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FULL PAPER

The synthesis and investigation of phthalazinones as anti-tubercular agents

Kristiana T. Santoso,[a,b,d] Chen-Yi Cheung,[c] Kiel Hards,[c,d] Gregory M. Cook,[c,d] Bridget L. Stocker,[a,b,d,*] and Mattie S. M. Timmer[a,b,d,*]

Abstract: A series of 2- and 7-substituted phthalazinones was synthesised and their potential as anti-tubercular drugs assessed via *Mycobacterium tuberculosis* (mc^{2}6230) growth inhibition assays. All phthalazinones tested showed growth inhibitory activity (MIC < 100 μM), and those compounds containing lipophilic and electron-withdrawing groups generally exhibited better anti-tubercular activity. Several lead compounds were identified, including 7-((2-amino-6-(4-fluorophenyl)pyrimidin-4-yl)amino)-2-heptylphthalazin-1(2*H*)-one (MIC = 1.6 μM), 4-tertbutylphthalazin-2(1*H*)-one (MIC = 3 μM), and 7-nitro-phthalazin-1(2*H*)-one (MIC = 3 μM). Mode of action studies indicated that selected pyrimidinyl-phthalazinones may interfere with NADH oxidation, however, the mode of action of the lead compound is independent of this enzyme.

Introduction

Phthalazinones exhibit a diverse range of pharmaceutical properties, such as anti-cancer,[1-3] anti-inflammatory,[4-6] anti-bacterial,[1,7] anti-oxidant,[1] and anti-neurodegenerative[8] properties, with mechanisms of action that are as varied as the diseases they are used against.[9-12] For example, the clinically used anti-cancer drug oliparib (**1**, Figure 1) inhibits the poly(ADP-ribose)polymerase,[9] while azelastine (**2**) is an antihistamine preferentially taken up by lung and alveolar macrophages.[10] Anti-bacterial phthalazinones include amide **3**, which inhibits *Francisella tularensis*, a potential bioterrorism agent, with a minimum bactericidal concentration (MBC) of 0.94 μM,[13] and oxadiazole **4**, which is anti-bacterial against *Escherichia coli* and *Salmonella typhi*.[14] Although the mode of action of anti-bacterial phthalazinones has not been studied, we reasoned that phthalazinones might exhibit anti-tubercular activity due to their ability to mimic the benzoquinone moiety in menaquinone (MK), a key intermediate in the mycobacterial respiratory chain of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB).[15]

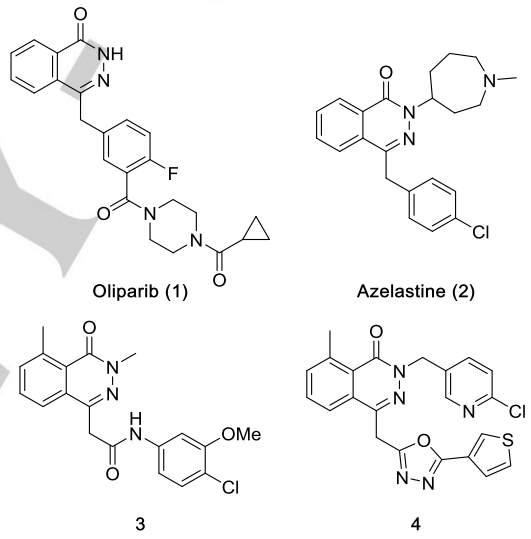

Figure 1. Representative phthalazinones.

The mycobacterial respiratory chain is a potentially rich source of antimycobacterial drug targets,[15] as is perhaps best illustrated by the recent discovery of bedaquiline (BDQ, **5**, Figure 2), the first FDA-approved drug for the treatment of TB in over forty years.[16-18] BDQ targets the F_1F_0-ATP synthase, acting as an uncoupler by dissociating the transmembrane proton transfer from phosphorylation reactions responsible for ATP generation. Other TB drug targets within the respiratory chain include the cytochrome bcc complex (inhibited by Q203, **6**),[19] cytochrome bc1 (lansoprazole, LPZ, **7**)[20] and, of particular interest to us, the respiratory type II NADH dehydrogenase (NDH-II).[21] NDH-II is absent from mammalian mitochondria yet it is essential for growth in mycobacteria as it catalyses the transfer of electrons from NADH to MK via the aid of a flavin co-factor (FAD).[22,23] There are three distinct binding sites in NDH-II: the first and second domains, which are situated in the aqueous environment of the cytoplasm and which bind NADH and FAD, respectively; and a hydrophobic

