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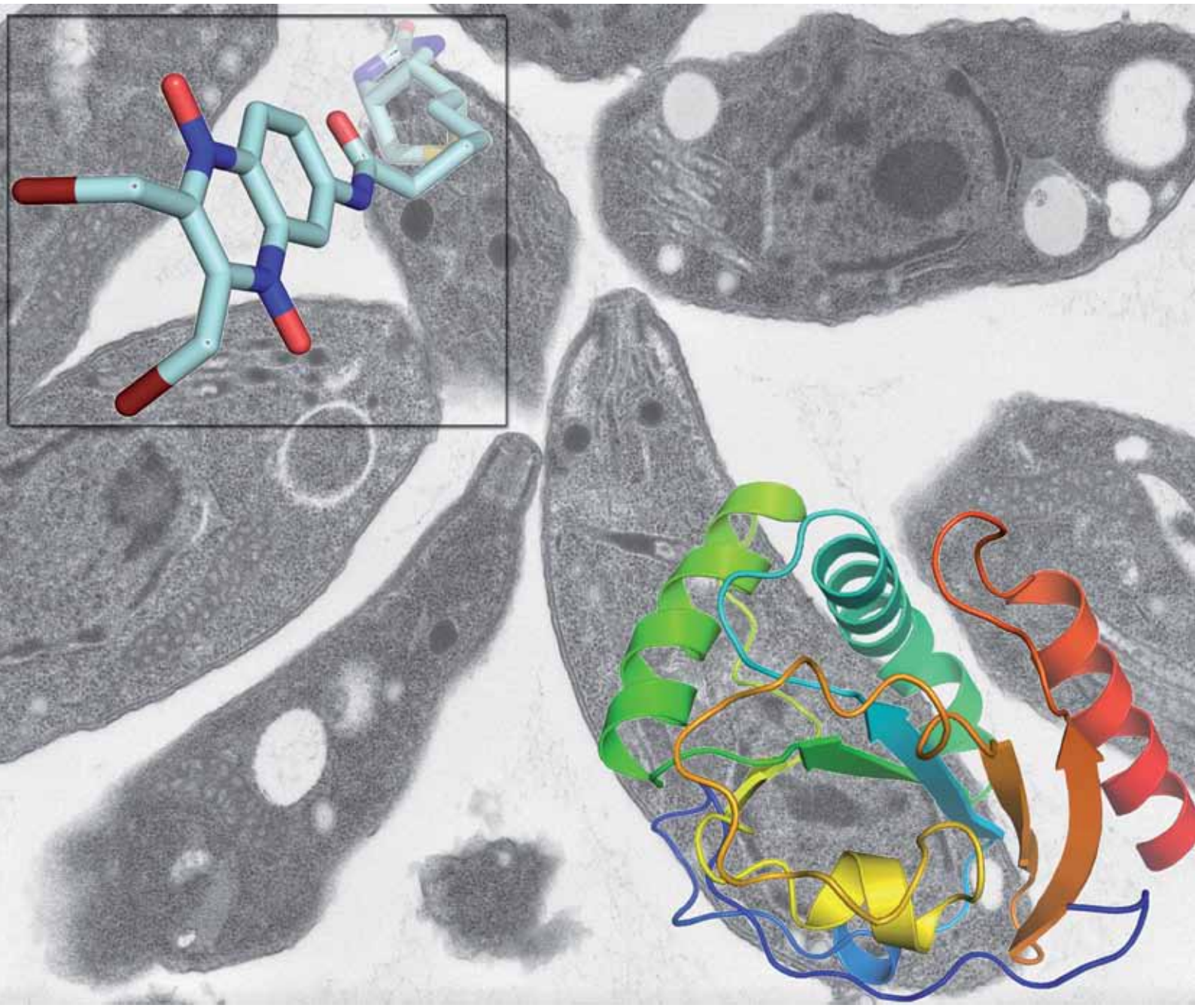

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**FULL PAPER**

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Highlights in Chemical Biology

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# Identification of conoidin A as a covalent inhibitor of peroxiredoxin II†

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Conoidin A (**1**) is an inhibitor of host cell invasion by the protozoan parasite *Toxoplasma gondii.* In the course of studies aimed at identifying potential targets of this compound, we determined that it binds to the *T. gondii* enzyme peroxiredoxin II (TgPrxII). Peroxiredoxins are a widely conserved family of enzymes that function in antioxidant defense and signal transduction, and changes in PrxII expression are associated with a variety of human diseases, including cancer. Disruption of the TgPrxII gene by homologous recombination had no effect on the sensitivity of the parasites to **1**, suggesting that TgPrxII is not the invasion-relevant target of **1**. However, we showed that **1** binds covalently to the peroxidatic cysteine of TgPrxII, inhibiting its enzymatic activity *in vitro.* Studies with human epithelial cells showed that **1** also inhibits hyperoxidation of human PrxII. These data identify Conoidin A as a novel inhibitor of this important class of antioxidant and redox signaling enzymes.

## Introduction

Small molecules that covalently modify their target protein(s) are of considerable clinical importance. A recent review lists 19 prescribed drugs, including the β-lactam antibiotics, that act through irreversible covalent protein modification.[1] Specific covalent modifiers of protein function can be useful tools for studying basic cell biological processes.[2–7] Broad specificity small molecules have also recently been used in chemical proteomics to modify sub-proteomes covalently.[8,9] Perturbation of protein function by covalent modification relies on the ability of a small molecule containing an electrophilic functional group to form an irreversible, covalent bond with a target protein nucleophile. In many examples, it is the sulfur atom in an activated cysteine residue that acts as the nucleophile, although carboxylic acids (aspartic/glutamic acid or the C-terminus of the protein), alcohols (*e.g.*, activated serine) and amino-containing groups (histidine, lysine, arginine or the N-terminus) can all react with electrophiles, depending on the effect of the protein microenvironment on the nucleophile protonation state.[10]

We have previously shown that Conoidin A (**1**) (Fig. 1) is an inhibitor of host cell invasion by the human pathogen *Toxoplasma gondii.*[11] In the course of our attempts to identify potential targets of **1** in *T. gondii*, reported here, we discovered that this compound is a covalent inhibitor of *T. gondii* peroxiredoxin II (TgPrxII). Peroxiredoxins (Prxs) are a widely conserved family of enzymes that function both in antioxidant defense and in redox signaling pathways. The latter involves the regulated and localised production of $H_2O_2$ and other reactive oxygen species (ROS). Prxs serve a critical role in converting these ROS signals into a cellular response.[12]

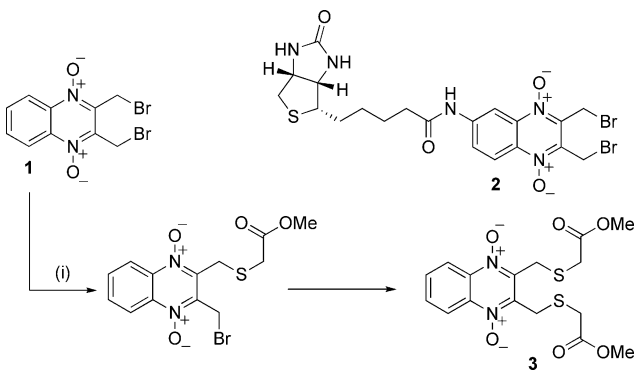


**Fig. 1** Structure of Conoidin A (**1**) and its biotinylated derivative, **2**. Also shown is the mechanism of reaction of **1** with a model thiol nucleophile: (i) triethylamine ($Et_3N$), mercaptoacetate, r.t. 30 min.

