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Selenium- and tellurium-containing redox modulators with distinct activity against macrophages: possible implications for the treatment of inflammatory diseases

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ABSTRACT

Various selenium- and tellurium-containing molecules are able to modulate the intracellular redox state of cells, an effect which may be used for the (selective) targeting of cancer cells, which are naturally under oxidative stress (OS). As macrophages also generate an environment rich in Reactive Oxygen Species (ROS) and nitric oxide (•NO), they may represent an additional, prime target for such redox-modulating agents. A range of selenium and tellurium-containing quinones have therefore been synthesized and subsequently tested in macrophage culture. The tellurium agents were generally cytotoxic at very low concentrations, and their mode of action seemed to involve the upregulation of intracellular ROS levels. This redox-modulating effect was confirmed by simple yeast-based chemogenetic analysis in conjunction with in vitro redox assays and electrochemistry. Together, these studies point towards an intracellular build-up of superoxide radicals as the most likely cause of toxicity. In contrast, some of the selenium derivatives were less toxic and exerted a pronounced inhibitory effect on the formation of lipopolysaccharide-induced •NO production. Whilst the Te-analogues therefore may enable the resolute, effective and fairly selective targeting of macrophages, the selenium agents could act less severely, but equally effectively by interfering with inflammatory signalling molecules.

1. Introduction

The last two decades have witnessed a growing interest in selenium- and tellurium-containing agents with potential 'redox-modulating' properties.[1–6] This interest is based on the fact that many human diseases are associated with—if not caused by—oxidative stress (OS).[7–9] The latter represents an imbalance in the cellular redox homeostasis, which is characterized by an intracellular build-up of Reactive Oxygen Species (ROS), and frequently also diminished levels of reduced glutathione (GSH). OS is found, for instance, in many auto-inflammatory, immune and infectious diseases, in cancer, diabetes, glaucoma and neurodegenerative disorders to name just a few.[7,9] An increase in intracellular levels of ROS is also frequently observed in older people and even in the homeless, which links OS not only to various diseases but also to age and lifestyle.[10,11]

Not surprisingly, pharmaceutical research has long focused on antioxidants, which may counteract the damaging effects of ROS and related species. Within this context, selenium-based agents have obtained a certain prominence as they can mimic the activity of the selenocysteine-containing antioxidant enzyme glutathione peroxidase (GPx); selenocysteine is also found in the human antioxidant enzyme thioredoxin reductase (TrxR).[12] With the notable exception of the Se-based agent ebselen,[13] however, most of these Se-compounds have not (yet) progressed to clinical trials. In contrast, tellurium-containing compounds have hardly been considered as potential drugs, as tellurium is not an essential trace element, seems to be 'alien' to the human body and is often associated with outright toxicity.[6]

Only recently, have first attempts been undertaken to employ tellurium agents as redox modulators in the context of cancer therapy.[3,5,6,14,15] Here, the ability of such tellurium (and selenium) agents to significantly change the pre-existing redox balance in the

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cell towards more oxidizing conditions is used to push oxidatively stressed cancer cells over the critical 'redox threshold' and induce cancer cell death by apoptosis.[3,16] As normal, healthy cells often contain less ROS compared to cancer cells, these redox-modulating agents may act fairly selectively against cancer cells, which bode well for possible therapeutic applications.[17] During the last couple of years, research into such redox-modulating agents has seen considerable progress: The first animal studies have been completed successfully, there is some evidence of selective action against patient-derived chronic lymphocytic leukaemia cells (CLL cells) compared to healthy peripheral blood mononuclear cells (PBMCs), and we now have a clearer insight into the possible biochemical mode(s) of action which often involve the cellular thiolstat (e.g., β-tubulin) and various regulatory pathways associated with it.[5,18]

Whilst a number of important questions still need to be answered as far as the anti-cancer activity of such agents is concerned, a new and equally important target for redox-modulating agents has been emerging in the context of inflammatory diseases (e.g., rheumatoid arthritis (RA)).[19–21] Macrophages, like cancer cells under OS, generate a highly oxidizing environment, which can cause severe damage not only to invading microorganisms (such as bacteria), but also to the surrounding tissue. In the case of RA and related inflammatory diseases, such macrophages are 'out of control' in two aspects. Firstly they generate ROS, nitric oxide (•NO) and cytokines for no apparent reason (and hence damage the human joint, causing severe inflammation), and secondly, they are resistant towards the rather large amounts of ROS and •NO that they generate.

One may therefore speculate that redox-modulating agents brought into contact with such macrophages could use the high local ROS levels to inflict severe damage and hence kill these cells fairly effectively—yet also selectively. Indeed, selectivity may be expected to result from the gradient in ROS concentration, which is highest in, at or near the ROS generating macrophages themselves and lower in the surrounding tissue. If this were the case, most macrophages could be destroyed rather selectively by redox-modulating agents. Subsequently, ROS and cytokine levels would be expected to decrease and the inflammation may ultimately subside. This rather unconventional strategy of 'fighting fire with fire' may ultimately be superior to conventional antioxidant strategies, which only counteract existing ROS but cannot interfere with ROS formation itself.[22]

Besides simply destroying macrophages, redox modulators may also target either the production or stability of •NO, a redox sensitive inflammatory signalling molecule, which is produced by an inducible enzyme, inducible •NO synthase (iNOS), upon activation of the innate immune system. Redox modulators may therefore also act as anti-inflammatory agents via disruption of the •NO signalling network.

Here, we provide initial evidence that certain compounds containing tellurium and quinone moieties are able to prevent the proliferation, and/or induce cell death of macrophages in cell culture when applied at (sub-)micromolar concentrations, most likely via a redox-mediated mechanism. Whilst the sulfur and selenium analogues generally are less cytotoxic, some of them are able to prevent the synthesis of •NO by macrophages and hence may be employed to disrupt pro-inflammatory cellular signalling.

2. Results/discussion

2.1. Selection and synthesis of compounds

In order to investigate the potential use of redox-modulating selenium and tellurium agents, several suitable agents were selected. The chemical structures of these compounds are shown in Fig. 1. Previous studies have indicated that multifunctional agents, which combine a 'ROS generating' quinone moiety with a catalytic, 'ROS using' chalcogen group are particularly effective (and selective) in cancer cell-based test systems.[5,23] Hence compounds containing selenium as well as tellurium, benzoquinone as well as naphthoquinone, were chosen for this study.