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membrane-anchoring domain, which contains the quinone binding site (Q-site).[24,25] To date, the antimycobacterial drug clofazimine (**8**) and our recently identified quinolinequinones, such as QQ8c (**9**), have been shown to target NDH-II thereby leading to increased NADH oxidation activity and bactericidal levels of ROS.[21,26,27]

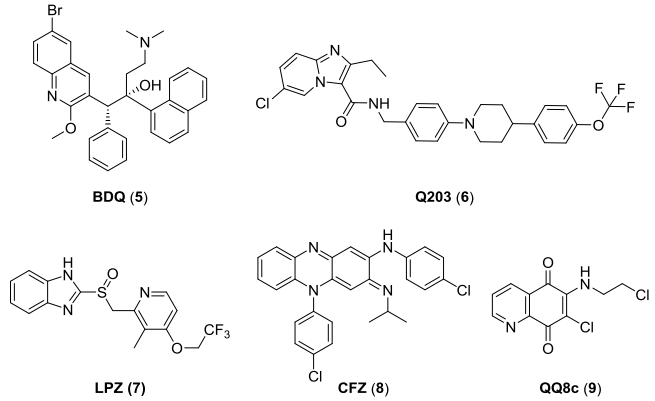

Figure 2. Representative drugs that target the mycobacterial respiratory chain.

The co-crystallisation of NDH-II with MK has proven challenging and has only been achieved with the yeast equivalent of the enzyme.[28] Notwithstanding, docking studies of MK and other quinones in the presence of FAD in NDH-II have demonstrated that quinones fit well into the Q-site.[24] Moreover, a hydrogen bond between one of the carbonyl oxygen atoms of the quinones and an amine proton on the FAD isoalloxazine ring, as well as a distant edge-to-face π-π interaction between MK and the FAD isoalloxazine ring, were observed. Co-crystallisation of *Caldalkalibacillus thermarum* NDH-II with a known inhibitor, 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), revealed similar binding interactions.[25] Accordingly, we proposed that the ketone-containing bicyclic planar phthalazinone scaffold could be accommodated between the hydrophobic residues Q317 and I379 in the Q-site,[24,25] with the ketone oxygen participating in hydrogen bonding interactions with the ring nitrogen of the FAD isoalloxazine ring. Moreover, with a pKa ≈ 8.88,[29] phthalazinones are able to carry charge through the cell membrane and thus act as an uncoupler. Accordingly, we proposed to synthesise a series of phthalazinones (**I**, Figure 3) and to test the ability of these derivatives to inhibit the growth of *M. tuberculosis* (mc^{2}6230) and in addition, target NDH-II.

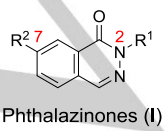

Figure 3. Target phthalazinone structures.

Results and Discussion

Modelling:

To determine whether the phthalazinones could be accommodated in the NDH-II Q-site, molecular docking of 2-methylphthalazin-1(2*H*)-one (**10**) was undertaken using USCF Chimera/DOCK6 (Figures 4).[30] Different molecular orientations with similar binding scores were observed (Figure 4B), however, in all instances, phthalazinone **10** was wedged between the hydrophobic residues Q317 and I379 with a similar binding orientation to that reported for MK and HQNO.[24,25] Hydrogen bonding was also evident between phthalazinone **10** and guanidine residue R382 (Figure 4B). A similar interaction was observed for the docked structure of MK into the Q-site, although the authors queried the plausibility of this interaction as R382 is embedded in the cell membrane.[24] A hydrogen bond between phthalazinone **10** and the ring nitrogen in FAD was also observed for certain binding modes (Figure 4C). Further analysis of the NDH-II Q-site also indicated that the pocket should be able to accommodate substituents at the 2- and 7-positions, thus providing scope to potentially improve the anti-bacterial activity of this class of compounds. As quinolinyl-pyrimidines have been found to interact with NDH-II,[31] we also reasoned that the addition of a pyrimidine to the phthalazinone scaffold might assist with NDH-II binding by way of a hydrogen bond between the pyrimidine amine and a glutamic acid residue (E324) in the Q-site.[24,25]

