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The benzimidazole based drugs show good activity against *T. gondii* but poor activity against its proposed enoyl reductase enzyme target

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ABSTRACT

The enoyl acyl-carrier protein reductase (ENR) enzyme of the apicomplexan parasite family has been intensely studied for antiparasitic drug design for over a decade, with the most potent inhibitors targeting the NAD^+ bound form of the enzyme. However, the higher affinity for the NADH co-factor over NAD^+ and its availability in the natural environment makes the NADH complex form of ENR an attractive target. Herein, we have examined a benzimidazole family of inhibitors which target the NADH form of *Francisella* ENR, but despite good efficacy against *Toxoplasma gondii*, the IC_{50} for *T. gondii* ENR is poor, with no inhibitory activity at 1 μM. Moreover similar benzimidazole scaffolds are potent against fungi which lack the ENR enzyme and as such we believe that there may be significant off target effects for this family of inhibitors.

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Fatty acid biosynthesis (FAS) is essential for cellular maintenance and survival making it an attractive target for drug design. Importantly, there are distinct differences between the eukaryotic and prokaryotic FAS pathways.[1] The Eukaryotic system, termed FAS I, uses one large polypeptide complex, but Prokaryotes use a series of discrete monofunctional enzymes termed the FAS II pathway to achieve the same goal.[2] Enoyl acyl-carrier protein reductase (ENR), which carries out the final stage of FAS II synthesis, has been the main focus of drug development programs in this area. This has resulted in a range of potent ENR inhibitors being developed such as isoniazid, the diazaborine family and the common antibacterial triclosan.[3–6] The potency and easy synthesis of triclosan has resulted in it being found in a range of common household items such as toothpastes, mouthwashes and chopping boards. Common to all of these potent ENR inhibitors is that they bind to the NAD^+ complexed form of ENR, despite the NADH co-factor having a higher affinity than NAD^+ for ENR and likely being equivalently prevalent in nature.[7] Moreover, the NADH/NAD^+ ratio has been shown to have a significant effect on the potency of isoniazid, an ENR inhibitor in *Mycobacterium tuberculosis*.[8] There are very few examples of inhibitors which have been designed against the NADH form of ENR. However, a benzimidazole family of inhibitors designed as such has modest activity against *Francisella tularensis* (*F. tularensis*) displaying an IC_{50} value of 300 nM.[9,10] The co-crystal structure of a benzimidazole/NADH/ENR complex shows the inhibitor makes no π-stacking interactions with the bound cofactor, a feature common to triclosan and its derivatives, but instead makes a hydrogen bond to the conserved catalytic Tyr and NADH cofactor.[10] The potency of the benzimidazole family has been shown for *F. tularensis*, *Escherichia coli*, *Bacillus anthracis*, *Yersinia pestis*, *Staphylococcus aureus* and *MRSA*, but to date no antiparasitic testing has been carried out.[9]

Despite its eukaryotic nature, *Toxoplasma gondii* (*T. gondii*) uses a typical FAS II pathway making it susceptible to inhibitors of ENR.[11] This pathway is located in the apicoplast organelle and

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not surprisingly apicomplexan parasites that also retain an apicoplast such as *Plasmodium* (*species*) and *Eimeria* (*species*) possess this pathway and are known to be susceptible to ENR targeted drugs.[11–13] Although FAS II and ENR play an important role in liver-stage development of *P. falciparum* it is not essential for the survival of blood stages where triclosan would therefore appear to have an off-target effect.[14,15]

The development of a novel set of ENR/NADH inhibitors against *F. tularensis* has provided a new potential therapeutic avenue for the development of *T. gondii* inhibitors. Here we demonstrate the benzimidazole family of compounds shows no inhibition of *T. gondii* ENR (TgENR) at 1 μM. However, the same inhibitors show promising activity with a MIC_{50} value of between 1 and 10 μM against two different strains of *T. gondii* parasites cultured in vitro. The ability of these compounds to curtail *T. gondii* growth, but not affect ENR activity, suggests that they have an off-target effect. Consistent with this idea, a structurally similar compound Chlormidazole is active against fungi which have a type I and not a type II fatty acid biosynthesis pathway and lack an ENR homologue, indicating an alternative primary target in fungi, most likely 14 alpha methylase. Although *T. gondii* lacks this enzyme, data mining of the PubChem compound library has shown a number of similar scaffolds with different targets which may explain some of the off-target affects evident against *T. gondii*.

