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Towards multifunctional antioxidants: synthesis, electrochemistry, *in vitro* and cell culture evaluation of compounds with ligand/catalytic properties

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Numerous human diseases are linked to a biochemical condition known as oxidative stress (OS). Antioxidants are therefore becoming increasingly important as potential disease prevention and therapeutic agents. Since OS is a multi-stressor event, agents combining a range of different antioxidant properties, such as redox catalysis and metal binding, might be more effective and selective than mono-functional agents. Selenium derivatives of aniline and pyridine combine redox activity with metal binding properties. These multifunctional agents have a distinct electrochemical profile, and exhibit good catalytic activity in the glutathione peroxidase mimic and metallothionein assays. They also show antioxidant activity in a skin cell model of UVA-induced stress. These compounds might therefore provide the basis for novel agents combining two or more distinct antioxidant properties.

Introduction

Oxidative stress (OS) is a biochemical condition associated with numerous human diseases, ranging from rheumatoid arthritis and neurodegenerative diseases to inflammation and cancer.[1,2] It is characterised by a significantly increased concentration of intracellular oxidising species, such as reactive oxygen species (ROS), which is often accompanied by the simultaneous loss of antioxidant defence capacity. Effective antioxidants able to counteract OS are therefore becoming increasingly important in disease prevention and therapy. Among them, compounds with glutathione peroxidase (GPx)-like activity are particularly interesting, since they *catalytically* remove oxidative stressors, and can therefore be applied in small quantities. In addition, catalysts are able to respond to their redox environment, hence providing selectivity for cells under OS. Not surprisingly, one such catalyst, ebselen, has already made its way into clinical trials.[3]

OS is not, however, a single stressor event, and a wide range of stressors, such ROS (*e.g.* oxygen-based radicals, peroxides), nitric oxide, peroxynitrite and free metal ions are known to form part of its biochemical makeup. To respond to this complex multi-stressor environment more effectively, compounds can be envisaged that combine a range of antioxidant activities in one, chemically simple molecule. Since ROS and adventitious, free metal ions, such as iron and copper, play a major role in OS, antioxidants able to interact with several of these species might be particularly active. Ultimately, such compounds might not only be very effective, but also quite selective for the particular OS stress environment they are tailored to.

Here we present initial results from *in vitro* and cell culture studies of chemically simple agents that combine redox, catalytic and metal binding sites, and might therefore act as multifunctional antioxidants.[4] We are able to show that the dichalcogen derivatives of either pyridine, aniline or quinoline combine the redox activity of the chalcogen moiety with the metal binding properties characteristic of their nitrogen and chalcogen groups.

Results

Selection and synthesis of multifunctional agents

Sulfur and selenium compounds are known to play a major role in antioxidant reactions. While sulfur agents can serve as electron donors, organoselenium agents frequently possess GPx-like activity. In addition, sulfur and nitrogen agents have excellent metal binding properties, and can bind adventitious metal ions such as copper and iron. As a consequence, dichalcogen derivatives of known metal binding agents were chosen for this study, since they promise to be redox active under physiological conditions, form thiols and selenols upon reduction *in vivo*, catalyse the reduction of ROS, and also interact with redox active metal ions. The chemical structures of these compounds are shown in Fig. 1.

Compounds **1** and **3** were successfully synthesised according to the method of Bhasin *et al.* (see Experimental section).[5] The other compounds were commercially available. These compounds were then evaluated for the following antioxidant activities: (a) electron donor/acceptor properties, (b) catalytic activity, (c) metal binding properties and (d) protection of cells from UVA-radiation. These studies required a combination of chemical and biochemical techniques, such as electrochemistry, protein and metal binding assays, and ultimately cell culture studies. In the following, the results are presented according to the potential antioxidant properties investigated.

Redox properties of dichalcogens

Generally, each of the compounds studied by Cyclic Voltammetry (CV) on the mercury electrode exhibited one characteristic, reversible electron transfer typical of a thiol/disulfide or selenol/diselenide couple. In line with previous findings, potentials for the latter were around 100 to 200 mV more negative compared to the thiol/disulfide couple. The redox couples had an anodic and cathodic peak (*E*pa and *E*pc, respectively) in the