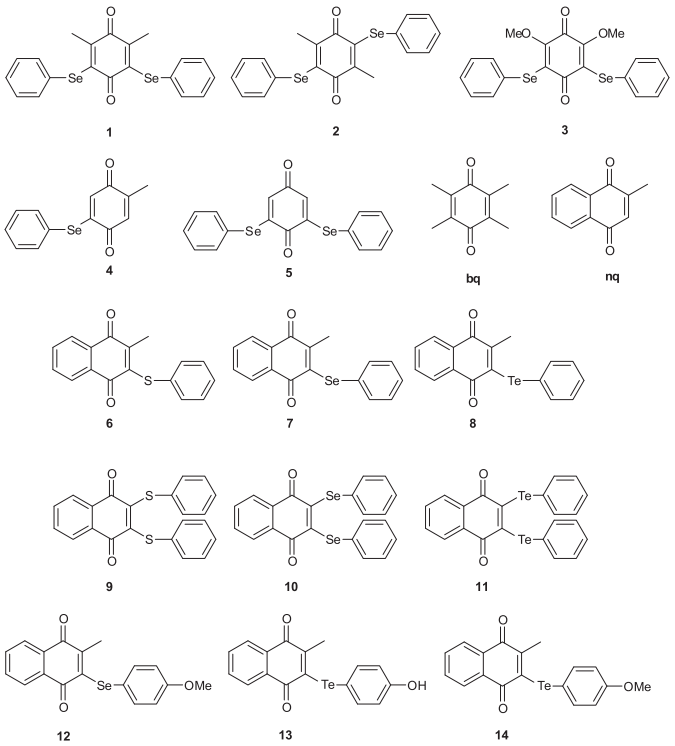


Fig. 1. Chemical structures of organochalcogens and reference compounds used in this study. These compounds are multifunctional and were selected on the basis of previous results obtained in cancer cells. Structures containing two (redox active) chalcogen centres in addition to a quinone redox centre are of particular interest. Compounds **1**–**4** have not been reported in the literature before and their synthesis is described in the experimental section.

Some of these compounds were already known in the literature and could be synthesized according to established literature methods, while others have been produced for the first time following specifically adapted synthetic procedures. Where appropriate, these synthetic avenues are discussed in the experimental section. Ultimately, 14 compounds (four of them novel) have been obtained in sufficient amounts and purity and were used for this study. The tellurium analogues of compounds **1**–**3** have also been synthesized and characterized chemically, but they were not of sufficient purity at the time to be employed in biological cytotoxicity assays.

2.2. Cytotoxicity in macrophage culture

Whilst compounds such as **8** and **11** have been studied in cancer cell models before,[5] there is no data available on possible cytotoxic effects of these compounds on macrophages. As part of our studies, we have therefore measured the impact of compounds **1**–**14**—together with 'chalcogen-free' benzoquinone and naphthoquinone controls—on RAW 264.7 macrophage viability using the MTT assay. Among the various compounds tested, the tellurium compounds, in particular, exhibit a significant cytotoxic effect, reducing cell viability in a concentration dependent manner and with IC_{50} values in the sub-micromolar range (Fig. 2). Table 1 summarizes the IC_{50} values obtained in the macrophage viability assay

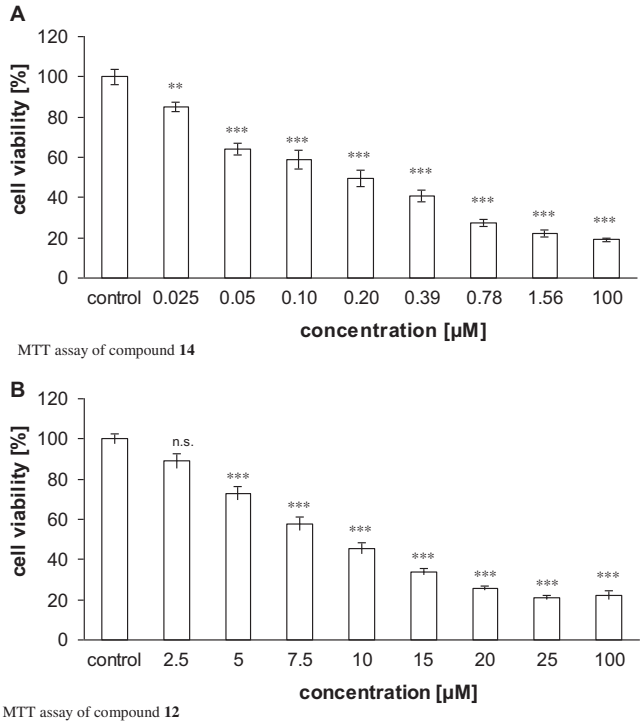


Fig. 2. Impact of compounds on macrophage viability measured in the MTT assay. Cells were incubated with the relevant compound for 24 h. Panel A shows the concentration dependence of cytotoxicity induced by the tellurium compound **14**, which is among the most active compounds studied in this assay. This compound is active in the submicromolar range (IC_{50}=160 nM). In contrast, the selenium analogue, compound **12**, is considerably less cytotoxic, with an IC_{50} value of 9.2 μM. Shown are the significances calculated with the Bonferroni test compared to control.

Table 1
Summary of the various selenium- and tellurium-containing compounds used as part of this study and IC_{50} values obtained in the macrophage survival assay

Compound	Chalcogen	IC_{50}
1	Se	20.46
2	Se	24.95
3	Se	26.11
4	Se	5.27
5	Se	43.77
bq	—	27.56
nq	—	8.88
6	S	5.97
7	Se	7.71
8	Te	0.30
9	S	15.15
10	Se	20.32
11	Te	0.30
12	Se	9.20
13	Te	1.20
14	Te	0.16

after 24 h. Compounds **8**, **11** and **14** are particularly active, with IC_{50} values of 0.30, 0.30 and 0.16 mM, respectively. In contrast, the sulfur and selenium analogues were considerably less toxic, with IC_{50} values in the micromolar range (e.g., compounds **9** and **10** with an IC_{50} of 6.0 and 7.7 μM, respectively).

These findings agree with previous reports, which generally point towards a considerably higher toxicity for the tellurium compounds in mammalian cells when compared to the corresponding selenium and sulfur analogues.[24,25] Interestingly, the cytotoxicity of **8** and **11** against macrophages is also at least 10 times higher when compared to values obtained by us for a range of cancer cells or healthy cells.[5] This finding is particularly significant, as it seems to identify macrophages as (the) prime target of such tellurium-based redox agents. If confirmed in more complex systems, such as animals, such a selective activity against macrophages at compound concentrations below 1 μM would be rather exciting, as it may provide a new avenue to target macrophages with considerable precision.

