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Optimisation of Conoidin A, a Peroxiredoxin Inhibitor

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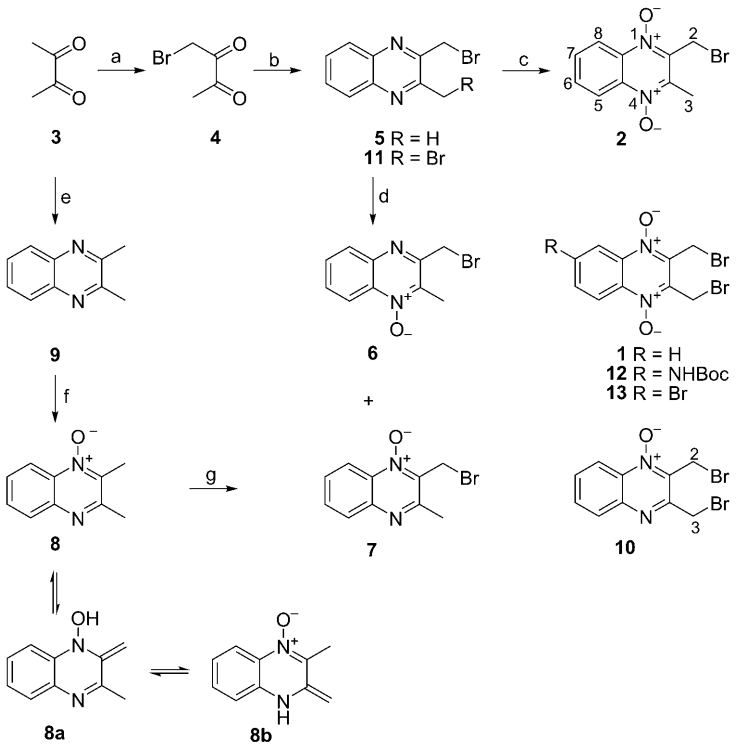
We recently reported that conoidin A (**1**) (Scheme 1) inhibits the function of the peroxiredoxin TgPrxII,[1] an understudied protein from the apicomplexan parasite *Toxoplasma gondii*.[2] **1** targets TgPrxII both in vitro and in live parasites and also inhibits the hyperoxidation of two mammalian peroxiredoxin homologues (PrxI and PrxII) in cells.[2] As the biology and chemistry associated with mammalian peroxiredoxins has entered a new phase, with the discovery that these proteins are involved in complex intracellular signalling cascades and are overexpressed in several cancers, inhibitors of peroxiredoxins are of increasing interest.[3,4] To date, there are only a few examples of Prx inhibitors in general[5] and the *Toxoplasma* enzyme TgPrxII in particular. To our knowledge, **1** is the first reported inhibitor of TgPrxII.

As no crystal structure of TgPrxII exists, we decided to explore how changes to the structure of conoidin A (**1**) affected its biological activity. Our goal was to develop a model for the binding mode of **1** to TgPrxII, thus aiding future inhibitor design and optimisation. Whilst peroxiredoxins remain controversial drug targets, mostly due to their high abundance in cells,[6] optimised Prx inhibitors that can be used to study Prx function are certainly of interest.[7] Our previous studies with conoidin A (**1**) showed that only one of the two available alkylating groups reacts with TgPrxII.[2] This led us to prepare conoidin B (**2**) (Scheme 1), a mono-bromo-analogue of **1**. Additional analogues were also prepared to generate further structure–activity relationship (SAR) data. Here we report our results culminating in proposed binding modes for the conoidins with TgPrxII.