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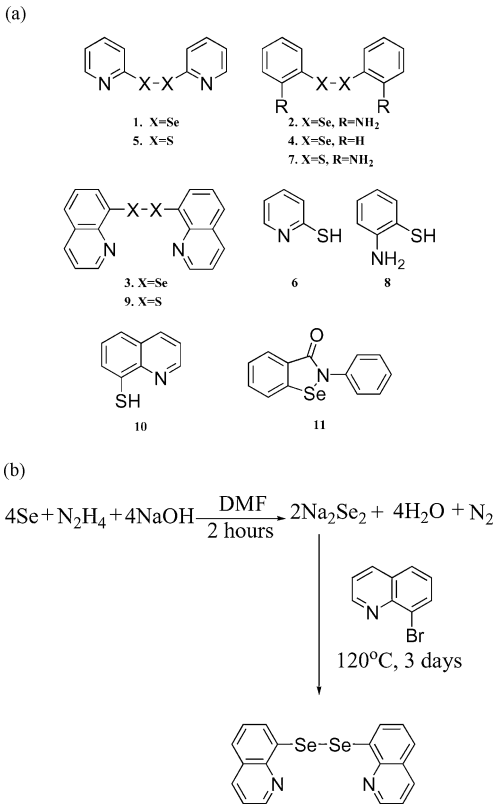

Fig. 1 (a) Chemical structures of compounds used in this study. (b) Synthetic pathway for diselenides.[5]

biologically relevant range between 0 and −900 mV *vs.* SSE, approximately corresponding to a one electron transfer process ($\Delta Ep_{,1/2} = 3.53\ RT/nF = 90.6/n$ mV, see Table 1). In all cases, the current *vs.* scan rate plots for the dichalcogen reduction processes were linear (R^2 between 0.973 and 0.999 for scan rates between 100 and 500 mV/s), characteristic of electrochemically active species adsorbed on the mercury electrode surface.

When the three classes of compounds, *i.e.*, pyridine, aniline and quinoline derivatives, were compared, the pyridine agents (**1** and **5**) exhibited the most positive $E^{0\prime}$ (*i.e.*, (*E*pa + *E*pc)/2) values (−511 mV for selenium and −305 mV for the sulfur analogue). In contrast, the aniline derivatives (**2** and **7**) had the most negative values (−851 mV for selenium and −741 mV for the sulfur analogue), implying that the aniline derivatives were the most 'reducing' of the compounds, and therefore the most promising candidates for antioxidant activity.[6,7] Interestingly, ebselen, used here as a benchmark control, exhibited irreversible electrochemical behaviour under these conditions (*i.e.*, at a mercury electrode), with an *E*pa value of −703 mV.

Overall, these studies confirmed that all dichalcogens and their reduced analogues were redox active, and able to undergo reversible oxidation and reduction reactions within the physiologically relevant potential range. Additionally, the results obtained by CV hinted at a ranking order of good reducing agents, with **2** topping this list.

Peroxidase-like catalytic activity

Compounds were then evaluated in *in vitro* assays indicative of potential antioxidant activity, with focus on catalytic activity and interactions with pro-oxidant metal ions (Table 1). Catalytic, GPx-like activity was first measured in the thiophenol (PhSH) peroxidation assay.[8] The latter is a well established and easy to use assay measuring the formation of PhSSPh from PhSH in the presence of H_2O_2. As expected, the selenium, but not the sulfur compounds were active in this assay.[6,7] While the former, once reduced, undergo selenol/selenenic acid catalysis, sulfur

Table 1 Electrochemical and *in vitro* studies. Experimental details for the electrochemical studies are given in the text ($E_{1/2}(Cu^{2+}) = 7$ mV). For the MT assay, compounds (1 μM) were incubated with MT (0.5 μM) and *tert*-butyl hydrogen peroxide (500 μM) in the presence of PAR (100 μM) in HEPES–Na^+ (20 mM, pH 7.4). The reaction was monitored spectrophotometrically in a continuous assay at 500 nm for 60 min at 25 °C. Maximum zinc release from MT was calculated by incubation with 10 μM ebselen and the amount of zinc release for different compounds compared to this theoretical value. For the PhSH assay, compounds (100 μM) were incubated with PhSH (1 mM) and H_2O_2 (2 mM) in methanol. The reaction was monitored spectrophotometrically at 305 nm for 10 min at 25 °C ($n = 3$). N.a., not applicable; n.s.d., no significant difference between samples with and without peroxide