Prxs use a reactive, peroxidatic cysteine residue to reduce ROS such as $H_2O_2$, resulting in the formation of a cysteine sulfenic acid (Cys-SOH). Two models have been proposed to explain how oxidation of the peroxidatic cysteine functions in redox signaling. The "floodgate" model proposes that Prxs serve to buffer fluctuations in ROS levels; hyperoxidation of Prx (*i.e.,* the further oxidation of Cys-SOH to sulfinic acid [Cys-$SO_2H$] or sulfonic acid [Cys-$SO_3H$] by locally high concentrations of

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$H_2O_2$) leads to Prx inactivation, local accumulation of $H_2O_2$ and activation of redox signaling pathways.[13] Alternatively, the "smoke alarm" model proposes that oxidation of a Prx alters its structure and oligomerisation, in turn affecting its ability to interact with and regulate the function of other signaling proteins including kinases, phosphatases and transcription factors.[14]

Changes in human PrxII expression are associated with cancer, cardiovascular dysfunction and neurodegeneration,[12] and there is significant interest in the therapeutic potential of drugs that target the Prxs. For example, increased expression of PrxII is associated with non-responsiveness to radiation therapy in cancer patients, and treatment of cancer cells with antisense RNA directed against PrxII increases their radiosensitivity.[15] We demonstrate here that **1** inhibits the enzymatic activity of TgPrxII, through covalent binding to its peroxidatic cysteine. The compound also inhibits the hyperoxidation of mammalian PrxII in response to oxidative stress. These data suggest that **1** and its derivatives may represent a useful new set of cell-permeable inhibitors for studying this important class of enzymes.

## Results

### A biotinylated analog of 1 binds to many proteins in parasite extracts

To identify potential targets of **1** in *T. gondii*, we prepared a biotinylated analog,[20] **2** (Fig. 1). Although **2** was found to be inactive in cell-based *T. gondii* invasion assays (data not shown), structure–activity relationship (SAR) data showed that other analogs of **1** with modifications at the same position retained biological activity,[20] suggesting that the biotin group either renders **2** membrane-impermeant or somehow prevents it from reaching its cellular target(s) within the parasite. To circumvent this problem, **2** was incubated with parasite lysates. Parasite proteins were then resolved by SDS-PAGE, transferred to nitrocellulose and probed with streptavidin-horseradish peroxidase (HRP; Fig. 2A). We observed covalent binding of the biotinylated compound to a large number of parasite proteins under these conditions (*e.g.*, see Fig. 2B, –iodoacetamide).

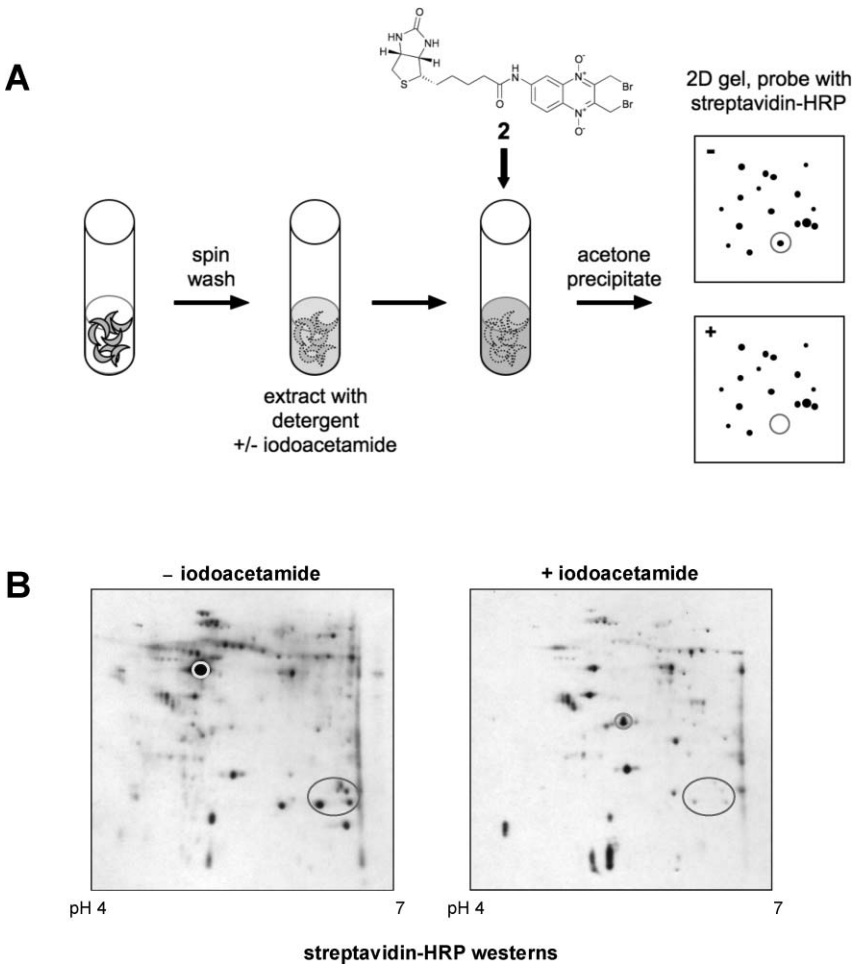


**Fig. 2** Binding of **2** to parasite proteins through free thiols and other nucleophiles. (**A**) Schematic of the *in vitro* labeling approach. (**B**) Influence of iodoacetamide on parasite protein labeling by **2**. Parasite protein lysates were extracted either in the absence (left) or presence (right) of iodoacetamide. Proteins were then labeled with **2** and resolved by 2D gel electrophoresis (first dimension isoelectric focusing on pH 4–7 IPG strips). Biotinylated proteins were visualised by streptavidin-HRP western analysis. Labeled proteins included protein disulfide isomerase (left panel [–iodoacetamide], small circle), protein phosphatase 2C (right panel [+iodoacetamide], small circle), and a prominent triad of proteins whose labeling is markedly reduced following iodoacetamide treatment (large oval). The triad corresponds to the candidate target proteins identified by the *in vivo* blocking approach (see Fig. 3).