Scheme 1. Synthetic routes used to prepare analogues of conoidin A (**1**). The numbering system used is indicated. *Reagents and conditions*: a) $CuBr_2$, 18-crown-6, $CHCl_3$, reflux, 60 h, 37%; b) 1,2-phenylene diamine, THF, 0 °C→RT, 17 h, 37%; c) *m*CPBA (2 equiv), CH_2Cl_2, RT, 20 h, 50%; d) *m*CPBA (1 equiv), CH_2Cl_2, RT, 20 h, 75% (**6**:**7** formed in ratio 6:1); e) 1,2-phenylene diamine, AcOH, reflux, 1.5 h, 90%; f) *m*CPBA (1 equiv), CH_2Cl_2, RT, 20 h, 65%; g) Br_2 (0.9 equiv), CH_2Cl_2, reflux, 2 h, 75%.

The synthesis of compound **2** and its analogues **6** and **7** was achieved as shown in Scheme 1. Compound **2** was prepared by conversion of butane-2,3-dione (**3**) to the brominated diketone **4**.[8,9] Subsequent condensation of **4** with 1,2-phenylenediamine gave high yields of the mono-bromo quinoxaline **5** that was oxidised (*m*CPBA, 2 equiv) to give **2**. The use of limited amounts of *m*CPBA led to a mixture of the two mono-*N*-oxides **6** and **7**,[10] as it was thought that pure samples of these two analogues would enable the role of the *N*-oxide functional groups to be explored. ^{1}H NMR analysis of the crude reaction mixture indicated that oxidation occurred preferentially at *N*4 in compound **5** to give **6** as the major product (**6**/**7**; 6:1). This presumably results from the lowered electron density at *N*1 due to the nearby bromine. However, pure **6** was obtained by recrystallisation, and an alternative route to **7** was devised that relied on the selective bromination of mono-*N*-oxide quinoxaline **8**, readily accessed from **3** via **9**. The structural assignment of **7** was based on X-ray crystallographic analysis.[11] The bromination presumably occurs adjacent to the *N*-oxide through reaction of bromine with the likely more abundant tautomer **8a**, in which the *N*-hydroxyenamine has increased reactivity towards electrophiles compared to the enamine present in the alternative tautomer **8b**. The increased reactivity of the enamine in **8a** can be rationalised in an analogous manner to the alpha effect.[12]

Subsequently the inhibition of TgPrxII by **2** and **5**–**7** was assessed. The activity of recombinant TgPrxII (rTgPrxII) is assessed using a glutamine synthetase protection assay.[1a] Using

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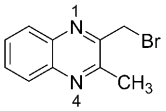
this assay with a 5 min pre-incubation time of compound **2** with the enzyme,[13] **2** was shown to inhibit rTgPrxII in vitro with an IC_{50} value of 42.3 ± 2.5 μM (figure S1 A in the Supporting Information, c.f. IC_{50} (**1**) $= 25.1 \pm 0.8$ μM[2]). Analysis of the incubation of rTgPrxII with **2** using electrospray ionisation mass spectrometry led to an observed increase in molecular weight of rTgPrxII of 187.9 Da corresponding to the addition of one molecule of **2** accompanied by loss of a bromine atom (figure S1 B in the Supporting Information; theoretical mass shift for formation of rTgPrxII:**2** complex with loss of bromine = 188.0 Da). In addition, tryptic digest and MALDI MS/MS experiments supported the view that **2** inhibits rTgPrxII by covalent modification of the active site Cys 47 (figure S1 C in the Supporting Information), as does **1**.[2,14]

IC_{50} values were determined for analogues **5**–**7** and competition experiments with a model thiol, methyl mercaptoacetate (Table 1), were carried out to assess differences in their inherent reactivity. Comparison of the IC_{50} values for **5** and the di-*N*-oxide analogue **2** showed that by incorporating the two *N*-oxide functional groups in **2**, an overall reduction in biological activity occurred even though **2** reacts significantly faster with a model thiol than **5** does. In addition, removal of the *N*4-oxide in **2** to give conoidin C (**7**) led to an increase in potency against the enzyme despite the fact that **7** reacts at a very similar rate to **2** with a model thiol.[14] The relative potency of **2** and **7** is consistent with a detrimental interaction of the *N*4-oxide with the protein. This effect can also be seen when the potency of **5** and **6** are compared. These compounds differ only because **6** contains a *N*4-oxide moiety, but **5** is a significantly more potent inhibitor of rTgPrxII than **6** despite the fact that **5** is less reactive than **6** towards a model thiol.[14] A preference for **6** to adopt an unproductive binding mode may also explain the relative potencies of **5** and **6**. These results clearly indicated that the potency of the analogues cannot be explained exclusively based on their chemical reactivity and that, as expected, the details of their interaction with the active site of the protein are important. It was therefore decided to use computational modelling techniques to provide some insights into the nature of these interactions.

