Redox catalysts as sensitisers towards oxidative stress

Niroshini M. Giles[a], Nick J. Gutowski[b], Gregory I. Giles[a], Claus Jacob[a,*]

[a] *School of Chemistry, University of Exeter, Stocker Road, Exeter EX4 4QD, UK*
[b] *Peninsula Medical School, Universities of Exeter and Plymouth, and Royal Devon and Exeter Hospital, Barrack Road, Exeter EX2 5DW, UK*

Received 27 November 2002; accepted 12 December 2002

First published online 9 January 2003

Edited by Julio Celis

Abstract The predominance of oxidative stress in many tumour cell environments provides a means to selectively target these cells via protein oxidation. The zinc fingers of transcription factors utilise cysteine thiols for structural zinc coordination. Redox control of DNA binding regulates transcription and therefore the overall rates of proliferation, apoptosis and necrosis in the carcinoma. We report here the adverse effects of glutathione peroxidase (GPx) mimics towards zinc finger motifs and PC12 cell survival. Nanomolar catalyst concentrations facilitated H_2O_2-induced oxidation of an Sp1 transcription factor fragment. In PC12 cells GPx catalysis triggered a significant increase in cell death, correlating with severity of oxidative stress. As a consequence, we conclude that GPx mimics are potential chemotherapeutic agents.

Key words: Oxidative stress; Redox catalysis; Glutathione peroxidase mimic; Zinc finger protein; PC12 cell

1. Introduction

Cellular production and removal of reactive oxygen species (ROS) need to strike a critical balance and as such the cell employs several antioxidant systems that effectively quench and neutralise these toxic molecules. In contrast, a host of human disorders, ranging from inflammatory and neurodegenerative to viral and proliferative diseases, are associated with oxidative stress, a biochemical condition where the production of ROS is increased while antioxidant defence is decreased [1,2]. A raised level of ROS has also been observed in certain animal and human cancer cells [3,4] and it has been shown that antioxidant enzyme levels in these cells are low. For example, human kidney, prostate and six forms of human lung carcinomas have lower antioxidant enzyme staining levels [5,6].

A disturbed redox environment could be exploited to specifically attack these types of cancer cells whilst leaving cells with a healthy redox balance unaffected. Fernandez-Pol et al. have recently suggested that agents which can regulate the zinc finger structure of transcription factors (e.g. Sp1) and hence influence DNA interactions have the potential to control viral and cancerous diseases [7]. While these authors have proposed the use of zinc-chelating agents to attack the zinc–sulfur complex, oxidation of the thiol ligands might be the more promising approach [8]. ROS rapidly oxidise and subsequently inactivate redox-sensitive proteins, severely damaging membranes and DNA. Theoretically, it should be possible to *catalyse* the oxidation of the zinc finger, preventing DNA binding, transcription, protein synthesis and proliferation and hence induce tumour cell death *without* excessive use of oxidants or chelators. Since redox-sensitive targets and ROS are already present in these cells, but do not rapidly react with each other, a redox catalyst merely *facilitating* a hindered oxidation process would dramatically *sensitise* these cells to already existing 'internal' oxidative stressors by promoting their reaction with zinc finger and other thiol dependent proteins.

The selenoenzyme glutathione peroxidase (GPx) catalyses the reaction of peroxides or peroxynitrite with glutathione (GSH), reducing the oxidative stressor with concomitant formation of oxidised glutathione. Catalytic antioxidants that mimic the action of GPx can be formed synthetically by placing a selenium or tellurium atom in an organic framework. These mimics can undergo the same reaction cycle as GPx, but lack the enzyme's substrate specificity and are able to accept any cellular thiol. As such they can function as either pro-oxidants or antioxidants, depending upon the cellular redox state.

This paper demonstrates the ability of nanomolar concentrations of redox catalysts to dramatically increase the damaging effects of the ROS hydrogen peroxide on a zinc finger protein Sp1 fragment. The most active compound sensitised cultured PC12 neuronal cancer cells to the effects of H_2O_2. The activity of this agent is the result of a synergistic effect of peroxide with catalyst as the latter was non-toxic in the absence of oxidative stress conditions.