Compound	*E*pa/mV ± 10	*E*pc/mV ± 10	$E^{0\prime}$	No. of e^- transferred	CV: $\Delta E^{0\prime}$ of ligand on addition of Cu^{2+}/mV	DPP: Cu Complex $E_{1/2}$/mV ± 10	MT assay, Zn release (%) ± 5	Thiophenol assay, V_0/nM s^{-1} ± 5%
1	−470	−551	−511	0.6	−50	+305	78	250
2	−823	−878	−851	0.7	−3	+104	46	167
3	−717	−751	−734	1.0	Complete depression of peak	+254	42	600
4	−743	−799	−771	1.0	−26	+171	32	67
5	−281	−329	−305	0.6	−225	+351	N.s.d.	N.s.d.
6	−291	−348	−320	0.8	−204	+351	3	10
7	−702	−780	−741	0.6	−40	+142	9	N.s.d.
8	−702	−747	−725	0.7	−23	+115	9	N.s.d.
9	−637	−683	−660	1.4	Depression of peak	+283	11	N.s.d.
10	−603	−626	−615	1.2	Complete depression of peak	+274	0	0
11	—	−703	N.a.	—	Depression of peak	—	28	167
Control—no compound	N.a.	N.a.	N.a.	—	N.a.	N.a.	15	8

compounds generally evade the sulfenic acid state and tend to remain in—or return to—the disulfide state, which is not catalytically active.[9] There was no clear trend in activity among the selenium compounds **1**–**4**, with the quinoline derivative **3** being the most active, and the others having an activity comparable to the one of ebselen (Table 1).

Catalytic activity was therefore further investigated in the metallothionein (MT) assay that measures catalytic zinc release from the zinc/sulfur protein in the presence of peroxide.[10] This assay is biologically more relevant than the PhSH assay (choice of protein, buffer, pH) and is generally also more robust and reliable.[11] As in the PhSH assay, the selenium compounds were more active than the sulfur ones. Among the former, the pyridine derivative **1** was the most, and the quinoline derivative **3** the least active (78 and 42% zinc release, respectively). Interestingly, selenium compounds **1**–**3** were significantly more active than ebselen in this assay (28% zinc release), confirming good catalytic activity *in vitro*.

Interactions with copper ions

In addition to ROS, free, adventitious metal ions able to generate oxygen radicals are frequently associated with OS. Among them, non-protein bound copper plays a major role because of its ability to convert peroxide into hydroxyl radicals in a Fenton-type reaction.[12] Chelators such as desferrioxamine are therefore frequently used as 'indirect' antioxidants, *i.e.* compounds that are not redox active themselves but interfere with the redox reactions of others.[13]

Interactions between the compounds and Cu^{2+} were therefore studied by differential pulse polarography (DPP) and UV/VIS spectrophotometry.[14] DPP of the copper ion detects changes in the metal's redox behaviour upon complexation, and is indicative of the ligand's indirect antioxidant properties. Changes in UV/VIS spectra of ligands can be used to calculate an apparent metal binding constant.

A summary of the results is shown in Table 1. Among the compounds tested, the pyridine derivatives (**1** and **5**) caused the largest shift in the Cu^{2+} reduction potential ($\Delta E_{1/2} = +298$ mV for **1** and +344 mV for **5**). This effect is illustrated for **1** in Fig. 2. Importantly, the shift in the copper reduction potential to more positive values was accompanied by shifts in the oxidation and reduction potentials of the ligand to more negative values ($\Delta E^{0\prime} = -50$ mV for **1**, −225 mV for **5**). In contrast, the aniline derivatives showed the smallest shift in copper reduction potential ($\Delta E_{1/2} = +97$ mV for **2** and +135 mV for **7**), and also the smallest change in the electrochemical potential of the ligand ($\Delta E^{0\prime} = -3$ mV for **2** and −40 mV for **7**).

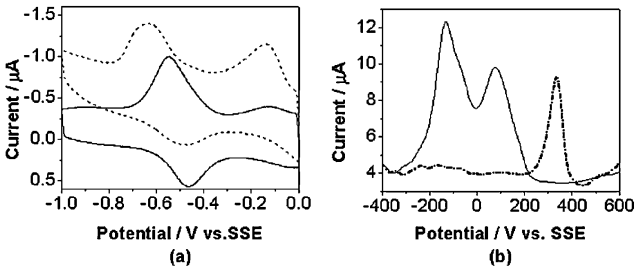

Fig. 2 (a) Cyclic voltammogram of **1** in the absence (solid) and presence (dashed) of Cu^{2+}. (b) Differential pulse polarogram of Cu^{2+} in the absence (solid) and presence (dashed) of **1**. Experimental details are given in the text. The voltammogram display follows the IUPAC convention.