Table 1. IC_{50} values for conoidin B (**2**) analogues against rTgPrxII, and the relative reactivity of the analogues with a model thiol.



Compd	*N*1	*N*4	IC_{50}[a] [μM]	Competition assay[b]
2	N^+O^-	N^+O^-	42.3 ± 2.5	= 1
5	N	N	32.9 ± 1.5	3
6	N	N^+O^-	105.6 ± 5.2	2
7	N^+O^-	N	16.6 ± 0.9	= 1

[a] Pre-incubation time of inhibitor with enzyme = 5 min in all cases. For inhibition curves see Reference [14] [b] The thiol competition experiment was carried out using the model thiol, methyl mercaptoacetate. Order of reactivity: **1**, fastest; **3**, slowest.[14]

Whilst no crystal structure of TgPrxII exists, two computational models of TgPrxII in the "closed" and "open" state have been reported in the literature.[1a] Regeneration of the "closed" model for monomeric TgPrxII based on the crystal structure of human PrxVI (PDB code: 1PRX) was carried out by us using the SWISS-MODEL server. Overall the model of TgPrxII was very similar to human PrxVI with a superimposition of the two structures showing a root mean square deviation (rmsd) of 0.54 Å over 221 Cα atoms. Figure 1 a shows a view of the model emphasising the active site containing the key Cys 47 residue.

The program GOLD was used to dock the conoidin analogues in the active site of TgPrxII. In brief, a 10 Å radius sphere around the Cys 47 residue was defined as the ligand-binding pocket and the sulfur atom of Cys 47 was specified as the covalent linking atom. For each analogue, a bromine atom in the inhibitor was replaced by the sulfur atom of Cys 47. Ten independent optimisation runs were then carried out and yielded ten GOLD docking solutions, which were ranked according to their GOLD score. For all the analogues assessed, the highest scoring poses were found to be very similar to that shown in Figure 1 b obtained using conoidin B (**2**). The inhibitor is oriented in such a way that the *N*1-oxide group is placed in a shallow pocket close to the active site Cys 47 residue. The model suggests that the *N*1 oxygen may accept two hydrogen bonds from the hydroxy group of Thr 44 and the backbone NH of Val 46 (the highest scoring pose obtained for **2** was very similar in this region to that shown in Figure 1 d for **12**). In the case of **6**, if the *N*4-oxide oxygen atom is positioned in this pocket, the reactive alkylating group is forced to be remote from the active site Cys 47 sulfhydryl group. The C3 methyl group of **2** may reside inside the active site, with the C6 and C7 carbon atoms pointing out into solution. It is interesting that the model also predicts that the *N*4-oxide is positioned in a hydrophobic region of the protein consisting of the residues Ile 125 and Val 127 (Figure 1 b). An inability to stabilise the highly electron-rich *N*4-oxide oxygen atom or the inability of the protein to accommodate, for example, water molecules that may be hydrogen bonded to this functional group in bulk solvent, may explain why the presence of the *N*4-oxide was found to be detrimental to activity (c.f., activity of conoidin B (**2**) vs conoidin C (**7**), Table 1).