2. Materials and methods

2.1. Materials

The zinc finger peptide fragment resembling the zinc finger of transcription factor Sp1 KFACPECPKRFMRSDHLSKHIKTHQNKK [9] (ZFPF) was ordered from the Peptide Synthesis Unit IBMS (University of Southampton, Southampton, UK). All chemicals required for electrochemistry, in vitro zinc finger assay, atomic absorption and cell culture were either analytical or tissue culture grade and purchased from Sigma-Aldrich Company (Poole, UK). Redox catalysts were synthesised as described previously [10]. Rat adrenal PC12 cell lines were purchased from ECACC (Salisbury, UK). Chelexed (i.e. 'metal free') nitrogen-purged buffers were used for the ZFPF oxidation assay. All experiments were performed in at least triplicate and results are given as means.

*Corresponding author. Fax: (44)-1392-263434.
E-mail address: c.jacob@ex.ac.uk (C. Jacob).

Abbreviations: GPx, glutathione peroxidase; ROS, reactive oxygen species; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ZFPF, zinc finger peptide fragment

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(02)03890-5

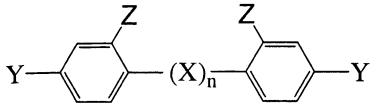
2.2. Methods

The zinc form of ZFPF (Zn_1-ZFPF, molecular weight = 3396 Da) was prepared according to an established metal-incorporation procedure [11]. The concentration of peptide was determined by parallel measurement of thiol (spectrophotometric 2,2′-dithiodipyridine assay) and amino content (spectrophotometric ninhydrin assay). The number of zinc ions bound per ZFPF was determined by atomic absorption spectroscopy. Cyclic voltammetry was performed on a 100 B/W workstation (BAS, Chichester, UK). Voltammograms of organochalcogens (50–100 μM) were recorded as previously described [10].

For the ZFPF stability assays, Zn_1-ZFPF (5 μM) and H_2O_2 (250 μM) were incubated in the presence of varying concentrations of catalyst in Tris–HCl (20 mM, pH 7.4) at 37°C for 10 min. The reaction was terminated by a 2-min incubation with catalase to remove excess H_2O_2. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was added (100 μM final concentration) and the reaction of the remaining ZFPF thiols was monitored at 412 nm ($\varepsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$) using a UV/VIS spectrophotometer.

For the cell survival assays, rat adrenal PC12 cells were cultured in RPMI 1640 suspension according to Kearns et al. [12], supplemented with glutamine (2 mM) and gentamicin (250 U/ml). Cells were fed three times a week and subcultured every 7 days. For experimentation, undifferentiated cells were plated at 100 000 cells (100 μl) per well and pre-incubated for 1 h with test compounds (see Table 1: 10 μM) at 37°C and 5% CO_2, in a Sanyo CO_2 incubator, followed by addition of varying concentrations of H_2O_2 (50–500 μM) and overnight incubation. A H_2O_2 dose–response curve in the absence of test compounds and a 'catalyst only' incubation at the highest catalyst concentration used were established as controls. The effect of H_2O_2 (200 μM) on the standard GPx mimic ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one, 10 μM) was also examined. Cell viability was measured using the standard MTT assay [13] and a Dynex technologies MRX microplate reader. Individual treatments consisted of six wells per experiment and experiments were replicated at least four times.

Table 1
Correlation between the oxidative capacity (IC_{50}) of compounds **1**–**8** and *E*pa



Comp	X	n	Y	Z	IC_{50} /nM	*E*pa/mV
1	Se	1	OCH_3	H	636	+753*
2	Se	2	H	H	31	+1141
3	Se	2	H	NH_2	19	+733
4	Se	2	OCH_3	H	21	+1000*
5	Te	1	OH	H	16	+299*
6	Te	1	OCH_3	H	35	+368*
7	Te	2	OCH_3	H	18	+578*
8	Te	2	H	H	18	+653
ebs	-	-	-	-	110	+1044*

The cyclic voltammograms were recorded at a scan rate of 200 mV/s. For the in vitro experiments, the Zn_1-ZnF (5 μM) was incubated with catalysts (nM range) and H_2O_2 (250 μM) in Tris–HCl (20 mM, pH 7.4) at 37°C for 10 min. Catalase (1 nM), followed by DTNB (100 μM), was added to the mixture and the formation of TNB^- measured at 412 nm.
Experimental error 10% ($n = 3$).
*Literature value [10].