2.3. Tellurides: redox modulation as possible mode of action

The cytotoxicity assays support the general hypothesis that macrophages, like cancer cells under OS, may represent targets of redox-active compounds; they do not, however, explain why and how such compounds may act on these cells. In order to obtain some basic and certainly *preliminary* insights into the possible mode(s) of action, we have therefore used cell-based assays to measure ROS and ˙NO production in these cells.

In the case of the tellurium compounds, the 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA)-based assay for intracellular ROS has been employed and a small increase of intracellular ROS levels (around 1 to 1.3-fold) could be observed for some of the tellurium-containing compounds, which reached a plateau after 40 min of incubation and then diminished again (data not shown).

Since intracellular ROS levels in macrophages were difficult to follow and quantify, a yeast-based chemogenetic screen has been employed as a more reliable and also more informative analytical alternative. The latter relies on a range of yeast mutants, which are deleted in various, mostly *redox-related* enzymes, such as superoxide dismutase (SOD), catalase (CAT) or glutathione disulfide reductase (GR). Each of these mutants is exposed to the test compounds, and mutants particularly sensitive to the compounds point towards a possible link between the (missing) enzyme and its cellular role(s) on the one hand, and the biochemical action of the compounds tested on the other. Importantly, for both, the enzymes of the glutathione metabolism and glutathione reductase, the yeast genome contains only one gene for the particular enzyme; and for the superoxide dismutases and catalase, the yeast genome has only one gene for a cytosolic (*sod1*) and a mitochondrial (*sod2*) enzyme and only one for cytosolic catalase, respectively.[26] This easy genetic accessibility of particular biochemical key players, as well as an easy experimental handling of yeast, turns this organism into a reasonable and advantageous system for chemogenetic screening.[27] As yeast features a cellular redox biochemistry, which is comparable to human cells (but less complex), *certain* comparisons are possible. Nonetheless, one should always be careful not to overstrain the analogy between this fungus and human cells.

Interestingly, the yeast-based chemogenetic analysis confirms a significant cytotoxicity for the most active tellurium compounds **8** and **14** (compound **11** was not tested in this assay). Some of the selenium compounds show a similar cytotoxicity, highlighting differences in susceptibility towards selenium- and tellurium compounds, which seem to exist between yeast cells on the one hand and macrophages on the other. As far as the different cytotoxicity of selenium and tellurium compounds in yeast is concerned, it is possible that yeast cells are either more sensitive towards selenium compounds or can cope better with tellurium compounds as may be expected from previous data obtained for human cells. Such considerations are interesting in the context of antifungal activity and should be investigated further in the future.

Compounds **8** and **14** are particularly toxic against strains lacking mitochondrial manganese-containing superoxide dismutase (*sod2*Δ) and, in some instances, are also toxic against strains lacking a cytosolic zinc, copper SOD (*sod1*Δ) (Fig. 3). Perhaps surprisingly, the screen also shows that mutants lacking cytosolic catalase (*ctt1*Δ) or glutathione reductase (*glr1*Δ) are less affected. Furthermore, mutants that are affected by/impaired in glutathione

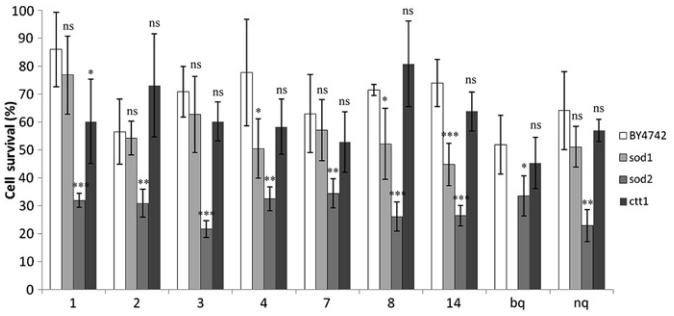


Fig. 3. Simple chemogenetic screen of selected compounds (at 100 μM concentration, 16 h incubation time) in a yeast-based cell survival assay. Besides the wild-type strain BY4742, mutants deficient in *sod1*, *sod2* and cytosolic catalase (*ctt1*) have been employed. Whilst most compounds show a moderate cytotoxicity against the wild-type strain, they are particularly toxic against the *sod2* mutant strain, pointing towards a possible link between the compounds' biochemical mode(s) of action and the intracellular role(s) of *sod2*. The latter includes the removal of superoxide radical anions. Shown are the significances calculated with Student's *t*-test compared to control.

biosynthesis show an ambivalent picture: Whilst the *gsh1*Δ mutant shows a high sensitivity to most (but not all) of the tested compounds, sensitivity of the *gsh2*Δ mutant is much less pronounced. Overall, data on the *gsh1*Δ and *gsh2*Δ mutants are fairly inconclusive and at this stage does not point towards a specific causality between the compounds tested and glutathione synthesis in general (data not shown).

It should be mentioned that inorganic selenite (SeO_3^{2-}) is known to affect DNA integrity, and hence DNA damage caused by selenium and tellurium agents may be another major cause of cell death. Therefore, a mutant deficient in homologous recombination (*rad52*Δ), the main pathway responsible for repairing DNA double-strand breaks, was also screened. This mutant was not sensitive to any of the compounds tested. This finding clearly counts against significant DNA damage or lack of DNA repair caused by the organoselenides and tellurides investigated as part of this study and is in sharp contrast to the known toxic effects of SeO_3^{2-} on DNA integrity.[28] Such issues surrounding selenium and tellurium compounds and their association with DNA damage and repair are of major interest as far as chalcogen toxicity is concerned—and form the topic of our ongoing investigations.

Although it is difficult to derive at finite conclusions based on these still limited results, it nonetheless seems that SOD activity (predominantly in the mitochondria, i.e., SOD2) is important for the defence of the cells against the effect(s) of the tellurium compounds tested. Hence, an involvement of superoxide radical anions ($O_2^{\bullet-}$)—possibly generated by some of the quinone compounds and exerting its damaging activity at or in the mitochondria—can be hypothesized (as various quinones are known to generate ROS). In contrast, as catalase deficient strains are less affected, H_2O_2 does not seem to play a major role in the activity of the compounds, which is striking because the SOD-dependent superoxide degradation results in the formation of H_2O_2.[29] One should note, however, that the yeast catalase in question is located in the cytosol, and therefore the H_2O_2 generated by *sod2* as part of the detoxification of $O_2^{\bullet-}$ may be detoxified in a catalase-independent manner (for instance by a mitochondrial peroxiredoxin).[30]

Nonetheless, one must be careful not to over-interpret these still preliminary sets of data. Firstly, the mutants lacking SOD or catalase may behave differently because $O_2^{\bullet-}$ and H_2O_2 may possess a different cytotoxicity in yeast (if one of these ROS is less cytotoxic than the other, then the mutant lacking the corresponding defence enzyme(s) may also be less affected). Such issues have formed the subject of several studies in the past and it seems that a general link between the SOD and catalase mutants on the one hand and the involvement of $O_2^{\bullet-}$ and H_2O_2 in the cytotoxic effects of certain compounds on the other is still valid.[31,32] Nonetheless, such studies need to be taken with some caution.