Studies with model substrates have shown that **1** can react with both thiols and amines[21] (see also below). To determine whether the biotinylated proteins in the parasite extract had reacted with **2** through a thiol(s) or an alternative protein nucleophile(s), duplicate parasite samples were extracted either in the presence or absence of iodoacetamide prior to incubation with **2**. We predicted that extracts that had not been pretreated with iodoacetamide would show proteins labeled through both thiols and other nucleophiles, whereas extracts preincubated with iodoacetamide, which alkylates and thereby blocks free thiol groups, would reveal the subset of proteins labeled by **2** *via* protein-based nucleophiles other than thiols.

Different parasite proteins were indeed biotinylated to different extents in the presence and absence of iodoacetamide (Fig. 2B). The differences in labeling suggest that, under these conditions, the primary mechanism of reaction of **2** (and, by extension, **1**) is with protein-based thiols, although the compound is almost certainly able to react with other protein-based nucleophiles (Fig. 2B, +iodoacetamide). The observed rapid reaction of **1** with methyl mercaptoacetate, a model sulfur nucleophile, compared with a model amine (*n*-butylamine) or oxygen (methanol) nucleophile was consistent with this observation. When treated with an excess of methyl mercaptoacetate in the presence of triethylamine, **1** afforded the disulfide **3** (Fig. 1) in 93% isolated yield after only 30 minutes. This reaction did not proceed in the absence of base, suggesting that activated thiol nucleophiles are required.

The most prominent protein that becomes biotinylated in the absence, but not in the presence, of iodoacetamide (Fig. 2B, –iodoacetamide, small circle), suggesting that it is therefore labeled *via* a thiol, was identified by mass spectrometry as a putative protein disulfide isomerase (ToxoDB 27.m00003; see Table S1†). Among the proteins that appear to be labeled *via* a nucleophile other than sulfur, we identified a putative serine/threonine protein phosphatase[22] (ToxoDB 44.m00037, Fig. 2B, +iodoacetamide, small circle).

### Identification of potential target proteins by "*in vivo* blocking"

Previous work has shown that **1** exhibits a highly selective activity profile across a series of secondary assays related to *T. gondii* invasion. It inhibits the ionomycin-induced extension of an apical cytoskeletal structure of the parasite known as the conoid,[23] but has no effect on parasite motility or secretion.[11] The large number of proteins labeled by **2** in parasite extracts was inconsistent with this apparent biological specificity of **1**, suggesting that, in extracts, **2** has access to a variety of proteins that are normally inaccessible to **1** within live parasites. Attempts to improve the specificity of labeling by varying the extraction or labeling conditions were unsuccessful. We therefore developed an alternative "*in vivo* blocking" approach to identify potential target(s) of **1** (Fig. 3A). Live parasites were pre-incubated with the unlabeled compound, **1**, in order to bind/block its *in vivo* target(s). A control sample was incubated with an inactive analog, **4** (see ref. 24 and data not shown). Parasites were then washed to remove unbound **1** or **4** and lysed with detergent to make a total protein extract that was subsequently incubated with **2**. Although it was anticipated that **2** would label many proteins in the extract, as described above, the physiologically relevant target(s) would have already been bound by **1** in live parasites and should, therefore, remain unbiotinylated. We predicted that this would result in the loss of a biotinylated protein spot(s), corresponding to potential target proteins, in extracts from parasites pre-incubated with **1** compared to the control, **4**.

Parasite proteins labeled by the *in vivo* blocking approach were subjected to 2D gel electrophoresis, transferred to nitrocellulose and probed with streptavidin-HRP. In comparison with the control (Fig. 3B, left panel), the labeling of several biotinylated proteins was decreased in samples pre-incubated with **1** (Fig. 3b, right panel). While different changes were observed from experiment to experiment, one triad of proteins at ~25–30 kDa showed a consistent and reproducible decrease in labeling following pretreatment with **1** (Fig. 3B, circle). These three proteins were excised from gels and analysed by mass spectrometry. The two lower molecular weight spots were identified as two isoforms of peroxiredoxin II (TgPrxII: ToxoDB 583.m00002, 24.4 kDa, predicted pI 6.51), a protein previously demonstrated to participate in the parasite's oxidative stress response.[19] The two isoforms likely represent different oxidation and/or phosphorylation states of TgPrxII.[25,26] The third spot corresponded to a conserved hypothetical protein (ToxoDB 57.m00038, 25.7 kDa, predicted pI 6.95). These three proteins show significantly reduced labeling by **2** in the presence of iodoacetamide (Fig. 2B, large oval), indicating that they are labeled on thiols. Each of these proteins has multiple cysteine residues that could potentially react with **1** and **2**: TgPrxII contains 5 cysteines[19,27,28] and the hypothetical protein 57.m00038 contains 4 cysteines.[23]

### 1 inhibits TgPrxII activity *in vitro*

Given the importance of Prx proteins in intracellular signaling pathways in other systems, we tested whether the binding of **1** to TgPrxII affects its enzymatic activity. The activity of recombinant TgPrxII (rTgPrxII) was assessed using the previously reported glutamine synthase protection assay (see ref. 29 and the ESI†). Using a short preincubation time (5 minutes) the effect of varying concentrations of **1** on enzyme activity was determined. **1** shows clear, dose-dependent inhibition of rTgPrxII, with an $IC_{50}$ value of 25.1 ± 0.8 μM (Fig. 4A). Given the nature of the protection assay, the calculated $IC_{50}$ value is highly dependent on factors such as the preincubation time of **1** with rTgPrxII. A five minute preincubation was selected in order to provide consistency between this assay and the electrospray mass spectrometric analysis (see below: the use of longer preincubation times would be expected to result in a lower $IC_{50}$ value for **1**).

### 1 binds covalently to Cys47, the peroxidatic cysteine of TgPrxII

Previous studies have implicated Cys47 of TgPrxII as the key nucleophilic residue involved in catalysis by this enzyme.[26] The thiol functional group of Cys47 is proposed to attack one of the oxygen atoms present in hydrogen peroxide resulting in cleavage of the oxygen–oxygen bond and in the initial formation of an enzyme-based sulfenic acid.[30] Mutation of Cys47 in rTgPrxII to alanine leads to a loss of activity of the protein in the glutamine synthase protection assay.[27] To assess whether **1** reacts with Cys47 of rTgPrxII, thereby implicating this residue in the mechanism of inhibition of rTgPrxII by **1**, a series of mass spectrometry (MS) experiments were carried out.