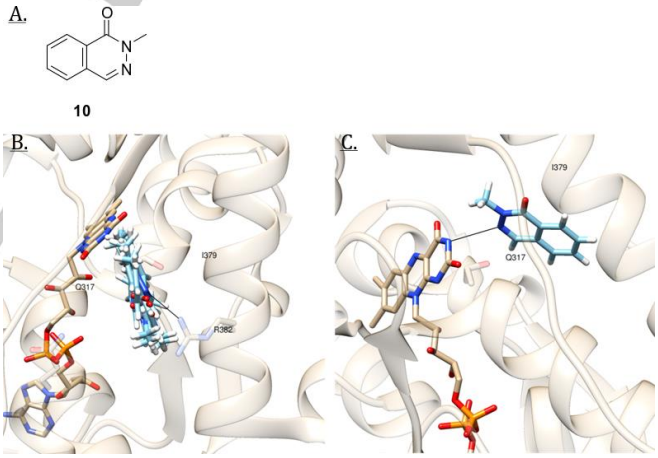

Figure 4. Docking studies of phthalazinone **10** in *C. thermarum* NDH-II (PDB: 6BDO),[25] calculated using USCF Chimera/DOCK6. A). Structure of 2-methylphthalazin-1(2*H*)-one (**10**). B). Phthalazinone **10** can adopt many different orientations in the binding tunnel, sandwiched between Q317 and I379. C). Positioning of a highly ranked conformer of **10** with a hydrogen bond to FAD.

Retrosynthesis:

A retrosynthetic route was devised (Scheme 1), whereby pyrimidinyl-phthalazinones functionalised at the 2- and 6′-positions (**II**) could be assembled from an appropriately functionalised chloropyrimidine **III** and 7-aminophthalazinone **I** (R^2 = NH_2). Here, phthalazinones bearing apolar alkyl and aromatic groups were proposed so as to increase the lipophilicity

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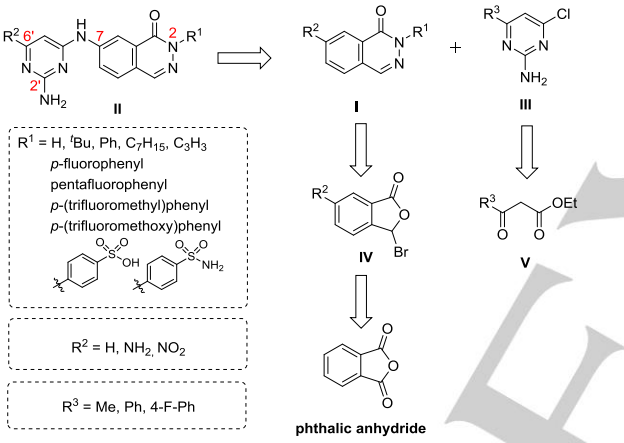
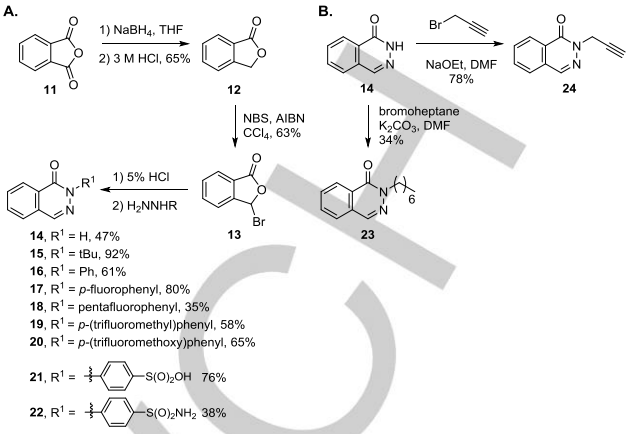
of the phthalazinone scaffold and assist with binding to the hydrophobic NDH-II binding site[24,25] while electron-withdrawing groups, such as fluorophenyl and sulfonamide, on the phthalazinone scaffold were thought to improve the ability of compounds to disrupt the mycobacterial respiratory chain.[32] In addition, the inclusion of a terminal alkyne substituent on the phthalazinone scaffold was proposed to increase the anti-tubercular activity of the compounds as several anti-tubercular agents containing alkyne groups have previously been reported.[33,34] The phthalazinones **I** could be synthesised by condensation of 3-bromo-6-nitro-isobenzofuranone **IV** with various hydrazines, followed by reduction, while the chloropyrimidines **III**, equipped with phenyl or methyl groups, were envisioned to be available from the condensation of functionalised beta-ketoesters (**V**) with guanidine. Furanone **IV** (R^2 = NO_2) itself can be prepared via the reduction of phthalic anhydride, followed by nitration and subsequent benzylic bromination. Using this same route, a library of phthalazinones **I** (where R^2 = H) could also be synthesised from phthalic anhydride.