Secondly, mitochondria are not the only source of $O_2^{\bullet-}$. It is therefore difficult to assign the effects observed here solely to an interaction with mitochondria or SOD2. Alternative, maybe more complex interactions are also possible (for instance involving NADPH oxidases) and cannot be ruled out at this point.[33]

The same considerations also apply to strains whose GSH synthesis is impaired or which are deficient in GR. Here, the results obtained so far count against the direct involvement of GSH or glutathione disulfide (GSSG) in the mechanism of the compounds' cytotoxic action. One should note, however, that other redox-modulating agents targeting the cellular thiolstat, such as allicin, do have a pronounced effect on strains lacking GR. This difference in toxicity, which apparently exists between most of the quinones tested on the one hand, and compounds such as allicin on the other, can be understood if one realizes that quinones can generate $O_2^{\bullet-}$ radicals, which *subsequently* oxidize cellular thiols, whilst allicin and related compounds themselves react with the cellular thiols.[20,34–37]

The possible involvement of $O_2^{\bullet-}$ has been confirmed in a simple cell-based luminescent lucigenin assay indicative of ROS generation. As part of this assay, the relative luminescence caused by the various selenium- and tellurium compounds is measured in the absence and presence of wild-type (BY4742) yeast cells. ROS generation based on simple chemical reactions (e.g., reactions with molecular oxygen) may be detected in the absence of cells, whilst increases in ROS levels due to biochemical processes (e.g., enzymatic conversions, inhibition of antioxidant enzymes) only become apparent in the presence of the cells.

As Fig. 4 indicates, none of the compounds tested generates significant levels of ROS in the absence of cells under the experimental conditions used in this assay (with the possible exception of the naphthoquinone **14**, which does seem to generate a small amount of ROS by its own). In contrast, the naphthoquinone menadione and the naphthoquinone-containing tellurides **8**, **14**

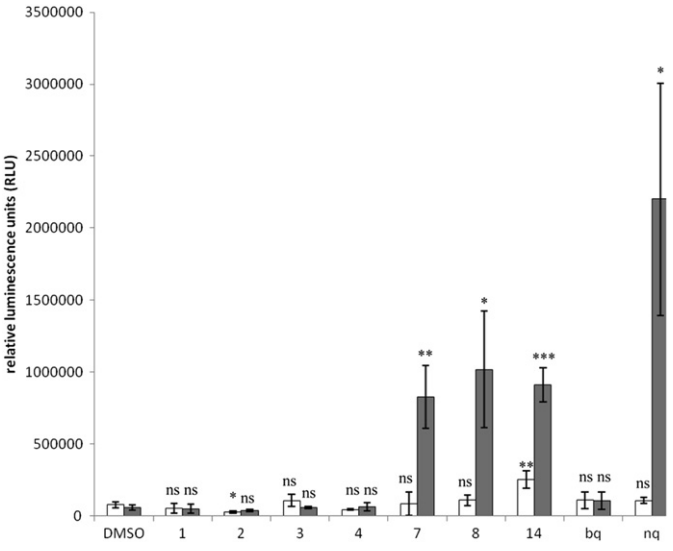


Fig. 4. Lucigenin-based luminescent yeast assay to estimate the formation of intracellular ROS in response to the compounds tested (incubation time 16 h). Whilst none of the compounds—with the possible exception of compound **14**—generates ROS on its own (white bars), significant levels of ROS can be measured for naphthoquinone and the naphthoquinone derivatives in the presence of cells (grey bars). Interestingly, the benzoquinone derivatives (and bq itself) under these conditions do not seem to generate any significant amounts of ROS. Statistical significances have been calculated with the Student's *t*-test and refer to the respective DMSO control.

and the selenide **7** cause a sharp increase in relative luminescence in the presence of cells. This increase in ROS levels is particularly significant at compound concentrations of 100 μM. Interestingly, the benzoquinones are notably less active than the naphthoquinones in this assay.

It should be emphasized that ROS formation is almost certainly due to the quinone moiety of the multifunctional compounds tested, and that the chalcogen (selenium or tellurium) moiety plays a subordinate role in ROS generation—but not in the *processing* of the quinone-generated ROS, which ultimately explains the differences in cytotoxicity between the selenium and tellurium compounds observed in the macrophages.[24,25,38]

The notion of quinones (primarily naphthoquinones) as ROS generating agents, and the apparent differences between the generally more active naphthoquinones and the less active benzoquinones, are supported by electrochemical results. Here, cyclic voltammetry in conjunction with a glassy carbon working electrode has been used to estimate the electrochemical potentials of the most active and some of the reference compounds, focussing on the quasi-reversible hydroquinone/quinone redox pair (see Experimental section for more details). Overall, $E_{1/2}$ values of around −190 mV to +100 mV have been obtained for the benzoquinones and (significantly more negative) $E_{1/2}$ values of −250 mV to −180 mV for the naphthoquinones, confirming the distinct differences in redox behaviour, which exist between these two classes of quinones. For comparison, benzoquinone bq shows an $E_{1/2}$ value of −192 mV, while naphthoquinone nq shows an $E_{1/2}$ value of −270 mV.

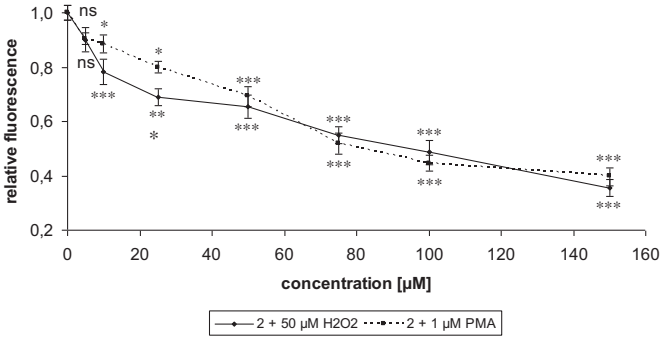
Interestingly, the anodic oxidation potentials *E*pa, which are indicative of the ease of oxidation of the reduced hydroquinones, and hence determine the ease of electron transfer from the hydroquinones to O_2 and the ease of $O_2^{\bullet-}$ formation (or H_2O_2), are generally lower (i.e., more reducing) for the naphthoquinones in comparison to the benzoquinones. The 'chalcogen-free' naphthoquinone nq, for instance, exhibits an *E*pa of −190 mV (IC_{50}=8.9 μM), while the corresponding benzoquinone bq shows an *E*pa of −3 mV (IC_{50}=27.6 μM). Similarly, the most cytotoxic (naphthoquinone) compounds **8** and **14** exhibit *E*pa values of −167 mV and −222 mV, respectively. These values are considerably lower than the ones observed for the (less active) benzoquinones, such as **4**, with an *E*pa value of +65 mV.