 

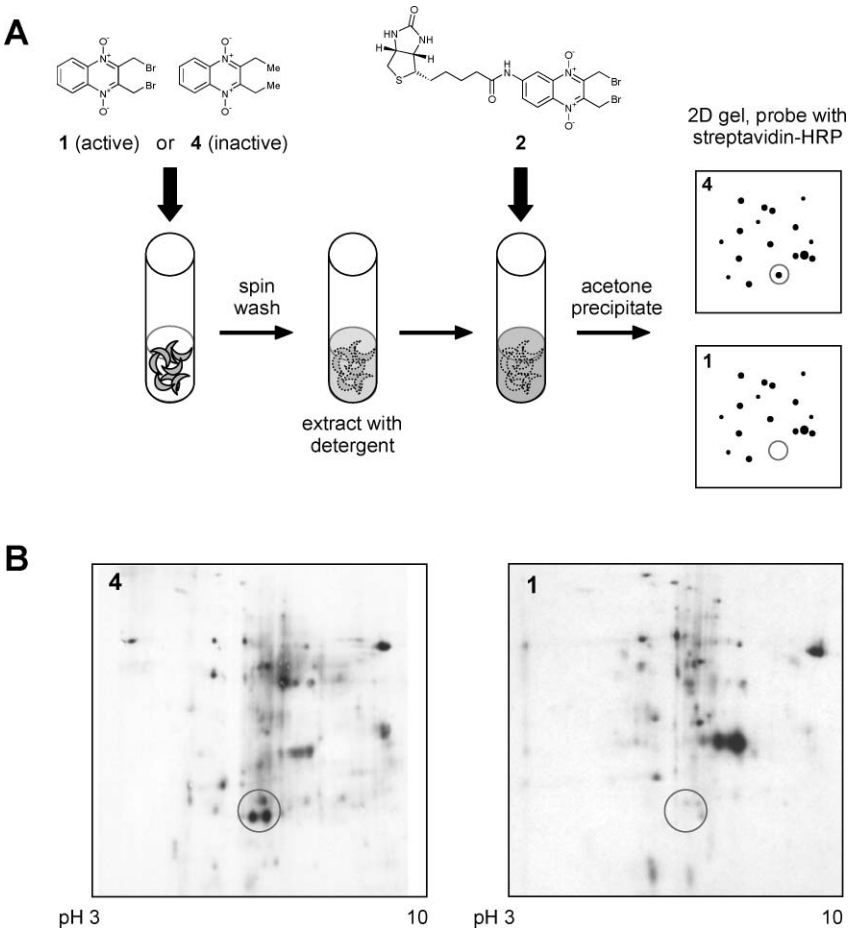


**Fig. 3** *In vivo* blocking approach to target identification. (**A**) Schematic of the *in vivo* blocking approach. (**B**) Unlabeled compound, Conoidin A (**1**), or a control inactive analog, **4**, was added to live parasites prior to extraction with detergent. The biotinylated analog, **2**, was then incubated with the resulting protein lysates. Total parasite proteins were resolved by 2D gel electrophoresis (first dimension isoelectric focusing on pH 3–10 IPG strips) and biotinylated proteins were visualised on western blots probed with streptavidin-HRP. Candidate target proteins are indicated by the circle; note that a broader pH gradient was used here than in Fig. 2, so the triad of potential target proteins focuses closer to the center of the pH gradient here than in Fig. 2.

Analysis of rTgPrxII using electrospray ionisation (ESI) methods gave one main species with an observed molecular weight ($M_r$) of 26924.7 Da (Fig. 4B(i), theoretical $M_r$ (rTgPrxII) = 26924.8 Da). Following a five minute preincubation of rTgPrxII with **1**, in a manner analogous to that used for $IC_{50}$ determination, MS analysis supported the formation of a rTgPrxII:**1** complex (Fig. 4B(ii)) although a relatively large signal corresponding to unmodified rTgPrxII was also present at this time point. As the analysis was carried out under conditions where only covalently linked complexes would survive, the formation of a rTgPrxII:**1** complex must result from covalent modification of rTgPrxII by **1**. In addition, the observed mass shift of 267.1 Da was consistent with addition of **1** to rTgPrxII accompanied by the loss of one of the two bromine atoms (theoretical mass shift for formation of this rTgPrxII:**1** complex = 267.0 Da). The observed loss of only one of the two bromines in **1** following extended preincubation times with rTgPrxII presumably stems from the lack of a second suitably nucleophilic residue in the active site of the enzyme that can react to displace the second bromine atom. This observation led to the proposal that addition of a thiol-containing compound (for example benzyl mercaptan, BnSH) after the initial preincubation with **1** would lead to the formation of a new enzyme–inhibitor complex. Addition of excess benzyl mercaptan to the rTgPrxII:**1** complex gave a mass shift of 312.1 Da with respect to rTgPrxII (45.4 Da with respect to the rTgPrxII:**1** complex, Fig. 4B(iii)). This second complex corresponds to the displacement of both bromine atoms in **1**; one by a nucleophile in rTgPrxII and the second by benzyl mercaptan (Fig. S3,† theoretical mass shift for formation of rTgPrxII:**1**:BnSH complex = 310.4 Da with respect to rTgPrxII; 44.6 Da with respect to the initially formed rTgPrxII:**1** complex). Interesting evidence to support the formation of a rTgPrxII:BnSH complex was also found (see ++ in Fig. 4B(iii) and ESI†). In contrast to the rTgPrxII:**1** complex, the rTgPrxII:**1**:BnSH complex was found to be stable over extended time periods. We therefore decided to perform tryptic digestion coupled with MALDI MS and MS/MS analysis using the rTgPrxII:**1**:BnSH complex to identify the protein residue involved in reaction with **1**.

TgPrxII contains five cysteine residues, only 3 of which (Cys47, Cys73 and Cys129) are expected to be within tryptic peptides of sufficient size to be observed during MS analysis of the digest (ESI†). All three of the expected peptides were observed upon MALDI MS analysis of a tryptic digest of rTgPrxII and