Johnson et al. reported that the 3,4-dichloro substituted benzimidazole **1** was potent against *F. tularensis* ENR (FtENR) complexed with NADH.[9] Hit compound **1** and two derivatives **2** and **3** were synthesized from commercially available 5,6-dimethylbenzimidazole and substituted benzyl bromides using NaH and KI in DMF, in moderate to good yields (Scheme 1). HPLC determined purity to be >95% for each compound.

The fibroblast host cell toxicity assays, inhibition assays and parasite replication assays were performed as previously described.[16–20]

Cell toxicity assays were carried out in PC3-Luc cells. Confluent cells were incubated with compounds **1**–**3** at 10 nM, 100 nM, 1 μM and 10 μM concentration in phenol red free DMEM (supplemented with 10% FCS, 1% L-glutamine and 1% penicillin streptomycin). At 48 and 96 h the cells were supplemented with 150 μg/ml D-luciferin potassium salt and imaged for 1 min in an IVIS Spectrum (Perkinelmer, USA).

To investigate if the benzimidazole compounds had a similar binding mode in TgENR as described for FtENR, co-crystallisation experiments were conducted.[10] In the first instance crystals were grown in the presence of 1.6 mM inhibitor, which was more than sufficient for the FtENR/NADH/Benzimidazole crystal structure. However, this was insufficient to produce a TgENR/NADH/Benzimidazole crystal complex. Instead, 3.2 mM of inhibitor was used and crystals were grown in the Morpheus crystal screen from Molecular Dimensions. Several different crystallisation conditions yielded diffracting crystals from which data was collected to identify any bound inhibitor. The highest diffracting crystals grew in condition G6 (0.1 M Na-Formate, NH_4-Acetate, Na_3-Citrate, NaK-Tartrate, Na-Oxamate, 1 M Sodium HEPES, MOPS pH7.5, 30% v/v P500MME_P20K). The crystals were flash frozen and data were collected on beamline I02 at the Diamond SRS. Full data collection and processing statistics are found in Table 1. The coordinates have been deposited within the protein data bank, accession number 4O1M.

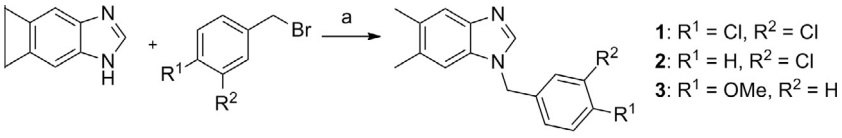
Table 1
Data collection and refinement statistics

	TgENR apo
Data collection statistics	
Space group	C121
Wavelength used (Å)	0.97949
Resolution range (Å)	30.0–2.0 (2.03–2.0)
Unique reflections	129,052 (6302)
Multiplicity	3.6 (3.7)
Completeness of data (%)	99.7 (99.6)
Mean I/σ (I)	6.5 (2.8)
Multiplicity	3.6 (3.7)
R_{merge} (%)	0.13 (0.5)
Refinement Statistics	
Resolution limits (Å)	30.0–2.0
R_{cryst} (%)	16.9
R_{free} (%)	19.9
Rmsd values	
Bond length (Å)	0.015
Bond angle (deg.)	1.54
Ramachandran plot[a]	
Most favoured (%)	97.9
Additionally allowed (%)	2.1
Generously allowed (%)	0.0
Disallowed (%)	0.0
Molecules in asymm. unit	6
Protein atoms	13,302
Co-factor atoms	264
Water molecules	415
Mean *B*-values (Å^2)	
Protein Mainchain/sidechain	9/13
Co-factors	17
Water molecules	15

Data was collected at station I02 at the Diamond synchrotron to 2.0 Å. Values in parentheses are for the highest resolution shell.
[a] Ramachandran values were determined in PROCHECK.[30]