Ultimately, these differences in *E*pa values (and $E_{1/2}$) may in part explain the somewhat higher ROS generating ability and biological activity observed for the naphthoquinones. One should note, however, that the most active naphthoquinones also contain tellurium, and a direct comparison between the benzoquinones and naphthoquinones is therefore not straight forward (the tellurium-containing benzoquinone analogues were not suitable for biological testing, see section 2.1). Hence, future studies may also consider anthraquinones as a possible alternative to the benzo- and naphthoquinones.

2.4. Selenium: from traditional antioxidant and GPx mimic to potent interceptor of ·NO-based cell–cell signalling

The results obtained for the tellurium compounds in the MTT cell survival test and the subsequent assays indicate that the behaviour of these compounds differs considerably from the one of the selenium compounds. In strong contrast to the tellurium compounds, the selenium analogues are generally considerably less toxic against macrophages and also do not seem to generate any significant amounts of ROS in the macrophages or yeast cells. Quite on the contrary, some of the selenium compounds actually reduce ROS levels induced by H_2O_2 or phorbol-12-myristat-13-acetat (PMA) in a concentration-dependent manner.

Fig. 5 illustrates this apparent ROS removing (i.e., 'antioxidant') activity. The relative fluorescence of the macrophages stressed with H_2O_2 or stimulated for internal ROS production with PMA evidently diminishes upon addition of compound **2**. This apparent antioxidant activity may have several causes, including a partial removal of macrophages (see below), inhibition of pro-oxidant enzymes or a genuine chemical 'neutralization' of various ROS. It is also possible that some ROS are removed catalytically in the presence of cellular thiols (the latter may become oxidized as part of this process). The figure allows the estimation of a rough 'IC_{50} value' for the inhibition of ROS formation, which is around 100–120 μM for compound **2**. This IC_{50} value is quite high when compared to the IC_{50} cytotoxicity values obtained for such compounds in the MTT assay (IC_{50} of compound **2** is 25 μM). The apparent 'antioxidant' effect illustrated in Fig. 5 therefore may not necessarily result from a true reduction of ROS levels in intact macrophages, but may—in part—be due to secondary effects, such as loss of viable, radical generating macrophages. Nonetheless, the effect can also be observed at lower concentrations, i.e., when cell survival is less affected.



ROS assay of compound **2**

Fig. 5. Compound **2** suppresses or removes ROS added externally (H_2O_2) or formed internally upon stimulation of macrophages with PMA. Data show relative fluorescence in cells incubated for 40 min with compound **2** in the presence of H_2O_2 or PMA compared to H_2O_2 or PMA treatment alone. Shown are the significances calculated with the Student's *t*-test compared to control.

Confronted with this rather interesting result, the impact of the selenium compounds on another reactive species, i.e., ·NO, was investigated. Nitric oxide plays a major role in the activity and signalling of immune cells. First of all, ·NO belongs to the Reactive Nitrogen Species (RNS), and therefore is often associated with OS.[39] Here, it can react with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$). ·NO also binds to certain metalloproteins, such as soluble guanylyl cyclase (sGC), and hence is involved in a range of beneficial health effects, such as vasodilation. Finally, ·NO is a signalling molecule involved in inflammatory processes. It controls, for instance, the activity of T lymphocytes and signal transduction, and also has a pronounced effect on mitochondrial events (some of which may result in apoptosis).[40]

In order to estimate the impact of our compounds on the ·NO production of macrophages, the latter were stimulated with 100 ng/ml lipopolysaccharide (LPS), which results in an increased production of ·NO. Fig. 6 illustrates the results obtained by the Griess assay for compound **3**, which are in strong contrast to the 'selenium-free' reference benzoquinone bq. Whilst bq does not seem to interfere with ·NO production, compound **3**, at a concentration of just 2.5 μM, prevents any notable ·NO production in the macrophage (at this concentration **3** is not toxic to the cells). Comparable results were obtained for compound **2**, which, at the same time, is also a good antioxidant in our assays (Fig. 5).

Some compounds, such as (the reduced form of) α-lipoic acid, are known to react directly with ·NO and also inhibit apoptosis of

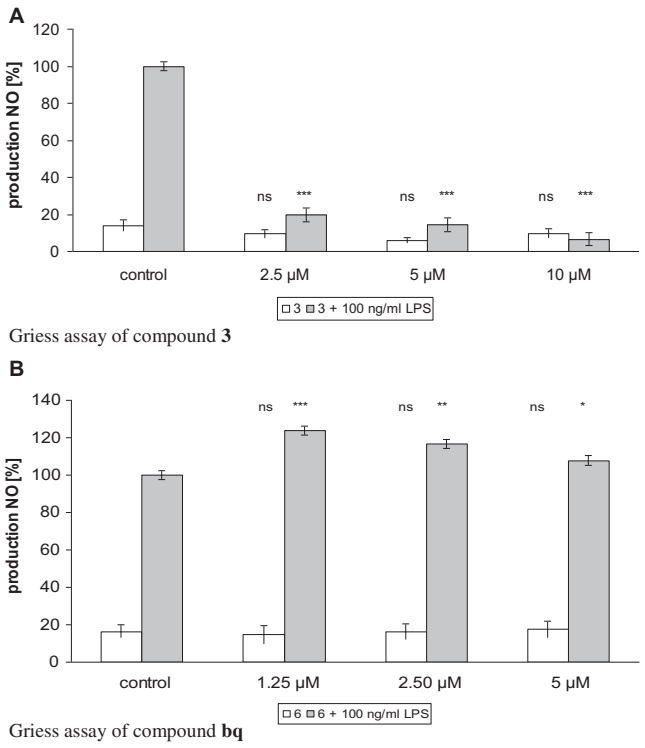


Fig. 6. Impact of some of the selenium compounds on •NO production of macrophages stimulated with LPS. Cells were incubated in the absence or presence of LPS with compounds for 20 h. Panel A illustrates the ability of compound **3** to almost completely suppress •NO production at concentrations in the low micromolar range. In contrast, the 'selenium-free' benzoquinone bq is unable to influence •NO production, highlighting the special role of selenium in this process. Whilst these results point towards a specific activity of the chalcogen in this process, it has not been possible to demonstrate a similar effect for the tellurium analogues, as these compounds are too cytotoxic. Shown are the significances calculated with the Student's *t*-test compared to control.