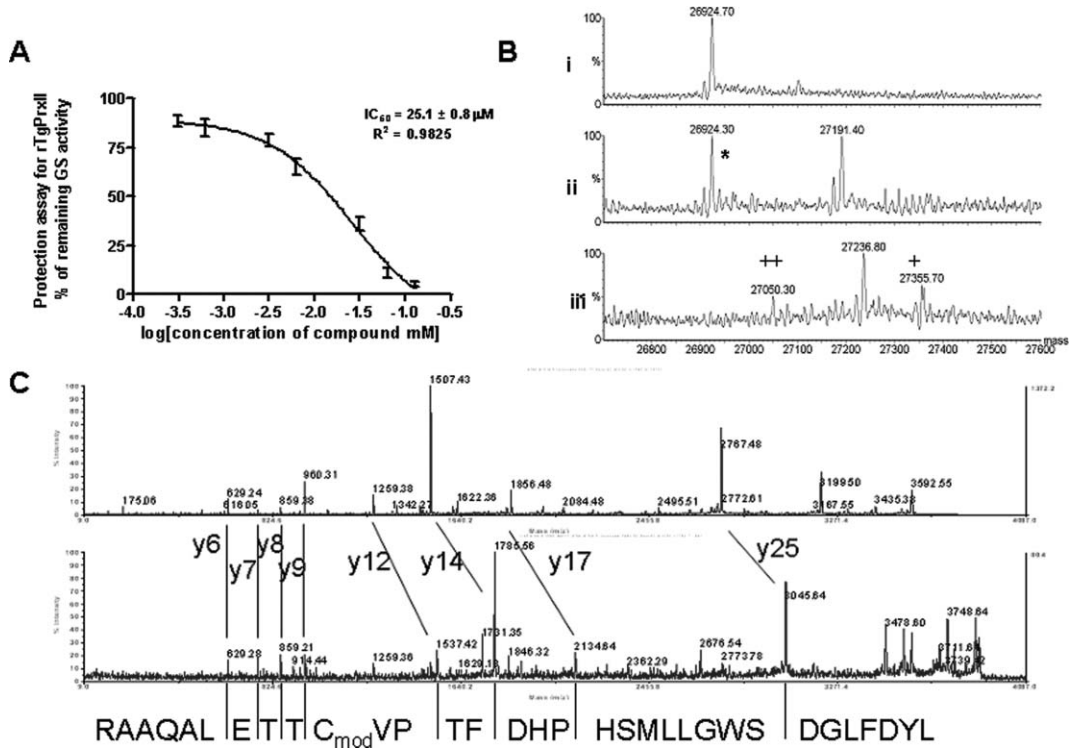


**Fig. 4** Conoidin A (**1**) binds to and inhibits recombinant TgPrxII *in vitro*. (**A**) $IC_{50}$ curve for TgPrxII inhibition by **1**; (**B**) ESI MS analysis of the reaction of TgPrxII with **1** and BnSH; (i) rTgPrxII ($M_r$ = 26924.7 Da); (ii) MS analysis of TgPrxII treated with **1** for 5 min; * unreacted TgPrxII ($M_r$ = 26924.3 Da); (iii) MS analysis of TgPrxII and **1** complex treated with BnSH; ++ BnSH adduct of unreacted rTgPrxII; + BnSH adduct of rTgPrxII:**1**:BnSH complex. (**C**) MS/MS analysis of the Cys47 containing tryptic peptide (LYDFLG DSWGLLMSHP HDFTPVCTTE LAQAAR) (i) in the absence of treatment with **1** and BnSH; (ii) after preincubation of rTgPrxII with **1** followed by BnSH.

MS/MS analysis confirmed their sequences (Fig. 4C(i) for Cys47-containing peptide). When the analysis was repeated using the rTgPrxII:**1**:BnSH complex, we obtained evidence to support the reaction of **1** at Cys47. MS/MS fragmentation of both the modified and unmodified Cys47-containing peptide gave a partial series of y ion fragments corresponding to the predicted sequence. Comparison of the ions obtained for the modified and unmodified peptide showed signals of the same mass up until y9, the last fragment that does not contain Cys47 (Fig. 4C(i), (ii)). The two spectra are then shifted relative to each other at the next y ion observed, y12. These data demonstrate that the modification occurs either on Cys47, Val46, or Pro45. While it is not possible from these data to prove definitively that **1** covalently modifies Cys47 of rTgPrxII, the only alternative explanation involves reaction of the backbone amides in the Cys47/Val46/Pro45 region. Given the observed reaction of **1** with model thiols described above, this makes significantly less chemical sense than the proposed reaction of **1** with Cys47. The observed mass shift (278.1 Da) for the modified Cys47-containing peptide differs from the theoretical mass shift (310.4 Da) expected for reaction of **1** at Cys47 followed by reaction with BnSH by a mass of 32Da. This difference has been assigned to the loss of both the oxygen atoms present in the *N*-oxide functional groups of **1** (when bound to rTgPrxII) during MS analysis based on a similar fragmentation observed for a structurally related compound under the MS conditions used here (data not shown).

These results are consistent with the view that Cys47 of TgPrxII is the peroxidatic cysteine, as proposed previously, and that *in vivo* labeling and inhibition of TgPrxII and rTgPrxII respectively results from covalent modification of Cys47 by **1**.

### Parasites lacking TgPrxII show no difference in sensitivity to 1 in invasion and conoid extension assays

The identification of TgPrxII as a potential target of **1** raised the intriguing possibility that redox signaling plays a previously unrecognised role in host cell invasion by *T. gondii*. To test directly whether binding of **1** to TgPrxII underlies the invasion-inhibitory effect of **1**, we generated a transgenic parasite line in which the single TgPrxII gene was disrupted by homologous recombination (see Experimental and Fig. 5A). The knockout parasites were viable, and showed no obvious growth defects in culture. No differences in the sensitivity of wild type and TgPrxII knockout parasites to various concentrations of **1** were observed in either the host cell invasion (data not shown) or conoid extension (Fig. 5B) assays.

### 1 inhibits hyperoxidation of human PrxII

The data above suggest that inhibition of TgPrxII by **1** is unlikely to be the mechanism by which **1** inhibits *T. gondii* host cell invasion and conoid extension. However, the data clearly identify **1** as a novel, covalent inhibitor of TgPrxII *in vitro*. To determine whether **1** can affect the Prx proteins of other species, we treated

 

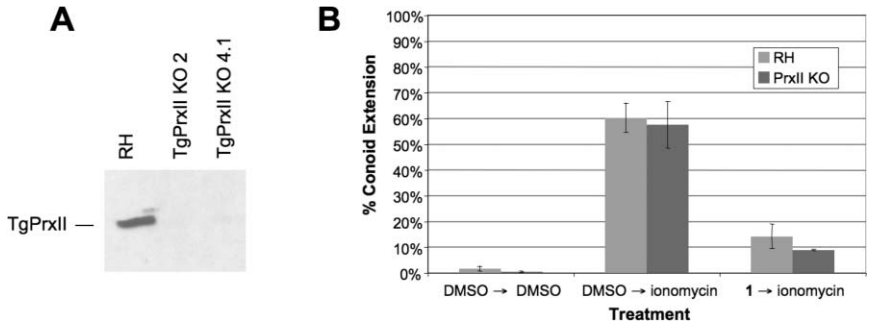


**Fig. 5** The sensitivity of parasites to Conoidin A (**1**) is unaffected in TgPrxII knockout and overexpressing parasites. (**A**) Western blot analysis of total protein extracts from RH strain parasites and 2 clonal TgPrxII knockout parasite lines (TgPrxII KO2 and KO4.1), probed with an anti-TgPrxII antiserum.[19] The lanes shown were from the same blot and were exposed and adjusted for contrast and brightness identically. (**B**) Conoid extension assays using RH and TgPrxII knockout parasites. Parasites were pretreated for 15 min with 100 μM **1** (or an equivalent amount of DMSO), followed by the addition of 10 μM ionomycin (or an equivalent amount of DMSO) and a further 5 min incubation. Ionomycin induces conoid extension, which is blocked to an equivalent extent by **1** in wild type and TgPrxII knockout parasites. The data shown represent the mean values from 3 independent experiments (± standard error); TgPrxII KO represents the combined data from the KO2 and KO4.1 knockout parasites.