Iterative cycles of model building and refinement were carried out to 2.0 Å resolution in COOT and REFMAC5 with PDB_REDO optimizing the refinement procedure, with resulting R_{fact} and R_{free} values of 0.17 and 0.20, respectively.[21–23] The resulting, refined map showed clear and continuous electron density for the bound NADH cofactor. However, there was only a small region of strong positive F_{obs}–F_{calc} density within the proposed binding site (Fig. 1A). Moreover, the position of this density is not consistent with the proposed binding site for the benzimide inhibitor (Fig. 1B). The inhibitor could not be refined either in a similar position to that seen in FtENR or in an alternative conformation. The features seen within the F_{obs}–F_{calc} map within the ENR binding site are consistent with the density associated with water and bound crystallization additives (Fig. 1C). Despite data for several crystallization conditions being collected and processed no evidence for benzimidazole binding could be seen, with all structures showing features consistent with those seen in Figure 1.

Since the inability to co-crystalise TgENR with the benzimidazole inhibitors is not in of itself evidence that the family are poor inhibitors, enzyme and cellular assays were used to investigate their potency. The enzyme assays showed no inhibitory activity



Scheme 1. Reagents and conditions: (a) NaH, KI, DMF, 0 °C warming to rt, 16 h, 40–60%.

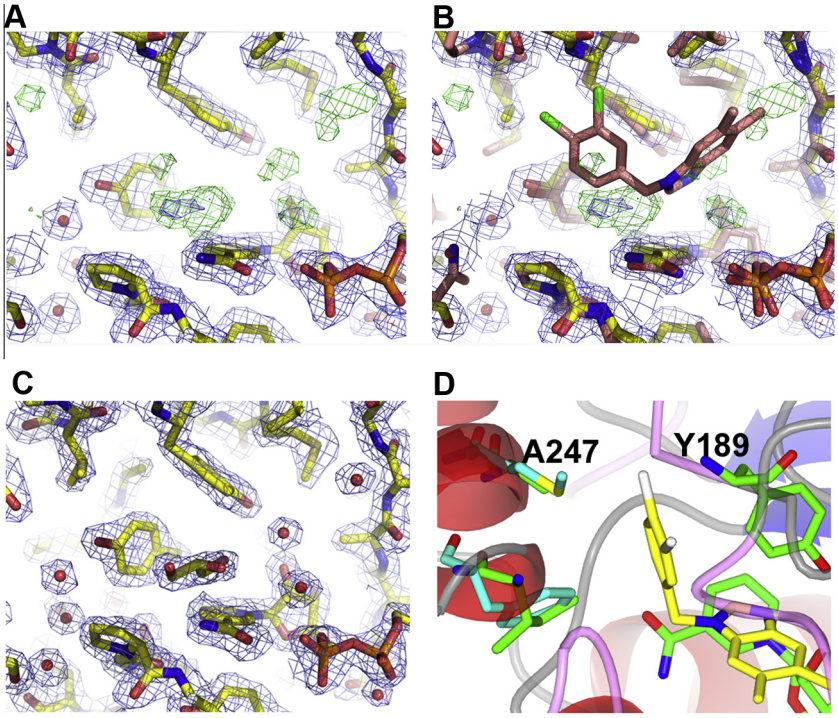


Figure 1. (A) Refined TgENR/NADH crystal structure with the $2F_{obs}$–F_{calc} density map (blue) and $1F_{obs}$–$1F_{calc}$ density map (green) contoured at 1.6σ and 4.0σ, respectively. (B) The same view as in (A) but with the FtENR/NADH/benzimide complex superimposed in red to show the predicted position of the benzimide inhibitor. (C) Representative density of the refined TgENR/NADH structure with waters and glycerol modeled into the inhibitor binding site with the map contoured at 1.5σ. For A–C, nitrogen, oxygen, chlorine, phosphorous and carbon are colored blue, red, green, orange and yellow (TgENR)/red (FtENR), respectively. (D) Overlay of FtENR (blue sidechains) and TgENR (green sidechains) inhibitor binding sites with bound inhibitor (yellow), NADH cofactor (green) shown in stick format. The position of the critical Tyr residue which forms a hydrogen bond to the bound inhibitor is shown along with those residues which are significant different between species.

even at 1 μM concentration for compounds **1**–**3** which showed that the benzimidazole compounds are very poor inhibitors of TgENR.