certain cells by a more indirect mode of action.[41,42] Therefore, in order to rule out any simple chemical interactions between •NO and the compounds tested, and hence to confirm a genuine interference with cellular processes, cell-free in vitro assays were carried out using diethylamine nonoate diethylammonium (DEA) and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) as chemical •NO donors. In these cell-free assays, none of the compounds tested interfered notably with •NO levels, pointing towards an interference of compounds such as **3** with the *biosynthesis* of •NO (and not with •NO itself). Indeed, there are reports in the literature, which point towards an inhibitory effect of quinones, sulfur (such as hydrogen sulfide) and selenium compounds on iNOS, and it may be possible that compound **3** acts as inhibitor of iNOS, which is the sole •NO generating synthase in macrophages. As the quinone bq is not active, this effect seems to be in large parts due to the presence of selenium. Indeed, iNOS is a haem-containing enzyme and strong inhibitory ligand binding effects of sulfur and selenium compounds at the haem centre are possible.[43–45] Interestingly, benzoquinones generally seem to be more active than naphthoquinones, which is in contrast to our observations with the tellurium compounds (see above).

One should note that the tellurium agents employed may also impact on the synthesis of •NO, yet these agents are also cytotoxic and hence such an effect has not been observed. The sulfur compounds tested were generally not particularly active in the •NO assay (data not shown).

If, how, and how strongly such chalcogen-containing compounds inhibit enzymes such as iNOS needs to be studied in more detail. There have been reports that the selenium compound ebselen inhibits isolated iNOS when employed in the low micromolar range. Yet this effect seems to disappear in the presence of thiols.[46] Inside the cell, matters seem to be even more complicated, as ebselen does not impact on iNOS expression, yet may interfere with iNOS activity or sequester •NO directly.[47] Furthermore, ebselen may also intercept intracellular redox signalling, hence acting on the •NO producing system more indirectly.[48]

From a pharmacological perspective, the rather dramatic effect exerted by low concentrations of **3**, as seen in Fig. 6a, is of special interest. Whilst macrophages are not killed at these concentrations, their ability to generate •NO is diminished, and hence some of their pro-inflammatory actions may also be reduced. If this effect is sufficient to reduce inflammation itself needs to be studied in more complex models, such as animals. Nonetheless, other agents able to interfere with the •NO production of macrophages, such as plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), have recently attracted considerable interest and are already under discussion in the context of a possible treatment of inflammatory diseases.[49] Future studies need to investigate the potential of these and related selenium compounds (such as ebselen) as modulators of macrophage •NO generation in considerably more detail. Available literature on this topic is still sparse and the various interactions of selenium compounds with •NO, •NO generation, •NO removal and peroxynitrite ($ONOO^-$) therefore provide a fertile ground for future studies.[46–48,50]

In any case, the selenium-based approach of interfering with •NO generation and cellular signalling represents a milder, and perhaps more sophisticated strategy towards anti-inflammatory treatment compared with the tellurium-based approach, which simply kills the macrophages. Nonetheless, the concentrations required in both scenarios differ (a couple of 100 nM of the tellurium agents are required to kill the macrophages compared to low micromolar concentrations of the selenium agents to suppress •NO production). Therefore statements referring to 'milder' effects, to more specificity and less side-effects are premature and need to be taken with some caution. Both strategies employ comparably low concentrations of agents, and both may be quite effective and selective.

3. Conclusions

Overall, our studies confirm that selenium- and tellurium-containing agents are not only active against cancer cells but also bear considerable potential in the field of inflammatory pathologies. The toxicity of some of the tellurium-containing agents manifests itself in the sub-micromolar range, i.e., at concentrations, which are considerably lower than the ones required for these compounds to kill cancer cells and healthy control cells.[5] Whether macrophages are really the prime targets of such compounds, and whether such an activity may be turned into a pharmaceutical application, still needs to be shown. At the same time, the underlying chemical interactions and biochemical processes remain vastly unexplored. Whilst antioxidant enzymes (primarily SOD), $O_2^{\bullet-}$ and redox processes appear to play an important role, the elucidation of such complex signalling pathways requires considerably more in-depth investigations in the future. Ultimately, the chemical design of these redox-modulating agents may also be refined further, for instance by exploring anthraquinone derivatives as possible alternatives.

Similarly, the perhaps rather surprising finding that some of the selenium agents, despite the presence of various quinone moieties in their respective structures, act as antioxidants and suppressors of •NO synthesis, bodes well for possible drug design but also poses some interesting questions. Compounds, such as **2** and **3**, which on the one hand prevent •NO formation by macrophages when used in

low micromolar concentrations and, on the other hand, also act as antioxidants (albeit at higher concentrations), may counteract some of the pro-inflammatory events associated with macrophages and the aggressive chemical and signalling molecules they release. It needs to be shown if this kind of 'double impact' on ROS and •NO levels is particularly beneficial or not.

Since tellurium compounds are generally more active in biological systems compared to their selenium analogues, the influence of (less cytotoxic) tellurium compounds on the macrophage-driven synthesis of •NO represents a topic, which also needs to be addressed in earnest as part of future investigations.

In any case, our studies provide ample opportunities for future interdisciplinary investigations in the field of selenium and tellurium chemistry and biochemistry, redox modulation, macrophage targeting and innovative anti-inflammatory drug design.

4. Experimental section

4.1. General procedure for the synthesis of compounds 1–17

Compounds **5**–**14** have been synthesized according to literature procedures.[5] Compounds bq and nq were purchased from Sigma–Aldrich. Compounds **1**–**4** have not been reported in the literature. The bromides 2-bromo-3-methyl-1,4-naphthoquinone, 2,5-dibromo-3,6-dimethyl-1,4-benzoquinone, 2-bromo-5,6-dimethoxy-3-methyl-1,4-benzoquinone, 2,6-dibromo-3,5-dimethyl-1,4-benzoquinone and 2-bromo-5-methyl-1,4-benzoquinone were prepared according to a literature procedure.[51]

4.2. General procedure

Under argon atmosphere, disulfide (or diselenide or ditelluride) (1 equiv) was dissolved in a mixture of 100 ml THF and 25 ml water. $NaBH_4$ (~4 equiv) was added to the yellow or orange solution and the mixture was stirred vigorously until it turned colourless. The appropriate haloquinone (1 equiv for dihaloquinone or 2 equiv for monohaloquinone) in THF (5 ml) was added and the formation of the desired product was monitored via thin layer chromatography (TLC). Afterwards the solution was stirred for further 15 min on air. The violet, dark red or orange coloured reaction mixture (depending upon the Te, Se or S counterpart of the product) was diluted with saturated aqueous NH_4Cl and extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by silica gel chromatography (mesh size 40–60 μm) using mixtures of petrol ether (40–65 °C) and ethyl acetate as specified for each compound below. Since the compounds might be sensitive to oxidation and light, they were stored under argon atmosphere and in the dark.