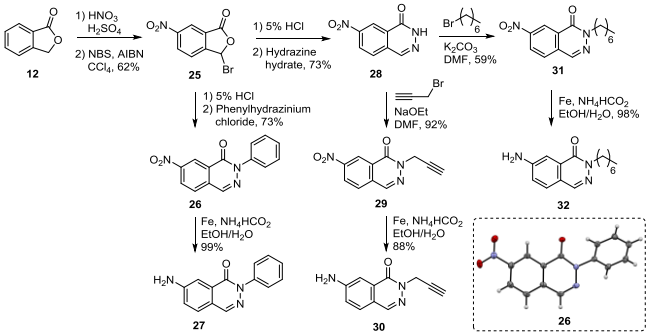
Scheme 1. Retrosynthesis for the preparation of pyrimidinyl-phthalazinones (**II**) and phthalazinones (**I**).

To synthesise the first library of phthalazinones, phthalic anhydride (**11**, Scheme 2) was reduced using sodium borohydride to give isobenzofuranone **12**, which then underwent a Wohl-Ziegler reaction[35] to give bromo-lactone **13**.[36] Lactone **13** was then treated with various hydrazines to afford phthalazinones **14**-**22** in good yields (34-92%, Scheme 2A). Here, ^{1}H and ^{13}C NMR spectroscopy confirmed the loss of resonances corresponding to H-3 and C-3 of bromide **13** (7.41 ppm and 74.8 ppm, respectively, in $CDCl_3$) and the appearance of resonances associated with the 4-positions of the phthalazinones (8.00 – 8.30 ppm, 136.0 – 139.0 ppm, respectively). In addition, IR spectra of phthalazinones showed absorptions between 1102–1120 cm^{-1}, typical of N-N stretches.[37] Phthalazinone **14** was also alkylated using heptylbromide and propargylbromide under basic conditions to give alkyl-phthalazinones **23** and **24**, respectively (Scheme 2B). The connectivity of the alkyl groups to the ring nitrogens was confirmed by HMBC, in which correlations between the *N*-CH_2 and the carbonyl carbons were observed for both **23** and **24**.



Scheme 2. Synthesis of first series of phthalazinones.

Next, the amino-phthalazinones were prepared (Scheme 3). Initially, it was envisaged that nitro-phthalide **25** could be synthesised by subjecting bromo-benzofuranone **13** to nitric acid, however, **13** was unstable under highly acidic conditions which necessitated the need to first nitrate isobenzofuran-1(3*H*)-one (**12**) before subjecting the intermediate, 6-nitrobenzofuran-1(3*H*)-one, to Wohl-Ziegler conditions to yield the desired phthalide **25**.[38] Next, bromo-phthalide **25** was reacted with phenylhydrazinium chloride to give phthalazinone **26**, of which a crystal structure was obtained to unequivocally confirm the configuration of the product (Scheme 3). The iron-mediated reduction of **26** then yielded the desired amino-phthalazinone **27**.[39] Similarly, treatment of nitro-phthalide **25** with hydrazine hydrate gave phthalazinone **28**, which was then alkylated using propargyl bromide or 1-bromoheptane to give propargyl-phthalazinone **29** and heptyl-phthalazinone **31**, respectively, in good to excellent yield. These two alkylated phthalazinones were then reduced, with 7-amino-2-propargyl-phthalazin-1(2*H*)-one (**30**) and 7-amino-2-heptylphthalazin-1(2*H*)-one (**32**) being obtained in 88% and 98% yield from their respective nitro-functionalised precursors.



Scheme 3. Synthesis of the amino phthalazinones.

Finally, the assembly of pyrimidinyl-phthalazinones was undertaken. First, the chloropyrimidines were synthesised via the treatment of guanidine carbonate (**33**) with either ethyl