This discrepancy between binding of benzimidazole to TgENR and FtENR was investigated by comparing the two binding sites. A number of sequence changes are observed including significant changes in the loop region Ser^{242}–Asp^{249} (TgENR numbering). A further change which may affect binding is the substitution of Met/Ala at the base of the binding pocket (Fig. 1D). The Methionine residue in FtENR makes packing interactions with the bound inhibitor and appears to be important for stabilising the inhibitor (Fig. 1D). The replacement with Ala in TgENR reduces the ability of this residue to co-ordinate benzimidazole binding. This is an important aspect of binding, as unlike with other ENR inhibitors the benzimidazole compound is not coordinated by forming stacking interactions with the bound co-factor.[10] A further change is the presence of Phe242 (TgENR numbering) which creates steric hindrance within the binding site, however we have previously shown that this residue is capable of flipping out of the binding site, to remove the steric hindrance.[24] These subtle changes will likely further reduce the potency of the benzimidazole family of inhibitors which have been shown to have only modest activity >100,000–300 nm against FtENR.[10] In the absence of a crystal structure we conducted docking simulations using AutoDock 4.2 of compound **1** to see if it could bind in a mode similar to that observed in FtENR.[25] The predicted binding mode of **1** within TgENR was similar to that of FtENR with no significant steric clashes when Phe242 is flipped out of the active site. However, it is clear from the enzyme assay data that this is not a potent interaction with 1 μM and 3.2 mM being insufficient for inhibition and co-crystallisation, respectively.

In conjunction with TgENR enzyme assays, parasite replication assays were also conducted using the RH strain (type 1) and Pru strain (type 2) *T. gondii* stably transfected with the yellow fluorescence gene (RH-YFP). This allows for parasite viability to be directly recorded from the relative fluorescence intensity. The fluorescence intensities were measured after 72 h using a Synergy H4 Hybrid Multi-Mode Microplate Reader with excitation/emission wavelength at 510/540 nm. Compounds **1**, **2** and **3** were effective against type 1 parasites with MIC_{50} values of 2.5 μM, 4 μM and >10 μM, respectively (Fig. 2A). Type 2 *T. gondii* were also inhibited with compounds **1** and **3** at 10 μM ($(p < 0.005)$ (Fig. 2B)). The difference in activity of compound **2** maybe due to subtle differences in the two types of strains used. This activity is far in excess of what was expected based on the effects of the same compounds on TgENR enzyme, suggesting that they have an additional off-target effect against *T. gondii*.

The promising parasite replication assay data shows that these compounds may be a promising lead for novel *T. gondii* inhibitors. To investigate their potential further, compounds **1**, **2** and **3** were tested for cellular toxicity on both fibroblast and prostate cancer cells (Fig. 2C and D). Importantly, none of the compounds showed significant toxicity, in either of the two different assays even at a concentration of 10 μM against mammalian cells (Fig. 2C and D). The low toxicity paired with the good MIC_{50} values make this family of inhibitors a promising avenue for inhibitor design, however the poor IC_{50} values makes TgENR an unlikely target. To further develop the benzamidazole family we looked for possible targets other than ENR in the PubChem database.

The PubChem search showed that similar small compound scaffolds have been developed which target Sterol 14 alpha-demethylase, Galanin receptor 3, Pteridine Reductase 1, transient receptor potential cation channel C4 (TRPC4) and aldosterone synthase (Table 2). The crystal structure for the *Trypanosoma brucei* Pteridine Reductase (3GN2) shows that the additional amine group of the