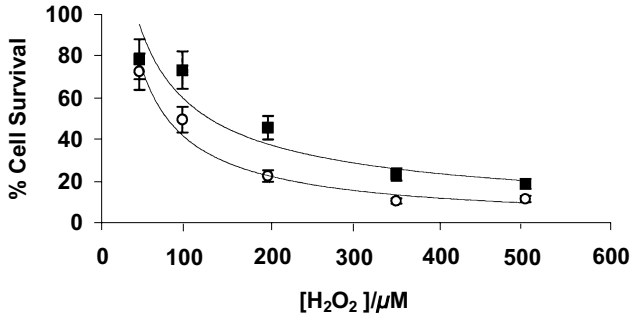


Fig. 1. Effect of monochalcogen **5** and H_2O_2 on PC12 cell survival. PC12 cells were treated with increasing concentrations of H_2O_2 only (50–500 μM, ■) and the experiment repeated in the presence of **5** (10 μM, ○). After an overnight incubation the % cell survival was measured using the MTT assay [13].

3. Results

3.1. Catalysis of zinc finger oxidation by a selection of organochalcogens

Hydrogen peroxide decreased the thiol concentration in the ZFPF sample indicating oxidation of the peptide. In the absence of additional redox agents, hydrogen peroxide (250 μM) oxidised 7% of the ZFPF (10 μM) thiols within 10 min. The presence of GPx mimics dramatically increased this oxidative effect of H_2O_2. Table 1 shows the IC_{50} values for a range of telluride and selenide catalysts obtained for the oxidation of the Sp1 zinc finger peptide in the presence of peroxide. Control experiments included incubations with methanol, H_2O_2 alone, and catalyst in the absence of H_2O_2, all of which had negligible effects on the measurements. Among the chalcogens tested, diselenides and both mono- and ditellurides dramatically enhanced the peroxide driven oxidation of ZFPF thiols, indicating that these agents are good thiol peroxidation redox catalysts in vitro. The monoselenide **1** and ebselen were significantly less active.

All of the chalcogen species assayed were redox active in the range of +300 to +1200 mV against the standard silver electrode. The tellurides possessed lower *E*pa values from +300 to +650 mV and the selenides were more resistant to oxidation with *E*pa values ranging from +700 to +1200 mV. Monochalcogens had a lower *E*pa, by approximately 225 mV, than their corresponding dichalcogens, as can be seen by a comparison of the analogous structures **1**, **4**, **6** and **7**. There was a general trend that compounds with a low oxidation potential, i.e. that are easier to oxidise, have a higher activity in the ZFPF assay (i.e. lower IC_{50} values) with an interesting correlation between the dichalcogenides (**2**–**4**, **7**, **8**, $R^2 = 0.77$). All of these compounds possessed one irreversible oxidation potential with the exception of **5**, which was unique in that it had several oxidation peaks, exhibited reversible electron-transfer behaviour at a relatively low potential (*E*pa = +299 and +821 mV, *E*pc = +255 and +763 mV) and was also the most effective redox catalyst, possessing the lowest IC_{50} value (16 nM). Cell culture studies have therefore focused on this versatile redox catalyst.

3.2. Catalysis of cancer cell death

PC12 cells were challenged by H_2O_2 to simulate the conditions of oxidative stress prevalent in malignant tumours [4]. Fig. 1 shows that increasing levels of the oxidative stressor

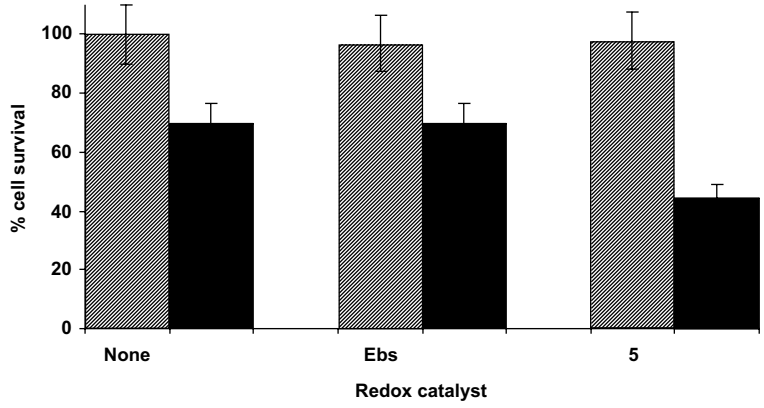


Fig. 2. Cytotoxicity and pro-oxidant effects of ebselen and **5**. PC12 cell viability was determined by the MTT assay in the absence (▨) and presence (■) of H_2O_2 (200 μM) after an overnight incubation. The pro-oxidant effects of ebselen (10 μM) and **5** (10 μM) were established under these conditions.