Analogous results were obtained when the docking was repeated using our original inhibitor, conoidin A (**1**). Interestingly, the modelling studies suggest that the TgPrxII active site can accommodate the additional large bromine atom in **1** (Figure 1 c). We have shown previously that when **1** reacts with TgPrxII, only one of the two bromine atoms is displaced. A possible rationalisation of this result is that in the proposed binding mode there are no nucleophilic residues positioned correctly for a second S_N2 reaction.[15] Our previous studies with **1** also showed that the initially formed TgPrxII:**1** complex could react rapidly with an externally added thiol nucleophile.[2] At present, it is difficult to rationalise this experimental observation with the proposed binding mode unless the pro-

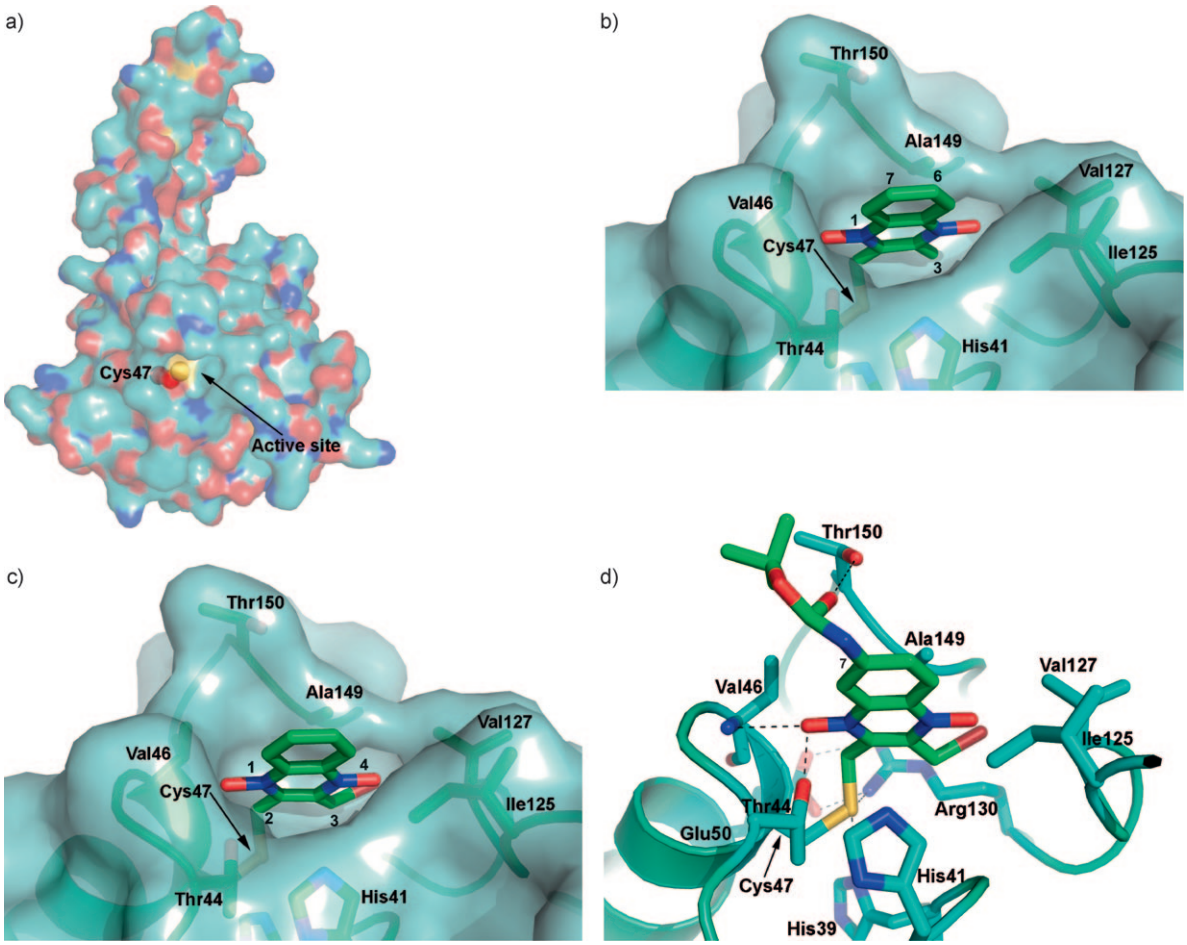
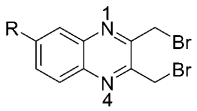