Binding constants

In the above experiments, the reduced and oxidised forms of the sulfur compounds had similar effects on the Cu^{2+} potential. Cu^{2+} binding was therefore independently confirmed for **6**, **8** and **10**, the compounds stable enough in their reduced states to give results. These studies relied on changes in the compounds' UV/VIS absorption spectra upon binding to Cu^{2+} (Fig. 3).

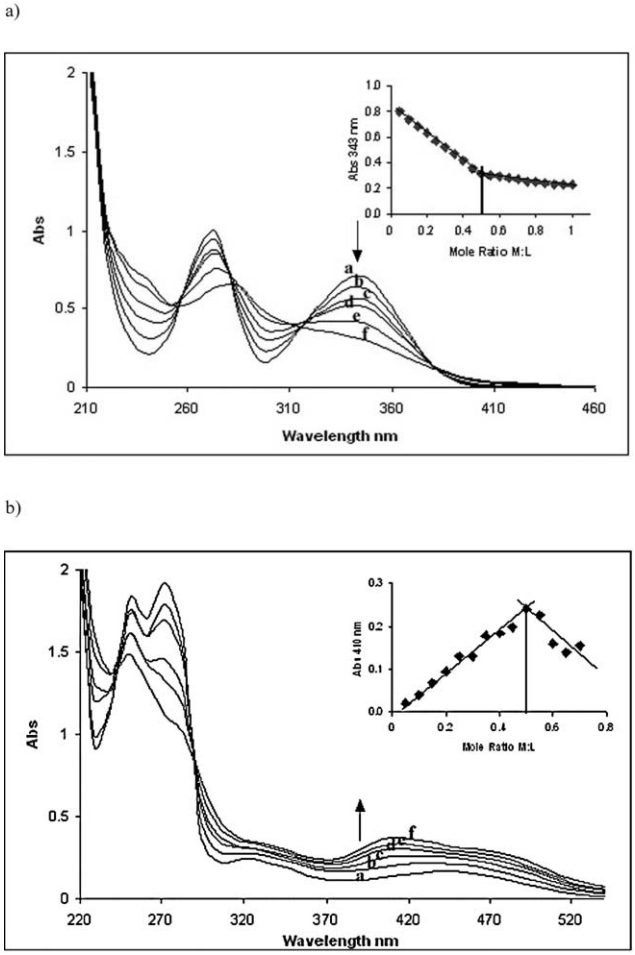

Fig. 3 Changes in the UV/VIS absorption spectra of (a) compound **6** and (b) compound **10** upon addition of increasing Cu^{2+} ($CuSO_4$) concentrations; (a–f) 0–50 μM Cu^{2+} in 10 μM increments. Spectra were recorded in 10 mM MOPS buffer (pH 7.0), at 25 °C. These changes in UV/VIS absorption form the basis for the estimation of copper binding stoichiometry and binding constants. Insert: mole-ratio plots to determine Cu^{2+} binding to (a) compound **6** and (b) compound **10**. Both compounds bind the copper ion with a 2 : 1 stoichiometry, indicating tetrahedral coordination around the metal.

For **10** (100 μM), a peak shift from 447 to 410 nm was observed in the presence of Cu^{2+} (5–50 μM), and the absorbance at 410 nm was used to determine the stoichiometic ratio by the 'mole-ratio' method (an extension of Job's method). For **6** (100 μM), a decrease in peak absorbances at 272 and 343 nm was observed in the presence of Cu^{2+} (5–50 μM), indicating the formation of an UV-transparent copper complex. For **8** (100 μM) a peak shift from 305 to 308 nm was observed upon addition of increasing concentrations of Cu^{2+} (5–25 μM), and was accompanied by decreases in peak absorbance at 224 and 261 nm. Unfortunately, **8** rapidly oxidises to the disulfide to give a peak at 328 nm, resulting in a more complex spectrum that although confirming Cu^{2+} binding to **8**, could not be quantitatively analysed by the 'mole-ratio' method.

The mole-ratio plots derived from the spectra of **6** and **10** are shown in Fig. 3 (insert). They indicate that **6** and **10** bind Cu^{2+} with a 2 : 1 ligand : metal stoichiometry, hinting at a tetrahedrally coordinated copper ion. Using changes in the UV/VIS spectra of the compounds upon addition of copper, copper binding constants were estimated to be 1.5×10^8 M^{-1} for **6** and 1.2×10^8 M^{-1} for **10** at 25 °C.