*4.2.1. Synthesis of 2,6-bis(phenylselanyl)-3,5-dimethyl-1,4-benzoquinone (**1**).* Compound **1** was synthesized from diphenyldiselenide (376 mg, 1.2 mmol) and 2,6-dibromo-3,5-dimethyl-1,4-benzoquinone (354 mg, 1.2 mmol) following the general procedure. Compound **1** was purified by repeated column chromatography on silica gel using petrol ether/ethyl acetate (95:5 v/v) as solvent, R_f=0.51. Yield 8.6%, red solid, mp 112 °C. ^{1}H NMR: δ=7.25–7.24 (m, 4H, H-a), 7.04–7.01 (m, 6H, H-b and H-c), 1.90 (s, 6H, CH_3) ppm ^{13}C NMR: δ=182.5 (1C), 180.0 (1C), 147.8 (2C), 144.1 (2C), 133.6 (4C), 129.6 (2C), 129.5 (4C) 128.0 (2C), 17.5 (2C) ppm. ^{77}Se NMR: δ=371.25 ppm. HRMS for $C_{20}H_{16}O_2Se_2$ (*m*/*z*): calcd: 477.95 (100%), 445.95 (92.2%), 443.95 (51.8%); found 447.9486 (100%), 445.9583 (93.10%), 443.9655 (55.60%).

*4.2.2. Synthesis of 2,5-bis(phenylselanyl)-3,6-dimethyl-1,4-benzoquinone (**2**).* Compound **2** was synthesized from diphenyldiselenide (978 mg, 3.0 mmol) and 2,5-dibromo-3,6-dimethyl-1,4-benzoquinone (949 mg, 3.2 mmol) following the general procedure. Compound **2** was purified by repeated column chromatography on silica gel using petrol ether/ethyl acetate (95:5 v/v) as solvent, R_f=0.50. Yield 23.4%, red solid, mp 112 °C. ^{1}H NMR: δ=7.45–7.44 (m, 4H, H-a), 7.24–7.22 (m, 6H, H-b and H-c), 1.99 (s, 6H, CH_3). ^{13}C NMR: δ=181.5 (2C), 148.1 (2C), 143.9 (2C), 133.7 (4C), 129.6 (2C), 129.5 (4C), 128.1 (2C), 17.8 (2C) ppm. ^{77}Se NMR: δ=367.34 ppm. HRMS for $C_{20}H_{16}O_2Se_2$ (*m*/*z*): calcd: 477.95 (100%), 445.95 (92.2%), 443.95 (51.8%); found 447.9483 (100%), 445.9556 (96.37%), 443.9646 (55.23%).

*4.2.3. Synthesis of 2,6-bis(phenylselanyl)-3,5-dimethoxy-1,4-benzoquinone (**3**).* Compound **3** was synthesized from diphenyldiselenide (470 mg, 1.5 mmol) and 2,6-dibromo-3,5-dimethoxy-1,4-benzoquinone (441 mg, 1.3 mmol) following the general procedure. Compound **3** was purified by repeated column chromatography on silica gel using petrol ether/ethyl acetate (95:5 v/v) as solvent, R_f=0.32. Yield 34.1%, black solid, mp 81 °C. ^{1}H NMR: δ=7.54–7.53 (m, 4H, H-a), 7.26–7.21 (m, 6H, H-b and H-c), 3.57 (s, 6H, CH_3). ^{13}C NMR: δ=182.6 (1C), 174.9 (1C), 156.9 (2C), 134.7 (4C), 129.3 (2C), 129.1 (4C), 128.7 (2C), 128.3 (2C), 60.7 (2C) ppm. ^{77}Se NMR: δ=355.35 ppm. HRMS for $C_{20}H_{16}O_4Se_2$ (*m*/*z*): calcd: 479.94 (100%), 477.94 (92.3%), 475.94 (51.6%); found 479.9597 (100%), 477.9580 (97.56%), 475.9626 (53.00%).

*4.2.4. Synthesis of 2-(phenylselanyl)-5-methyl-1,4-benzoquinone (**4**).* Compound **4** was synthesized from diphenyldiselenide (492 mg, 1.5 mmol) and 2-bromo-5-methyl-1,4-benzoquinone (158 mg, 0.8 mmol) following the general procedure. Compound **4** was purified by repeated column chromatography on silica gel using petrol ether: ethyl acetate (95:5 v/v) as solvent, R_f=0.51. Yield 21.1%, orange solid, mp 112 °C. ^{1}H NMR: δ=7.82–7.80 (m, 2H, H-a), 7.74–7.66 (m, 3H, H-b and H-c), 7.50 (s, 1H), 6.45 (s, 1H), 5.46 (s, 1H), 1.80 (s, 3H, CH_3). ^{13}C NMR: δ=185.2 (1C), 184.7 (1C), 147.3 (1C), 137.1 (2C), 132.7 (1C), 130.6 (1C), 130.4 (2C), 130.3 (1C), 16.1 (1C) ppm. ^{77}Se NMR: δ=414.08 ppm. HRMS for $C_{13}H_{10}O_2Se$ (*m*/*z*): calcd: 277.98 (100%), 275.99 (50.4%), 273.99 (18.9%); found 277.9823 (100%), 275.9825 (49.87%), 273.9939 (18.49%).

4.3. Cell culture

RAW 264.7 cells (murine macrophages, established from a tumour induced by the Abelson murine leukaemia virus) were cultured in RMPI medium containing 10% FCS, 5% glutamine, 5% penicillin/streptomycin and kept in an atmosphere containing 5% CO_2 and at a temperature of 37 °C.

4.4. Statistics

Unless stated otherwise, all experiments were performed three times and in triplicate. All graphs represent means±SE as error bars. Statistical differences were performed using independent two-sample Student's *t*-test in Microsoft Excel. MTT assays were analyzed using one-way ANOVA at a significance level of 0.001 followed by a Bonferroni test (OriginPro 8.6G Software, OriginLabs, Northampton, MA, USA). ***: $P<0.001$, **: $P<0.01$, *: $P<0.05$.