Figure 1. a) Molecular model of TgPrxII; b) Docking of conoidin B (**2**) in the active site of TgPrxII model; c) Docking of conoidin A (**1**) in the active site of TgPrxII model; d) Docking of conoidin D (**12**) in the active site of TgPrxII model.

tein undergoes a significant conformational change after reaction occurs at Cys47. A conformational change of this type has been documented previously for the PrxI subfamily of peroxiredoxins[16a] and proposed for both TgPrxII[1a] and the PrxVI subfamily.[16b]

Inhibition of TgPrxII by C6/C7-substituted analogues of conoidin A (**1**) was subsequently considered. Whilst the proposed binding modes must be viewed with some caution, additional modelling studies led us to two interesting predictions that could be addressed experimentally. First, previous studies using a model thiol nucleophile had shown that in analogue **10** (Scheme 1) the initial substitution reaction occurs at the C2 bromine atom adjacent to the *N*1-oxide functional group rather than at the methylene bromide substituent at C3 (data not shown). It would therefore seem likely that **10** should fit well with the predicted binding mode due to the presence of the *N*1-oxide and absence of this functional group at *N*4. Interestingly, this prediction turned out to be correct as analysis of **10** in the glutamine synthetase protection assay showed that **10** was twice as active as **1** (IC_{50} (**10**) $= 10.3 \pm 1.7$ μM vs IC_{50} (**1**) $= 23.1 \pm 0.8$ μM;[2] Table 2). In addition, it was observed that the absence of both *N*-oxide functional groups in **11** was detrimental to activity probably reflecting both the reduced chemical reactivity and the lack of an *N*-oxide functionality that helps correctly orientate the inhibitor in the binding site.

Table 2. IC_{50} values for conoidin A (**1**) analogues against rTgPrxII, and the relative reactivity of the analogues with a model thiol.



Compd	*N*1	*N*4	R	IC_{50}[a] [μM]	Competition assay[b]
1	N^+O^-	N^+O^-	H	23.1 ± 0.8	3
10	N^+O^-	N	H	10.3 ± 1.7	4[14]
11	N	N	H	41.4 ± 0.6	n.d.
12	N^+O^-	N^+O^-	NHBoc	8.1 ± 0.1	2
13	N^+O^-	N^+O^-	Br	19.9 ± 0.2	1

[a] Pre-incubation time of inhibitor with enzyme = 5 min in all cases. For inhibition curves see Reference [14]. [b] The thiol competition experiment was carried out using the model thiol, methyl mercaptoacetate. Order of reactivity: **1**, fastest; **4**, slowest.[14] n.d. not determined.

The second prediction was that if the proposed binding mode is correct then it may be possible to prepare C6/C7-sub-

stituted analogues of conoidin A (**1**) that have increased activity resulting from additional interactions with residues remote from the active site. For example, the GOLD score associated with an analogue of **1** in which an additional NHBoc substituent had been incorporated at the C6/C7 position of **1** (conoidin D, **12**) was predicted to be better than that of **1** itself (data not shown). We have previously reported the synthesis of **12** and **13**,[17] the C6/C7-NHBoc and -bromine substituted analogues of **1**, respectively. Whilst there was little difference in the biological activity of **13** compared to **1**, the incorporation of the NHBoc functionality did indeed lead to an increase in potency (IC_{50} (**12**) $= 8.1 \pm 0.1$ μM vs IC_{50} (**1**) $= 23.1 \pm 0.8$ μM;[2] Table 1), making **12** the most potent analogue prepared in this series to date. This result is currently rationalised by the potential for **12** to form an additional hydrogen bond with the hydroxy group in Thr150 that cannot be achieved by **1** (Figure 1d). Interestingly, competition studies with model thiols again showed that there was little correlation between inherent reactivity with a model thiol and observed activity against TgPrxII as **13** was found to be slightly more reactive towards methyl mercaptoacetate than **12** (Table 2).