human epithelial cells with varying concentrations of **1**, subjected these cells to oxidative stress, and analysed the oxidation state of PrxI, -II and -III by western blotting with an antibody that detects hyperoxidised Prx proteins. As can be seen in Fig. 6A, 5 μM **1** strongly inhibits the glucose oxidase-mediated hyperoxidation of mammalian PrxI and PrxII (but not PrxIII). The analogous *in vivo* experiment could not be performed with *T. gondii* because a parasite-specific antibody against hyperoxidised Prx proteins is not available and the mammalian antibody does not cross-react with the *T. gondii* Prx proteins (data not shown). Interestingly, when the compound-treated human epithelial cell samples were resolved under non-reducing conditions, **1** induced the formation of higher molecular weight complexes containing PrxII (Fig. 6B, arrow), at doses that correlated with the inhibition of Prx hyperoxidation.

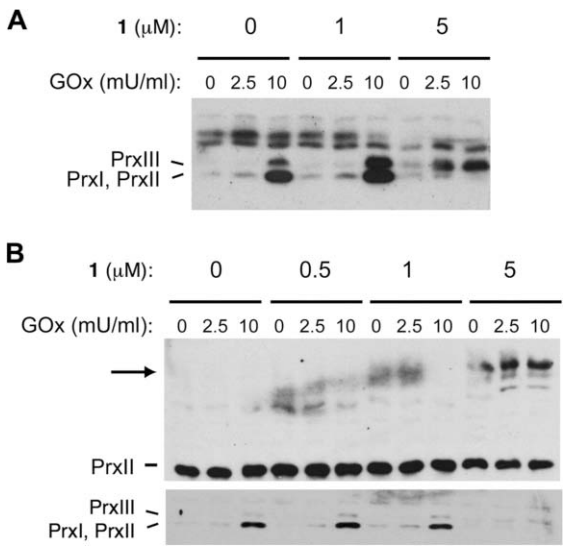


**Fig. 6** Conoidin A (**1**) inhibits Prx hyperoxidation and induces the formation of high molecular weight PrxII-containing oligomers in human epithelial cells. (**A**) Inhibition of Prx hyperoxidation by **1**. Human small airway epithelial cells were pre-incubated for 30 min with varying concentrations of **1**. Glucose oxidase was added at the indicated concentrations for an additional 1.5 h. 30 μg of total cell protein were loaded per lane, resolved by SDS-PAGE under reducing conditions, and probed with an antibody that recognises hyperoxidised Prx proteins; hyperoxidised PrxI and PrxII co-migrate under these conditions. (**B**) **1** induces the formation of higher molecular weight complexes containing PrxII (arrow). Samples were prepared as described above but resolved under non-reducing conditions. The top panel was probed with an anti-PrxII antibody and the lower panel was probed with the antibody that recognises hyperoxidised Prx proteins.

## Discussion

Prxs are ubiquitous enzymes that function in intracellular signaling and defense against oxidative stress. Prxs are divided into three classes: "typical" 2-Cys Prxs, "atypical" 2-Cys Prxs and 1-Cys Prxs.[26] While all Prxs use a reactive, peroxidatic cysteine residue to reduce ROS, resulting in the formation of a cysteine sulfenic acid (Cys-SOH), the three classes of Prxs differ in the mechanism by which the peroxidatic Cys-SOH is subsequently reduced during the catalytic cycle. Typical 2-Cys Prxs are obligate homodimers. In these enzymes, the Cys-SOH of the peroxidatic cysteine in one subunit is attacked by a resolving cysteine in the other subunit, generating an intersubunit disulfide bond. Oxidation of typical 2-Cys Prxs influences structural transitions between dimer, decamer and other oligomeric forms.[14] In the atypical 2-Cys Prxs, the peroxidatic and resolving cysteines are contained within a single polypeptide, and reduction of the peroxidatic cysteine occurs *via* an intramolecular disulfide bond. 1-Cys Prxs do not contain a resolving cysteine. In these enzymes recycling is mediated by other thiol-containing electron donors such as glutathione. TgPrxII is unusual, in that it shows homology to 1-Cys Prxs at the primary sequence level, but is reported to function like a typical 2-Cys Prx.[27]

We have identified Conoidin A (**1**) as a novel, cell permeable inhibitor of *T. gondii* PrxII. We have also demonstrated that **1** acts through covalent modification of Cys47, the peroxidatic cysteine of TgPrxII. Our studies showed that only one of the two electrophilic sites present in **1** is involved in the reaction with rTgPrxII (Fig. 4B and C). Previous work on the mechanism of reaction of **1** with nitrogen nucleophiles,[21] however, and the results presented here using a model thiol nucleophile (Fig. 1) indicate that it is chemically possible for reaction at both electrophilic sites in **1** to occur. In addition, the observed reaction of the rTgPrxII:**1** complex with benzyl mercaptan (Fig. 4B) provides direct evidence

that the second electrophilic site present in **1** is highly reactive in the presence of a suitable nucleophile. We therefore conclude that the observed "partial reaction" of **1** with rTgPrxII results from a lack of other suitably reactive residues in the vicinity of Cys47. Interestingly, this observation also implies that there is no suitable nucleophile from a second molecule of rTgPrxII available for reaction with the rTgPrxII:**1** complex, as formation of rTgPrxII dimers cross-linked by **1** would be predicted to occur. The lack of cross-linked dimer formation in the presence of **1**, together with the previously reported inability of glutathione, lipoic acid, thioredoxin and glutaredoxin to reduce the protein[28] casts doubt on the model that TgPrxII functions as a 2-Cys peroxiredoxin.[27]

