucose (10 mM) and tetraethylammonium chloride (20 mM). The osmolarity of the perfusate was adjusted to 300 mOsm by the addition of sucrose. Patch pipettes (5–7 MΩ resistance) were filled with a solution (pH 7.2) of the following solution: CsCl (130 mM), EGTA (1.1 mM), $MgCl_2$ (2 mM), $CaCl_2$ (0.1 mM), NaCl (10 mM), HEPES (10 mM) and Na_2ATP (2 mM). Currents were evoked by applying 200 ms ramps (−100 mV to +100 mV, 0.2 Hz) from a −80 mV holding potential, measured at the peak of the current–voltage relationship (+20 mV) and corrected for cell size to yield current densities (pA pF^{-1}, mean ± s.e.m.).[30] Cell viability was determined using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and at this concentration $A\beta P_{(1–40)}$ did not result in significant cell death.

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