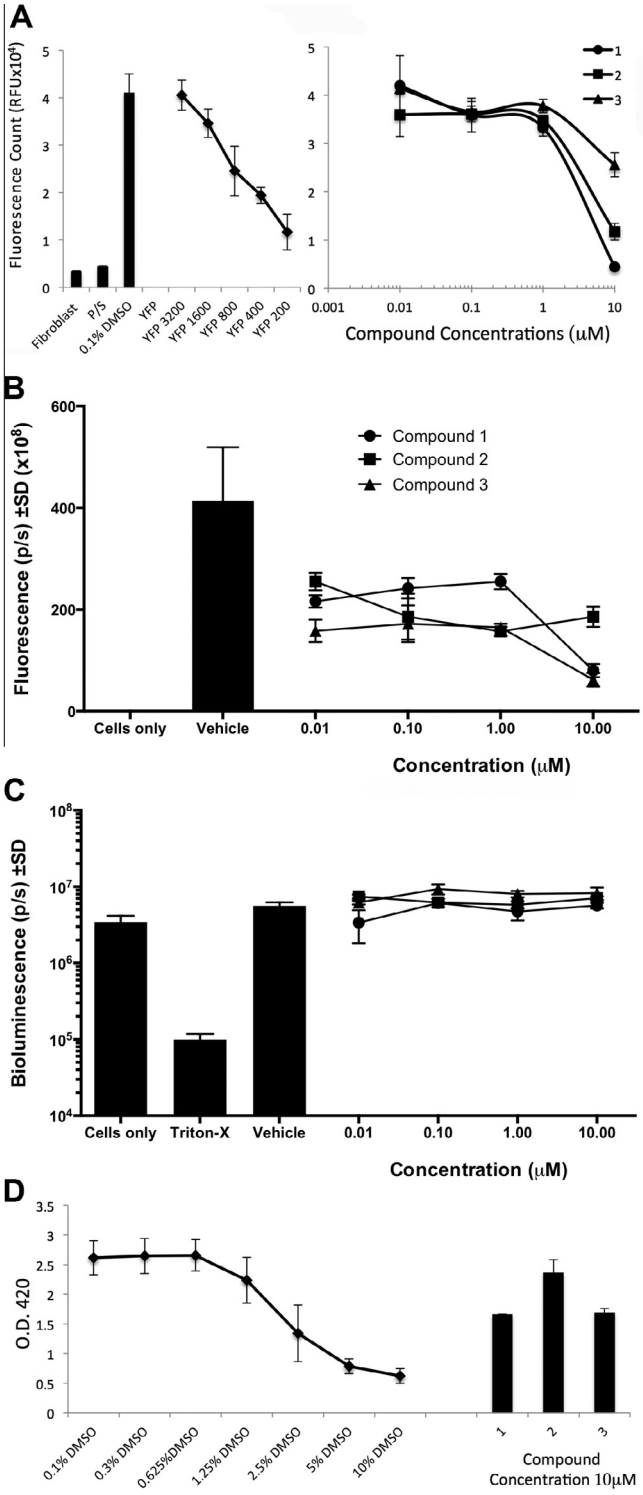
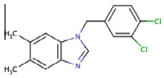
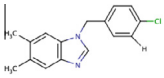
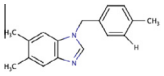
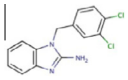
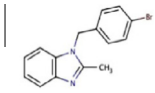
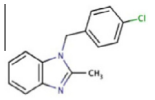
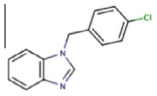
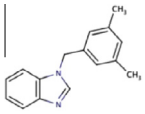


Figure 2. Efficacy and toxicity of compound **1**–**3** against *T. gondii*. (A) Growth of RH-YFP in human Foreskin fibroblasts (HFF) measured as fluorescence intensity. HFF were infected with differing numbers of RH-YFP tachyzoites as indicated, with uninfected control HFF also shown. HFF infected with 3200 RH-YFP tachyzoites treated with pyrimethamine/sulfadiazine (p/s) or 0.1% DMSO are additional positive and negative controls, respectively (left panel). The effect of compound **1**–**3** against *Toxoplasma* tachyzoites was determined by measurement of RH-YFP fluorescence as shown in the right hand panel. HFF were infected with 3200 RH-YFP and treated with compounds **1**–**3** at the concentration indicated. Toxicity was measured using WST stain. (B) The effect of compounds **1**–**3** on *Toxoplasma* growth was determined by infecting confluent fibroblasts with YFP expressing Pru strain *T. gondii* tachyzoites and measuring YFP florescence at 72 h. Compounds **1** and **3** effectively reduced parasite growth however at 10 μM ($p < 0.005$). (C) Compounds **1**–**3** were incubated with confluent luciferase expressing PC3-Luc cells to determine their toxicity against prostate cancer cells. 1% Triton X was included as a positive control for cytotoxicity. After 96 h luciferin (150 μg/ml) was added and cytotoxicity was determined by a reduction in bioluminescent activity from the cells. Neither compounds **1**–**3**, nor the vehicle control resulted in a reduction of bioluminescent activity from the PC3-Luc cells. (D) The viability of host HFF cells was assessed by WST-1 staining after 72 h of incubation of indicated compounds at 10 μM concentration. Effect of various concentrations of DMSO present in the HFF culture medium served as a standard toxicity curve.