Our initial interest in Conoidin A (**1**) stemmed from its identification as an inhibitor of host cell invasion and conoid extension by *T. gondii*.[11] The *in vivo* blocking experiment identified TgPrxII as a potential target of **1** in *T. gondii*, and given the key role that Prx proteins play in signal transduction in other systems, we decided to test the hypothesis that **1** inhibits conoid extension and invasion through an effect on this protein. We determined that TgPrxII knockout parasites show no change in sensitivity to treatment with **1**, strongly suggesting that inhibition of TgPrxII is not responsible for the invasion defect observed upon treatment of parasites with **1**. One caveat to this set of experiments is that *T. gondii* tachyzoites express four putative Prx proteins, TgPrxI-III and TgAOP (Genbank accession #FJ356079), a homologue of the *P. falciparum* protein PfAOP,[31] and it is possible that **1** targets one or more of these other Prx proteins in addition to TgPrxII. If the different Prx proteins serve redundant functions with respect to conoid extension and invasion, the disruption of any one Prx might not be sufficient to confer resistance to **1**. It should be noted that while TgPrxI and TgPrxII are cytosolic enzymes,[19,32] epitope-tagged TgPrxIII and TgAOP localise to the parasite mitochondrion and endoplasmic reticulum, respectively (see ref. 19 and L. Y. Kwok and D. Soldati, unpublished observations). It is therefore unlikely that either TgPrxIII or TgAOP plays a role in invasion or conoid extension. Further work will be required to determine if **1** inhibits TgPrxI, TgPrxIII and/or TgAOP, and whether a parasite in which multiple Prx proteins have been disrupted shows any reduction in sensitivity to the compound. We are also currently attempting to generate a parasite line in which the other potential target identified by *in vivo* labeling, 57.m00038, has been disrupted. While the predicted sequence of 57.m00038 contains no motifs or homologies that provide insights into its potential function, it is intriguing that this protein was recently identified in a conoid-enriched protein fraction during an analysis of the *T. gondii* conoid proteome.[23]

The *in vivo* blocking experiments showed that, in live parasites, **1** has access to only a small subset of the many proteins that can be biotinylated in cell lysates. Consistent with this observation, **1** exhibited a highly selective activity profile across a series of secondary assays related to *T. gondii* invasion.[11] The basis of this apparent *in vivo* specificity is unknown, but it could involve different local conditions in different parts of the cell (*e.g.,* pH, cation concentrations, reducing conditions), or perhaps concentration of the compound in (or its exclusion from) particular subcellular compartments. For example, **1** inhibits the hyperoxidation of mammalian PrxI and PrxII, with little or no effect on the hyperoxidation of PrxIII (Fig. 6); mammalian PrxI and PrxII are cytoplasmic, whereas PrxIII is primarily localised to the mitochondrion.[26] Potential substrates may also vary in their abundance and/or inherent reactivity. Further studies will be required to understand the differences between the *in vivo* and *in vitro* specificity of **1**.

In addition to its effect on the hyperoxidation of human PrxI/II, treatment of human epithelial cells with **1** resulted in the dose-dependent formation of multiple PrxII-containing high molecular weight complexes. Similar complexes were observed after treatment of *T. gondii* with **1** (data not shown). The nature of these high molecular weight complexes is currently unknown, but, based on their electrophoretic mobility they appear to be different from the disulfide-crosslinked dimers observed during the normal PrxII redox cycle (see ref. 26 and data not shown). Rather, they may represent dimers that have been covalently crosslinked by the binding of **1** to the peroxidatic cysteine on one subunit and an as yet undetermined residue(s) on the other subunit.

Dysregulation of Prx expression is associated with human disease, and pharmacological agents that can affect Prx function, particularly in an isotype-specific fashion, have significant therapeutic potential.[12] To this end, studies are currently underway to determine the structure of the human PrxII:**1** complex. Such studies will inform future synthetic efforts to optimise the affinity and specificity of this inhibitor towards the different Prx isoforms.

## Experimental

### Parasite and host cell culture

*T. gondii* tachyzoites (RH strain) were cultured in confluent monolayers of human foreskin fibroblasts (HFFs) grown in Dulbecco's Modified Eagle's medium (DMEM) (HyClone) containing 1% fetal calf serum (Invitrogen). Freshly egressed parasites (defined as the time when 75–80% of infected host cells had been lysed by parasite egress) were harvested by passage through a 26 gauge needle followed by filtration through a 3.0 μm Nuclepore track etch membrane filter (Whatman). Invasion assays were performed as previously described,[11] with the exception that manual rather than automated fluorescence microscopy was performed. Conoid extension assays were performed as previously described.[11]

### Reaction of 1 with methyl mercaptoacetate; synthesis of (3-methoxycarbonylmethyl-sulfanylmethyl-1,4-dioxy-quinoxalin-2-ylmethylsulfanyl)-acetic acid methyl ester, 3

To a solution of **1** (30.7 mg, 0.09 mmol) in neutral $CHCl_3$ (0.7 ml) was added methyl mercaptoacetate (28.3 μl, 0.20 mmol) and triethylamine (29.3 μl, 0.21 mmol). After stirring at room temperature for 30 minutes, the reaction mixture was washed with 2 M HCl (1 ml), water (1 ml) and brine (1 ml). The organic phase was dried and reduced *in vacuo* to yield a yellow oil. Purification by column chromatography (DCM:MeOH 97:3) gave the title product **3** as a yellow oil (33.5 mg, 0.084 mmol, 93%). IR (NaCl, thin film) $\nu$max/cm$^{-1}$: 1734 (s), 1332 (s), 1285 (m), 773 (m) cm$^{-1}$; $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 8.62–8.58 (m, 2H, H-5 and H-8), 7.85–7.81 (m, 2H, H-6 and H-7), 4.47 (s, 4H, 2 × Ar-$CH_2$-S), 3.67 (s, 6H, 2 × $OCH_3$), 3.54 (s, 4H, 2 × $CH_2$); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 170.6 (C=O), 141.7 (C4a and C8a), 137.0 (C2 and C3), 132.0 (C6 and C7), 120.4 (C5 and C8), 52.6 (MeO), 34.7 ($CH_2$S), 28.0 (C9 and C10); MS-ES$^+$ (m/z) 399 ([M + Na]$^+$, 100%);

 

HRMS-ES$^+$ (m/z) [M + Na]$^+$ calcd for $C_{16}H_{18}N_2O_6S_2Na$: 421.0509, found 421.0504.

### Labeling of parasite extracts with 2

Harvested parasites were diluted in Hanks Balanced Salt Solution (Invitrogen) buffered with Hepes, pH 7.0 (Invitrogen) (HH) to a final concentration of $6 \times 10^7$ parasites per ml. Parasites ($3 \times 10^7$) were pelleted by centrifugation (4 minutes, $1100 \times g$), washed with HH, and repelleted. Parasites were resuspended in extraction buffer (120 mM KCl; 20 mM NaCl; 20 mM Hepes, pH 7.0; 50 mM octyl-β-D-glucopyranoside; 1:500 Sigma P8340 protease inhibitor cocktail) or in extraction buffer containing 15 mM iodoacetamide and incubated for 1 hour on ice to give a total parasite extract. Parasite extracts were subsequently incubated with 50 μM **2** for 45 minutes at 25 °C. Parasite proteins were precipitated with acetone and solubilised for 2D gel electrophoresis as described below. The *in vivo* blocking experiment was carried out as described above except that, prior to extraction, the parasites were treated with either 100 μM **1** or **4** in HH for 45 minutes at 25 °C and no iodoacetamide was used prior to labeling the extracts with **2**.