Considered together, the electrochemical and *in vitro* studies confirm a range of possible interactions of compounds such

as **1**–**3** with various, chemically very different oxidative stressors present during OS. They also hint at a complex set of 'responses' these compounds show in the presence of stressors, such as shifts in electrochemical potentials, which might allow activation of antioxidant properties in the presence of OS. The most promising compounds (**1**–**3**) were therefore tested in a human skin fibroblast cell line (FCP7).

Protection against UVA radiation

An ultraviolet A (UVA, 320–400 nm) based assay was chosen since radiation-induced OS is associated with both, ROS *and* adventitious metal ions.[15] In addition, skin is easily accessible from a pharmacological point of view and provides a prime target for antioxidant use.

Fig. 4 shows the results obtained for **1**–**3** in the cell culture studies. A decrease in cell viability to approximately 60% of control cells was observed when cells were irradiated with a dose of 500 kJ m^{-2} UVA compared to that of control cells. Pretreatment with **1** and **2** significantly reduced this loss, most likely due to the antioxidant activity of these compounds (24% ± 5.6 and 27% ± 7.6 noted absolute increase in cell viability at maximum protective concentrations, respectively). Activity of both compounds clearly exceeded that of ebselen (16% ± 3.5 increase). In contrast to the cytoprotective effects offered by **1** and **2**, the quinoline derivative **3**, although slightly protective at 1 μM (9% ± 5), behaved as a pro-oxidant at higher concentrations (5 μM) when exposed to UVA irradiation, and dramatically enhanced UVA cell damage (reducing overall cell viability by another 42% ± 8.5). Such phototoxic responses are frequently reported for quinoline based drugs.[16]

Discussion

The use of antioxidants in disease prevention and therapy is often limited by the complex network of biochemical redox interactions which is characteristic of OS. Antioxidants able to neutralise just one (or a few) stressors are therefore unlikely to be either very effective or selective in cells under OS. In contrast, multi-functional agents, the redox and metal binding properties of which are tailored to closely mirror the redox environment posed by a specific form of OS, such as UVA-induced stress, promise to be considerably more effective.

Taken together, the results obtained in this study demonstrate that it is possible to design and synthesise chemically simple molecules that combine a range of important antioxidant properties. While the individual activities, such as GPx-like catalysis, already point towards good antioxidant behaviour, it is the combination of these different activities which provides the extraordinary protection from OS-related damage seen in the skin cell culture.

Compound **2**, in particular, is active in all of the various antioxidant assays employed. Compared to ebselen, it is also considerably more active in the skin cell culture and can therefore be considered as a leading compound for the development of more effective, multi-functional antioxidants. Interestingly, this compound has recently been shown to be protective against lipid peroxidation in brain tissue extracts from mice.[17] While these cell culture studies support our results, they provide little insight into the chemical basis for such high antioxidant activity, and do not hint at potential design criteria for improved antioxidant activity. In contrast, our electrochemical and *in vitro* results indicate that this activity might be due to a combination of redox catalysis and metal binding.

We have used CV in the past to gain a deeper understanding of the redox properties of chalcogen compounds.[6,7] The electrochemical behaviour of sulfur, selenium and tellurium compounds is somewhat more complex than that of metals and metal complexes, and can be performed on carbon as well as mercury electrodes.[6,7,9] The studies presented here have therefore employed CV on a mercury electrode to focus on the thiol/disulfide and selenol/diselenide redox couples. The results obtained by CV show that **2** has exceptionally low oxidation and reduction potentials, even when compared to structurally similar selenides **1** and **3**. The reduced form of the compound can therefore be expected to be a good reducing agent, one that would be readily reactive towards intracellular ROS such as hydrogen peroxide. In addition, reversibility of electron transfer also points towards the compound's ability to 'redox cycle', *i.e.*, to participate in redox catalysis.