There has been a surge in interest in the biological activities associated with the peroxiredoxins, both in mammalian and parasite systems. While there is a wealth of genetic techniques that enable the role of this protein family to be dissected, additional information can also be gained through the use of peroxiredoxin inhibitors. The relative lack of inhibitors of this protein class may explain why the chemical genetic approach is currently underused for the peroxiredoxins. In addition, the lack of inhibitors is one factor in the limited number of drug discovery reports linked to these proteins at present. Covalent modifiers of proteins are often viewed as less useful than reversible inhibitors as chemical tools or drugs[18] as they cannot be washed out of cell experiments or are too reactive to provide the required levels of selectivity. However, covalent inhibitors of proteins can provide useful starting points for tool or drug development.

The studies presented here describe our attempts to understand how conoidin A (**1**) binds and covalently modifies TgPrxII. A model of the binding mode of conoidin A (**1**) to TgPrxII is presented. Evidence in support of the proposed binding mode comes from our SAR studies on a range of novel conoidin A (**1**) analogues that have been prepared. Several problems associated with the fit of **1** into the active site of TgPrxII have been identified and, when corrected, have led to the preparation of more active analogues (conoidins B–D (**2**, **7** and **12**)). The ultimate goal of this programme is to use this information to convert the conoidins into a second generation of inhibitors that are highly potent noncovalent peroxiredoxin inhibitors. We believe these studies provide the first step along this path.

Experimental Section[14]

2-(Bromomethyl)-3-methylquinoxaline 1-oxide (7): Br_2 (0.41 g, 2.58 mmol) was added to a solution of **8** (0.50 g, 2.87 mmol) in anhyd CH_2Cl_2 (20.0 mL). The reaction was heated at reflux (2 h), cooled and washed with 10% [wt/vol] $Na_2S_2O_3$ (3×20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated in vacuo to give a pale yellow solid. Purification by flash column chromatography on silica gel (EtOAc:petroleum ether 40–60; 1:19→1:9) gave the desired product **7** as a pale yellow solid (0.54 g, 2.15 mmol, 75%); mp: 117–118 °C; IR (NaCl, Nujol): $\nu_{max} = 1506$ (w), 1341 (m), 1065 (m), 774 (m), 629 (m) (C-Br) cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 8.92$ (d, $J = 8.0$ Hz, 1H), 8.02 (d, $J = 8.3$ Hz, 1H), 7.67–7.78 (m, 2H), 4.78 (s, 2H), 2.68 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 163.0, 142.8, 139.6, 135.5, 130.6, 129.2, 118.8, 45.8, 13.9 ppm; MS (ES+): *m/z* (%): 252 (100%) $[M+H]^+$; HRMS (ES+): *m/z* $[M]^{+\bullet}$ calcd for $C_{10}H_9BrN_2O$: 251.9898, found: 251.9900.

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Keywords: conoidin A · inhibitors · molecular modeling · peroxiredoxin · *Toxoplasma gondii*

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[11] CCDC-740449 contains the supplementary crystallographic data for for **7** (excluding structure factors). These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
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[13] A standard pre-incubation time was selected due to the relatively large number of compounds studied here. As pointed out by a reviewer, we accept that a study of the dependence of inhibition on pre-incubation time would provide an improved comparison of the activity of the inhibitors.
[14] For a more detailed discussion see the Supporting Information.
[15] Reference [1a] proposes that His 39 deprotonates the active site Cys 47 and hence would be expected to be protonated. Arg 130 could be involved (see B. Hofmann, H. J. Hecht, L. Flohé, *Biol. Chem.* **2002**, *383*, 347 – 364) but is proposed to form a salt bridge with Glu 50.
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[18] Drugs that covalently modify their biological targets, such as the β-lactams, are typically only useful in cases where there is very high specificity for the target, or in the case of antimicrobials, where the target is either absent from the host or has a unique property with respect to the host isoform. We thank a reviewer for the suggestion to include this caveat.

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