4.5. MTT assay

The MTT assay was performed as described in the literature.[52] Briefly, cells were seeded in a 96-well plate at a density of 40,000 cells/100 μl and allowed to adhere for 6 h. Then cells were treated with test compounds in 2.5, 5, 10, 15, 20, 25 and 100 μM in the absence or presence of 30 μM H_2O_2. Cells were also incubated

with 0.2% DMSO, which was used as solvent for the tested compounds and served as control. After incubation for 24 h the medium was removed, 150 μl MTT (0.5 mg/ml in medium) were added and incubated for 40 min. Then MTT was removed and cells were lysed by addition of 200 μl DMSO. The absorbance was measured at a wavelength of 550 nm and 690 nm was used as reference wavelength. Each concentration was tested in three independent experiments and in triplicate. A preliminary experiment showed that the appropriate solvent control containing 0.2% DMSO had no statistically significant effect on the cell viability and thus all calculated cell viabilities are expressed relative to 0.2% DMSO. IC_{50} values were calculated using OriginPro8.6.

4.6. Griess assay

The Griess assay was performed according to a literature procedure.[53] Briefly, cells were seeded in a 96-well plate at a density of 80,000 cells/200 μl. After allowing the cells to adhere for 6 h, cells were treated with the test compounds in the presence or absence of 100 ng/ml LPS (ultrapure from *Escherichia coli*, K12 strain. TLR 4 ligand). DMSO in the appropriate concentration was used as control. After incubation for 20 h, 100 μl of the supernatant were transferred into a 96-well plate and 90 μl of sulfanilamide and 90 μl of *N*-(1-naphthyl)-ethylene-diamine were added to quantify nitrite as a metabolite of •NO. $NaNO_2$ was used for the standard curve on the same plate. The absorbance at 550 nm was measured using a microplate reader against the background of 690 nm. For the determination of cell viability, MTT assay was performed as described. In a preliminary experiment different concentrations of LPS (50 ng/ml to 1 μg/ml) were tested to determine the effective concentration of LPS. To make sure that the effects observed were not an interaction of the test compounds with Griess reagents themselves, the same assay was performed without cells[42] using the •NO donors diethylamine nonoate diethylammonium salt (DEA) or *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Briefly, 10 μM or 100 μM of test compounds were added to a solution containing 100 μM DEA or 100 μM SNAP and allowed to incubate for 15 min (DEA) or 7 h (SNAP). The amounts of •NO released by the •NO donors were not statistically significantly changed in the presence of the test compounds.

4.7. ROS assay

The ROS assay was performed as described in the literature.[54] Cells were seeded with a density at 80,000 cells/200 μl in a 96-well plate and allowed to adhere for 4 h. Then, 100 μl of the medium were removed and 100 μM of the test compounds were added to yield final concentrations of 5, 10, 25, 50, 75, 100 and 150 μM. Cells were incubated at 37 °C for 25 min. Afterwards the complete medium was removed, cells were washed with warm HBSS and 200 μl of 20 μM 2′,7′-dichlorodihydrofluorescein diacetate in HBSS were added. After incubation for 25 min, cells were washed with HBSS, 200 μl of HBSS were added and cells were stimulated with 1 μM PMA or 50 μM H_2O_2. Cells only treated with DMSO, dye and stimulus served as control. The fluorescence was followed for 40 min in a fluorescence reader (Wallac Victor 2) at a temperature of 37 °C using the excitation filter set at 485 nm and the emission filter set at 535 nm. In a control experiment, different concentrations of PMA and H_2O_2 were tested and 1 μM of PMA and 50 μM of H_2O_2 were found to differ significantly from the cells treated only with dye. Another control experiment, in which cells were only incubated with the test compounds and stimuli in the absence of dye, showed no fluorescence, proving that neither test compounds nor stimuli induced fluorescence by themselves.

4.8. Yeast strains and cultivation

Yeast strain BY4742[55] and its mutant derivatives (see table) were obtained from Euroscarf, University of Frankfurt, Germany (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) and grown overnight in liquid complete synthetic dropout medium (CSM; 7 g/l yeast nitrogen base; ForMedium, Hunstanton, Norfolk, UK; 0.8 g/l complete dropout, Vista, CA, USA; 40 g/l glucose, supplied by Carl Roth, Karlsruhe, Germany) with shaking (210 rpm) at 28 °C.

Mutant	ORF	Genotype
*gsh1*Δ	YJL101C	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YJL101c*::*kanMX4*
*gsh2*Δ	YOL049W	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YOL049w*::*kanMX4*
*glr1*Δ	YPL091W	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YPL091w*::*kanMX4*
*sod1*Δ	YJR104C	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YJR104c*::*kanMX4*
*sod2*Δ	YHR008C	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YHR008c*::*kanMX4*
*ctt1*Δ	YGR088W	*MATα*; *his3Δ1*; *leu2Δ0*, *lys2Δ0*, *ura3Δ0*; *YGR088wc*::*kanMX4*

4.9. Lucigenin-assay for ROS accumulation

The lucigenin assay was employed as a general method for quantification of ROS accumulation rather than as a specific probe for superoxide accumulation, since the specificity of lucigenin for $O_2^{\bullet-}$ has been called into question.[9,56] Overnight yeast cultures were adjusted to an OD_{600}=1 and treated with the test compounds, dissolved in DMSO, at end concentrations of 10 and 100 μM. Controls were treated with DMSO alone. The final concentration of DMSO in the cultures was 0.6 mM. Cultures were incubated for a further 16 h (28 °C, 210 rpm). The same treatment was performed with cell-free medium in order to exclude a chemical and cell-independent formation of ROS.

Lucigenin (bis-*N*-methylacridinium nitrate; Sigma–Aldrich, Steinheim, Germany) was dissolved in DMSO to a concentration of 50 μM. Lucigenin-solution (100 μl) was added to a 100 μl aliquot of yeast culture and the emission of photons by luminiscence was quantified over an interval of 10 s using a luminometer (Lumat LB9501, Berthold, Wildbad, Germany).

4.10. Quantification of cell survival

Yeast cells were grown overnight as described above. The culture was adjusted to an OD_{600}=1 and test compounds were added to final concentrations of 10 and 100 μM. Cells were incubated further for 16 h (28 °C, 210 rpm). Subsequently, dilution series of the yeast cultures were plated onto YPD plates (10 g/l yeast extract; Carl Roth, Karlsruhe, Germany; 20 g/l pepton; Duchefa, Haarlem, The Netherlands; 20 g/l glucose; Carl Roth, Karlsruhe, Germany; 2% agar) and incubated at 28 °C. The number of colony forming units (cfu) was counted. Cell survival after treatment with the test substances is expressed as a percentage of the control colony counts.

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