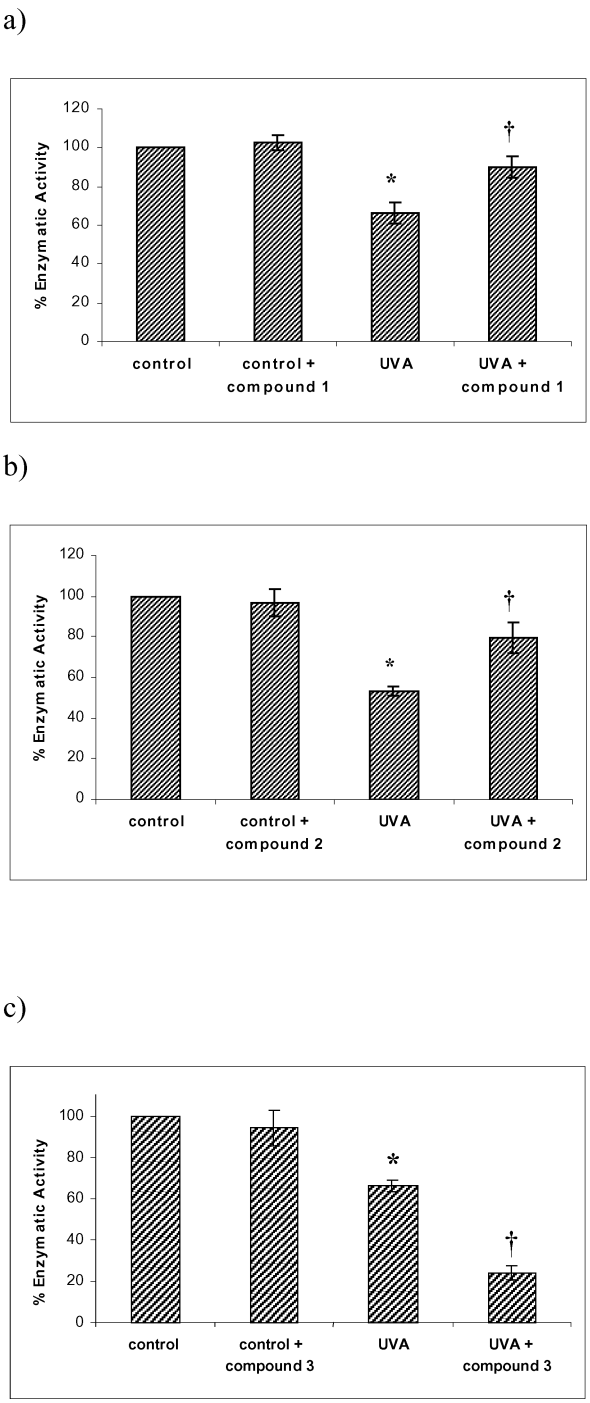

Fig. 4 Activity of **1**–**3** in a human skin fibroblast cell line. Cells were irradiated with UVA (500 kJ m^{-2}) following an 18 h pre-incubation period with and without **1**–**3** (5 μM for **1** and **3**, 1 μM for **2**) at 37 °C in a 5% CO_2 atmosphere. Control cells were treated similarly except that they were not irradiated. Cell viability was determined 4 h post irradiation using a standard colourimetric MTT assay. Results represent 3 independent experiments, each of which was performed in triplicate; $^{*}P < 0.05$: significant difference from untreated control; $^{\dagger}P < 0.05$: significant difference from UVA treated control.

These electrochemical findings are reflected in the catalytic assays. Not surprisingly, the selenium compounds are more active than the sulfur compounds, a trend already observed earlier.[7] Interestingly, compound **1**, the most oxidizing of the selenium agents studied, is also the most active in the MT assay. This is hardly surprising, since the catalytic, GPx-like peroxidation cycle involves a slow thiol/selenosulfide exchange step, when the selenosulfide is reduced back to selenol by a thiol (such as PhSH, MT or GSH). More oxidising selenium species might therefore result in a faster exchange reaction and might well be catalytically more active than more reducing ones.

MT is also known to lose zinc ions in the presence of metal binding agents.[18] Since compounds such as **1** can also interact with metal ions, their activity in the MT assay could be the result of a combination of different protein/compound interactions. This point obviously needs further investigation (see also below). In any case, it is unlikely that peroxidation catalysis alone explains why **2** is a good antioxidant in skin cell culture under UVA-induced stress, whilst **3** actually acts as a pro-oxidant.

The notion that compounds such as **1**–**3** act *via* a more complex mechanism is supported by the copper binding experiments. As mentioned in the introduction, OS is frequently characterised by increased concentrations of ROS and free, adventitious metal ions. While compounds such as GSH, vitamin E and many selenium compounds (such as ebselen) might defend against ROS, chelators like desferrioxamine have been used to neutralise the threat posed by redox-active metal ions participating in Fenton-type reactions. The results obtained here show that compounds like **2** integrate both of these antioxidant activities.