### 2D gel electrophoresis

Acetone-precipitated parasite proteins were resuspended in pre-warmed (50 °C) SDS buffer (0.3% [wt/vol] SDS, 200 mM DTT) and incubated in a sonicator bath for 20 minutes, followed by a 10 minute incubation at 55 °C. Samples were then incubated, with periodic vortexing, for 2 hours at 25 °C. Four volumes of octyl buffer (9.9 M urea, 4% [wt/vol] octyl-β-D-glucopyranoside, 100 mM DTT) containing 0.2% (vol/vol) ampholytes (pH 3–10 or pH 4–7 as appropriate) were added to each sample, followed by a one hour incubation at 25 °C. Bromophenol blue (0.1% wt/vol) was added and samples were centrifuged for 30 minutes at $13{,}000 \times g$ to remove insoluble material. Samples were used to rehydrate 11 cm pH 3–10 or pH 4–7 Immobiline DryStrip IPG strips (GE Healthcare) for 16 hours at 50V in a Protean IEF cell (BioRad). After rehydration, isoelectric focusing was performed as follows: conditioning, 250V for 15 minutes; voltage ramping, 8000V for 2.5 hours; focusing, 8000V for 35,000 VHrs. Focused IPG strips were equilibrated in 50 mM Tris, pH 8.8, 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS and 1% (wt/vol) DTT for 10 minutes at 25 °C, followed by 10 minutes in 50 mM Tris, pH 8.8, 6 M urea, 30% (vol/vol) glycerol, 2% (wt/vol) SDS, and 1.2% (wt/vol) iodoacetamide, and then loaded onto 12% SDS-PAGE gels for resolution in the second dimension.

### Streptavidin western blot analysis

Parasite proteins resolved by 2D gel electrophoresis were transferred to Protran nitrocellulose (Schleicher and Schuell) *via* semi-dry blotting (2 hours, 150 mA/gel). The membranes were blocked in 5% (wt/vol) powdered milk in TBS-T (150 mM NaCl, 20 mM Tris, pH 7.4, 0.1% [vol/vol] Tween-20), washed in TBS-T (3 × 10 minutes), and incubated for 1 hour with ECL Streptavidin-HRP conjugate (Amersham Biosciences, 1:25,000 in TBS-T). Blots were washed in TBS-T (3 × 10 minutes), incubated with ECL detection reagents according to the manufacturer's instructions (Amersham Biosciences) and immediately exposed to BioMax MR film (Kodak).

### Protein identification

2D gels were fixed (10 minutes in 50% [vol/vol] methanol, 10% [vol/vol] acetic acid), stained with colloidal Coomassie stain (1.6% [vol/vol] phosphoric acid, 0.08% [wt/vol] Coomassie brilliant blue G-250 [Biorad], 8% [wt/vol] ammonium sulfate, 20% [vol/vol] methanol) for >12 hours, and destained in double distilled $H_2O$. Protein spots of interest were excised from gels with a clean razor blade and subjected to in-gel digestion with a ProGest Investigator in-gel digestion robot (Genomic Solutions), using standard protocols.[16] Briefly, the gel cubes were cut into 1 mm$^2$ cubes, destained by washing with acetonitrile and subjected to reduction and alkylation before digestion with trypsin at 37 °C. The peptides were extracted with 10% formic acid and concentrated down to 20 μl using a SpeedVac (ThermoSavant). They were then separated using an UltiMate nanoLC (LC Packings) equipped with a PepMap C18 trap and column, using a 30 min or 60 min gradient (depending on the molecular weight of the sample being analysed) of increasing acetonitrile concentration, containing 0.1% formic acid (5–35% acetonitrile in 18 min or 35 min respectively, 35–50% in a further 7 or 20 min, followed by 95% acetonitrile to clean the column). The eluent was sprayed into a Q-Star Pulsar XL tandem mass spectrometer (Applied Biosystems) and analysed in Information Dependent Acquisition (IDA) mode, performing 1 sec of MS followed by 3 sec MS/MS analyses of the 2 most intense peaks seen by MS. These masses were then excluded from analysis for the next 60 sec. MS/MS data for doubly and triply charged precursor ions were converted to centroid data, without smoothing, using the Analyst QS1.1 mascot.dll data import filter with default settings. The MS/MS data file generated was analysed using the Mascot 2.1 search engine (Matrix Science) against MSDB May 2006, selecting for alveolates, or ToxoDB (http://www.toxodb.org/). The data were searched with tolerances of 0.2 Da for the precursor and fragment ions, trypsin as the cleavage enzyme, one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification and methionine oxidation selected as a variable modification. The Mascot search results were accepted if a protein hit included at least one peptide with a score above the homology threshold and the MS/MS interpretation accounted for the major peaks in the spectrum (results summarised in Table S1†).

### Generation of the TgPrxII knockout parasite lines

The knockout vector for TgPrxII was based on the chloramphenicol acetyltransferase selectable marker gene *5′TgPRXII-pTub5CAT-3′TgPRXII*, in which the 5′ flanking region (1740 bp) of *TgPrxII* was amplified by PCR from *T. gondii* genomic DNA with primers PrxII-1 (5′-CCGGGTACCAGTGGTGTGCGTTCGCG-3′) and PrxII-2 (5′-CCGGGGAACTCGAGTTTCATGC-3′) and cloned between the *Kpn*I and *Xho*I restriction sites of pTub5CAT in the previously described vector pT/230.[17] The 3′ flanking region (1724 bp) was amplified with primers PrxII-3 (5′-GGAGCGGCCGCCACTCACGGAATGG-3′) and PrxII-4 (5′-CCACCGCGGACCACATAGTGGGCACC-3′) and cloned between the *Not*I and *Sac*II sites. Stable transformants were generated in RH strain parasites as previously

described.[18] *TgPrxII* knockout parasites were identified by an indirect immunofluorescence assay and confirmed by western blotting using rabbit polyclonal anti-TgPrxII antibodies.[19] Knockout clones KO2 and KO4.1 were isolated by limiting dilution.

### Analysis of Prx hyperoxidation in mammalian cells

Human small airway epithelial cells were incubated for 30 min with **1**, glucose oxidase was added and the cells were incubated for an additional 1.5 h. Cell extracts were resolved by reducing and non-reducing SDS-PAGE, followed by western blotting with anti-Prx-$SO_2H/SO_3$ (Lab Frontier LF-PA0004) as previously described.[14]

### Other methods

Details of the glutamine synthase protection assay and mass spectrometry analysis can be found in the ESI.†

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