hydrogen peroxide reduce cell survival. This effect of peroxide is considerably enhanced by a physiologically relevant dosage of **5** (10 μM), which in the absence of H_2O_2 has no toxic effect on PC12 cells. A significant effect was observed even at the lowest concentration of H_2O_2 (50 μM) used in the study. Fig. 2 is a representation of the non-toxic nature of compound **5** in the absence of H_2O_2 and also shows the non-toxicity and inactivity of the standard drug ebselen, corresponding to its lower in vitro IC_{50} of 110 nM. The 2% methanol control had no effect on cell survival.

4. Discussion

The results obtained in the in vitro experiments indicate that chalcogen-based GPx mimics are effective redox catalysts. These agents enhance the (per)oxidation of thiols in the presence of the oxidative stressor hydrogen peroxide [14]. While GPx is rather specific for GSH thiols, an advantage in pro-oxidant therapy is that synthetic mimics lack this substrate specificity and can be used to attack zinc/sulfur complexes such as the zinc finger ZFPF. Oxidation of ZFPF has important biochemical implications. The mRNA levels and DNA-binding capacity of Sp1 increase in epithelial tumours [15] and Sp1 has also been implicated in lung cancer [16]. Catalysts, using the ZFPF of Sp1 – or indeed other redox-sensitive proteins [17] – as a target, therefore have the potential to destroy this finger (and similar redox-sensitive thiol proteins) in vivo and ultimately shut down cells with impaired redox balance, such as epithelial and lung cancer cells.

On the other hand, redox catalysts 'only' facilitate redox reactions under the control of an already existing cellular redox balance. These agents do not themselves change this balance and their activity is therefore determined by the environment in which they are placed. GPx mimics could therefore be considered as redox sensors, being able to distinguish on grounds of oxidative stress. If these assumptions were correct, catalysts should greatly potentiate the effects of already existing stressors but should not be toxic on their own.

The results obtained in cell culture clearly support our initial hypothesis. The cell culture activity of compound **5** is not

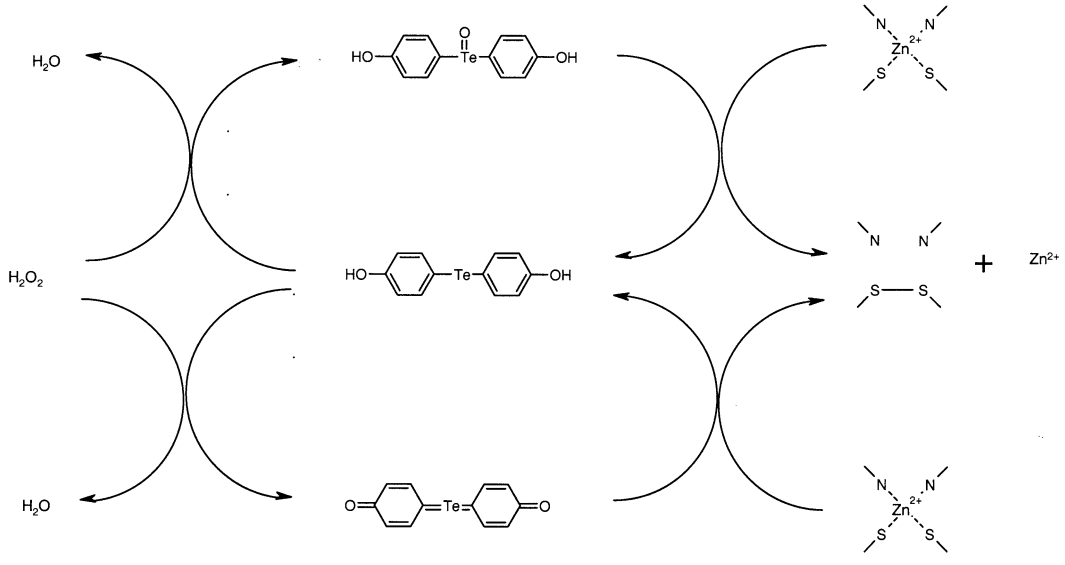


Fig. 3. Hypothetical catalytic cycles for GPx mimic **5**. **5** can be oxidised by hydrogen peroxide to form the telluroxide, which is then reduced by ZFPF, regenerating the telluride. Oxidation of ZFPF results in zinc ejection due to the formation of the disulfide. **5** may also operate via an alternative redox path, forming the telluroketone upon oxidation by hydrogen peroxide, which is then reduced by ZFPF.