As far as copper binding is concerned, the studies have revealed a number of interesting trends. Firstly, almost all of the compounds studied are able to shift the copper reduction potential to more positive values, and this shift is most pronounced for ligands with more positive oxidation and reduction potentials. A positive shift in the Cu^{2+} potential upon ligand binding has been observed before, and agrees with the stabilisation of the Cu^{+} oxidation state by the sulfur, selenium and nitrogen ligands.[19] The importance of the chalcogen group for metal interactions is underlined by the shifts observed for the control selenium compound **4**, while pyridine, aniline or quinoline did not interfere with the Cu^{2+} reduction potential. The shift of the copper potential implies a less reducing Cu^{+} ion, and this might be biochemically beneficial, since Cu^{+} is acting as an electron donor in the Fenton reaction.

Secondly, the copper/ligand interaction also leads to significant changes in the ligand's electrochemical properties, with shifts to more negative values most pronounced for the pyridine derivatives, which also cause the largest shifts of copper reduction potential. The fact that copper binding would therefore make the ligands more reducing is rather interesting, since it would (at least in theory) imply antioxidant activation by the pro-oxidant metal ion. This effect might ultimately provide the basis for increased efficiency and selectivity of compounds such as **1** and **2**. While efficiency would be increased by multifunctional properties, selectivity would be the result of copper-activated reduction by the ligand. The binding constants estimated for **6** and **10** support this notion. Compared to conventional chelators, such as desferrioxamine (copper binding constant 1.3×10^{14} M^{-1}) and EDTA (copper binding constant 5×10^{18} M^{-1}), metal binding to these chalcogen compounds is weak enough to allow metal sensing and exchange in a cellular environment.

Future studies will have to consider the precise nature of the emerging redox/metal binding relationship. For example, metal binding to the diselenides might well be triggered or enhanced by dichalcogen reduction, a process possible in cells with sufficient GSH concentrations. In addition, the metal complexes might exhibit their own catalytic behaviour, possibly even similar to metal-based dismutase mimics. These topics require further chemical and biochemical investigations.

Although the relationship between ligand redox behaviour, complexation and peak shifts is therefore only just emerging, the results obtained so far for **1** and **2** are rather promising. They support the notion of chemically simple, multifunctional agents, and underline the usefulness of techniques such as CV and DPP play in their analysis. Together with the cell culture studies, the *in vitro* results hint at a class of multifunctional agents whose antioxidant activity might well surpass the one of 'mono-functional' selenium or tellurium agents studied in the past.[6,7]

Conclusion

The study of compounds combining a number of distinct antioxidant properties provides the opportunity to refine and enhance antioxidant activity, whilst also potentially increasing selectivity for cells under OS. These are clear advantages over previously used agents, since they no longer require the administration of combinations of redox active *and* metal binding agents, and might also exploit the emerging synergy between redox and metal binding properties.

Future studies will obviously have to focus on the interplay of redox processes and metal binding and evaluate the precise underlying redox and metal exchange mechanisms. At the same time, complementary biochemical studies will also have to address the activity of such compounds in relevant cell models and on human skin. Nevertheless, it seems feasible that such chemically simple, yet multi-functional and biochemically active compounds might play a major role in future antioxidant research.

Experimental

Materials

Compounds **2**, **9** and **11** were purchased from Fisher Scientific (Leicestershire, UK). 2-Bromopyridine and 8-bromoquinoline were purchased from Sigma-Aldrich (Poole, UK). Selenium powder and hydrazine hydrate were purchased from Lancaster Synthesis UK (Lancashire, UK). Cell culture materials were obtained from Life Sciences Technologies (Paisley, UK), with the exception of foetal calf serum (FCS), obtained from PAA (Austria). All other reagents were analytical grade and purchased from Sigma-Aldrich (Poole, UK). Cd,Zn-metallothionein was purchased from Sigma, and Zn_7–MT was reconstituted and purified according to a standard method.[20]

Methods

Synthesis of compounds 1 and 3. Compound **1** was synthesised from selenium and 2-bromopyridine according to Bhasin *et al.*, and analytical results were found to be in accordance with published values.[5] Compound **3** was synthesised according to this procedure replacing 2-bromopyridine with 8-bromoquinoline and refluxing for 3 d. This compound has previously been synthesised following a different synthetic pathway. Analytical results agreed with literature values.[21]

Cyclic voltammetry (CV) and differential pulse polarography (DPP). CV and DPP were performed on a 100B/W workstation (BAS) at 25 °C. Cyclic voltammograms of the compounds (25 μM) were recorded in MOPS buffer (10 mM, pH 7) containing potassium nitrate (50 mM), in the presence and absence of $Cu(NO_3)_2$ (25 μM), using a mercury working electrode, an Ag/AgCl reference electrode (SSE) and a platinum wire counter electrode at a potential range between 0 and −800 mV. This buffer was chosen since it is known not to interact with metal ions.[22]