Table 2
Activity data for the benzimidazole based scaffold inhibitors

Notebook ID	Structure	IC_{50}	Target	Organism	*T. gondii* MIC_{50}
1		300 nM >1000 nM	Enoyl-ACP Reductase	*Francisella tularensis* *T. gondii*	3 μM
2		>1000 nM	Enoyl-ACP Reductase	*T. gondii*	4 μM
3		>1000 nM	Enoyl-ACP Reductase	*T. gondii*	>10 μM
4		400[a] nM	Pteridine Reductase 1	*Trypanosoma brucei*	ND
5		140 nM 668[b] nM	Galanin Receptor 3 Transient receptor potential cation channel C4 (TRPC4)	Homo Sapiens Homo Sapiens	ND ND
6		ND 200 nM	Sterol 14α-demethylase Galanin Receptor 3	Fungi, *Mycobacteria*, *Leishmania* & *Trypanosoma* Homo Sapiens	ND ND
7		635 nM 107 nM	Steroid 11-beta-hydroxylase Aldosterone synthase	Homo Sapiens Homo Sapiens	ND ND
8		188 nM 632 nM	Steroid 11-beta-hydroxylase Aldosterone synthase	Homo Sapiens Homo Sapiens	ND ND

[a] Value for Ki^{app} not IC_{50}.
[b] Value for EC_{50} not IC_{50}.

bound inhibitor (**4**) is essential in the formation of two hydrogen bonds with Asp^{161} which is absent in the benzamidizole compound.[26] This would make it an unlikely target within *Toxoplasma*. The Galanin receptor and TRPC4 have been shown to be targets of similar compounds **5** and **6** (Table 1). However, these two proteins are not present within *Toxoplasma*, making them unlikely targets. A further target is sterol 14 alpha-demethylase, which is inhibited by the antifungal drug Chlormidazole (**6**) (Table 2). Sterol 14 alpha-demethylase is found in a range of fungi and bacteria such as *Mycobacteria* species as well as the *Leishmania* and *Trypanosoma* parasites. However, it is absent from apicomplexan parasites with BLAST searches revealing only poor matches to putative cytochrome P450 enzymes in *T. gondii*. Moreover, *T. gondii*, as well as other apicomplexan parasites lack a cholesterol biosynthesis pathway, instead relying on scavenging mechanisms.[27] Further studies have found that different sterol 14 alpha-demthylase targeting drugs were more effective against a different cytochrome P450 CYP121 in Mycobacteria.[28] An additional sterol 14 alpha-demthylase inhibitor itraconazole is effective against *T. gondii*, however, an additional off target has not been found and it has been hypothesized that there may be several general non-specific targets in *T. gondii* which account for the cellular activity.[29] A similar scaffold has been shown to display activity against steroid 11-beta-hydroxylase and aldosterone synthase (**7**, **8**). Taken together this would suggest that despite the benzimidazole inhibitor family offering potential as a novel inhibitor against *F. tularensis* ENR it has a likely off target effect which accounts for the good MIC_{50} value established against the parasite in vitro but poor IC_{50} value observed against *Toxoplasma* ENR.

Further studies will be required to determine the exact target of the benzimidazole family of inhibitors and establish a clear structure-activity relationship (SAR). However, they present the potential for novel *T. gondii* inhibitors given their good anti parasitic activity and low toxicity against both prostate and HFF cells.

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