intrinsic to the compound itself, but is generated as a synergistic effect of the peroxide/catalyst interaction. These findings make GPx-like catalysts a distinct class of potential drugs that are different from 'continuously active' agents and 'triggered', suicide agents (which once activated are permanently active). As it has been established that antioxidant levels in certain human and animal cancer cells are low, it might be possible to use this particular cellular redox state, in conjunction with redox catalysts, to specifically promote cancer cell death.

We have previously shown that there is a relationship between electrochemical potential and chemical structure, which we have used to predict redox activity against the zinc/sulfur protein MT [10]. The mechanism of GPx mimics is considered to consist of chalcogen atom oxidation by ROS, followed by reduction by cellular thiols. In the case of **5**, the possibility of additional redox mechanisms, possibly involving a conjugated, quinone-like oxidised species (Fig. 3), might explain its exceptional catalytic activity. The elucidation of the precise mode of action of these redox agents in malignant cells is the subject of ongoing investigation.

In summary, these preliminary studies have investigated the use of novel organochalcogen catalysts as possible anti-cancer agents. Interestingly, the mechanism of action of these catalysts depends on their environment, as they have the possibility to function as antioxidant catalysts in normal cells [10] and pro-oxidants in cells exposed to oxidative stress. If these agents are also able to adjust their activity in response to their redox environment in vivo, they might provide access to an important category of drugs. While this work has focussed on cancer cells with a disturbed redox balance, similar conditions are also present during infection and inflammation. In addition, agents similar to **5** could be used to attack entities with a disturbed redox balance while protecting healthy cells in their role as antioxidants.

Acknowledgements: This work was financially supported by the Wellcome Trust, the Leverhulme Trust, the Alzheimer's Society (UK) and the University of Exeter. We would also like to thank Janet Holley, Catriona Collins, Karen Tasker, Kim Green, Hugh Pearson and Chris Peers for their help and advice.

References

[1] Halliwell, B. (1991) Am. J. Med. 91, 14–19.
[2] Sies, H. (1993) Eur. J. Biochem. 215, 213–219.
[3] Floyd, R., Kotake, Y., Hensley, K., Nakae, D. and Konishi, Y. (2002) Mol. Cell. Biochem. 234–235, 195–203.
[4] Droge, W. (2002) Physiol. Rev. 82, 47–95.
[5] Coursin, D., Cihla, H.P., Sempf, J., Oberley, T. and Oberley, L. (1996) Histol. Histopathol. 11, 851–860.
[6] Oberley, T. and Oberley, L. (1997) Histol. Histopathol. 12, 525–535.
[7] Fernandez-Pol, J., Hamilton, P. and Klos, D. (2001) Anticancer Res. 21, 931–958.
[8] Jacob, C., Maret, W. and Vallee, B.L. (1999) Proc. Natl. Acad. Sci. USA 96, 1910–1914.
[9] Lania, L., Majello, B. and De Luca, P. (1997) Int. J. Biochem. Cell. Biol. 29, 1313–1323.
[10] Giles, G.I., Tasker, K.M., Johnson, R.J.K., Jacob, C., Green, K.N. and Peers, C. (2001) Chem. Commun. 23, 2490–2491.
[11] Vašák, M. (1991) Methods Enzymol. 205, 41–44.
[12] Kearns, S. and Dawson Jr., R. (2000) Adv. Exp. Med. Biol. 483, 563–570.
[13] Mossman, T. (1983) J. Immunol. Methods 65, 55–63.
[14] Giles, G.I. and Jacob, C. (2002) Biol. Chem. 383, 375–388.
[15] Kumar, A. and Butler, A. (1999) Cancer Lett. 137, 159–165.
[16] Blaine, S., Wick, M., Dessev, C. and Nemenoff, R. (2001) J. Biol. Chem. 276, 42737–42743.
[17] Giles, G.I., Tasker, K.M., Collins, C., Giles, N.M., O'Rourke, E. and Jacob, C. (2002) Biochem. J. 364, 579–585.