Compounds (75 μM) were studied by DPP in the presence and absence of $Cu(NO_3)_2$ (75 μM), using the above buffer system and a glassy carbon working electrode (cleaned and polished with Al_2O_3 after each scan), at a potential range between +600

and −1000 mV, pulse amplitude 80 mV, sample width 45 ms and pulse width 100 ms. Quinoline derivatives required 50% methanol due to limited solubility in aqueous media. Buffers were purged with nitrogen for 30 min prior to use. Potentials are given *vs.* SSE and are standardised against ferrocene.[23] For the sulfur compounds, the disulfide and the reduced thiol analogues were studied.

Thiophenol and MT assays. The thiophenol (PhSH) and MT assays are spectrophotometric assays that can be used to determine the activity of compounds as peroxidation catalysts. While the PhSH assay directly measures the oxidation of PhSH in the presence of H_2O_2 in methanol, the MT assay monitors oxidative zinc release from the two zinc/sulfur clusters of MT (20 cysteine residues holding 7 zinc ions) in the presence of ^{t}BuOOH and a chromophoric dye. Both assays have their own advantages, and together, they provide a good indication of whether a new compound, when measured against known benchmark activity (*e.g.*, ebselen), is catalytically active or not.

For the PhSH assay, 100 μM of the sulfur or selenium compound was added to a 1 mM methanolic solution of PhSH and the reaction initiated by addition of 2 mM H_2O_2. PhSSPh formation was monitored at 305 nm for 10 min at 25 °C. Initial velocities (V_0) were calculated from the first 5–10% of the reaction.

For the MT assay, MT (0.5 μM) was incubated with ^{t}BuOOH (500 μM) and PAR (100 μM) in 20 mM HEPES–Na^+ buffer (pH 7.0, 25 °C) in the absence and presence of sulfur or selenium compounds (1 μM). Catalytic activity was assessed based on the total extent of zinc release after 60 min. Maximum zinc release occurs when all protein thiols (10 μM) are oxidised. This was measured using an excess ebselen, and activities are expressed as a percentage of this value.

Cell culture. Fibroblast monolayers (FCP7) were cultured routinely in Minimum Essential medium with Earle's salts (EMEM) supplemented with 2 mM L-glutamine, 50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 0.2% sodium bicarbonate and 15% FCS at 37 °C in 5% CO_2. Cells were used between passages 11 and 15. A standard colourimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to assess cell viability following UVA irradiation.[24] For this, cells were seeded in 3 cm dishes (5×10^4 cells/dish) in supplemented EMEM medium (2 ml) and the fibroblast monolayers were allowed to grow for 3 d in order to reach 80% confluency. Cells were pretreated with compounds (0.1–10 μM dissolved in DMSO) 18 h prior to the UVA irradiation process. The concentration of DMSO was kept constant at 0.1% of the medium in order to avoid cellular effects of the vehicle, and control cells were pretreated with DMSO alone. UVA irradiation was performed using a broad spectrum 4 kW UVA lamp (350–400 nm, Sellas, Germany) whereby cells were irradiated with 500 kJ m^{-2} UVA in Ca^{2+}–Mg^{2+} PBS according to an established literature procedure.[25] Following irradiation, cells were washed with PBS (1 ml) and the conditioned medium (1.5 ml) was added back to each dish. Cells were incubated further for 1 h at 37 °C, after which the MTT assay was performed as previously described.[26]

Abbreviations

^{t}BuOOH, *tert*-butyl hydroperoxide; $Cu(NO_3)_2$, copper nitrate; $CuSO_4$, copper sulfate; CV, cyclic voltammetry; DPP, differential pulse polarography; $E_{1/2}$, half-wave potential; *E*pa, anodic oxidation potential; *E*pc, cathodic reduction potential; $E^{0\prime}$, standard potential of redox couple; GCE, glassy carbon electrode; GPx, glutathione peroxidase; GSH, glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); H_2O_2, hydrogen peroxide; OS, oxidative stress; PAR, 4-(2-pyridylazo)-resorcinol monosodium salt; PBS, phosphate buffered saline; PhSH, thiophenol; PhSSPh, diphenyldisulfide; MT, metallothionein; MOPS, 3-[*N*-morpholino]propanesulfonic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SSE, standard silver electrode.

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