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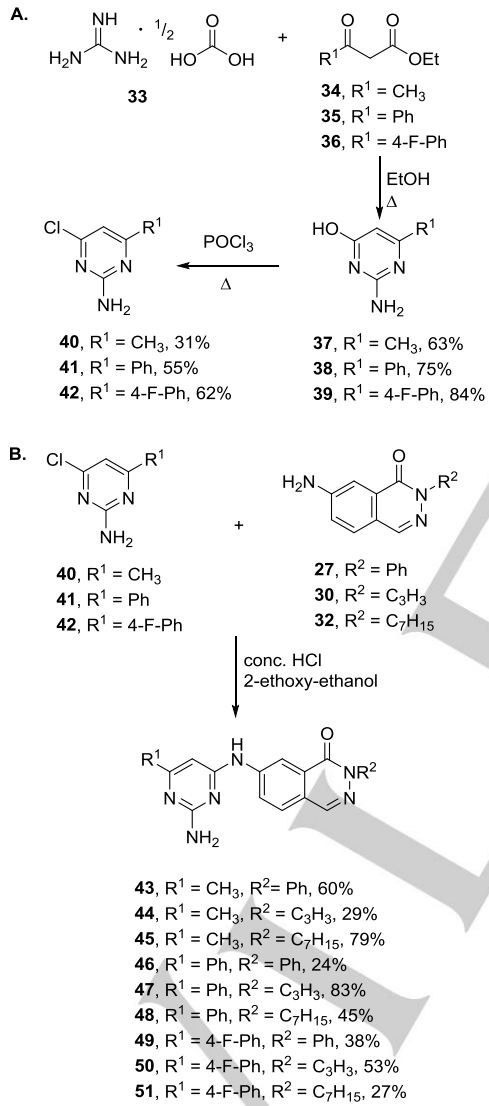
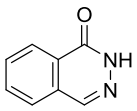
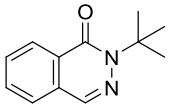
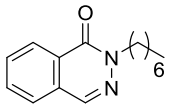
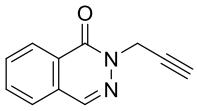
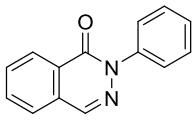
acetoacetate (**34**),[40] ethyl benzoylacetate (**35**),[41] or ethyl-4-fluorobenzoylacetate (**36**) to give hydroxypyrimidines **37, 38**, and **39**, respectively, in good yield (Scheme 4A). The hydroxypyrimidines were then treated with $POCl_3$ under heating with reflux to yield the corresponding chloropyrimidines **40**,[40] **41**[42] and **42**. Condensation of the chloropyrimidines with amino-phthalazinones **27**, **30** and **32** under acidic conditions then gave pyrimidinyl-phthalazinones **43**-**51** in moderate to good yields (Scheme 4B). Successful formation of pyrimidinyl-phthalazinones **43**-**51** was confirmed via NMR spectroscopy, which included an HMBC between the bridging amine NH and carbons at the 5'-, 8- and 6-positions.



Scheme 4: Synthesis of the pyrimidinyl-phthalazinones

Having completed the synthesis of phthalazinones and pyrimidinyl-phthalazinones, the compounds were then screened for their ability to inhibit the growth of *M. tuberculosis*, with the first line TB drug isoniazid (INH) being used as a positive control (MIC = 0.2 μM). Here, the unfunctionalised phthalazinone **14** exhibited an MIC = 50 μM (entry 1, Table 1), while the incorporation of an aliphatic chain at the 2-position led to an increase in inhibitory activity of the phthalazinones, with *tert*-butyl- (**15**), heptyl- (**23**) and propargyl- (**24**) phthalazinones all exhibiting low micromolar MIC values (3 μM, 6 μM, 12.5 μM, respectively, entries 2-4). Although the incorporation of lipophilic groups at this position significantly increased the potency of the compounds, there was no direct correlation between activity and the lipophilicity of the molecule as a whole, as expressed in terms of cLogP, the calculated logarithm of the n-octanol/water partition coefficient. Notably, the cLogP for the most potent compound, *tert*-butyl-phthalazinone **15** (cLogP = 1.848), was in between those of heptyl compound **23** (cLogP = 3.785) and unsubstituted phthalazinone **14** (cLogP = 0.155). Phenyl-substituted phthalazinone **16** was a poorer inhibitor (MIC = 100 μM, entry 5), although when electron-withdrawing groups were incorporated onto the phenyl ring (*e.g.* **17**-**22**, entries 6-11), lower MICs of 50 μM were obtained for all derivatives, except the one that contained a *p*-CF_3 group (**19**, entry 8), where an MIC = 12.5 μM was observed. Thus, these results imply that increasing the electron-withdrawing nature of the phthalazinones can increase the inhibitory activity of the compounds towards *M. tuberculosis*.

Table 1. Inhibition of *M. tuberculosis* by phthalazinones

Entry	Compound	MIC (μM)[a,b]
1	 **14**	50
2	 **15**	3
3	 **23**	6
4	 **24**	12.5
5	 **16**	100

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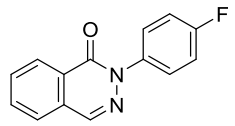
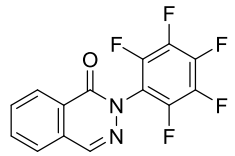
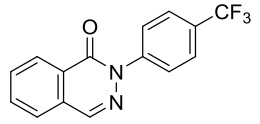
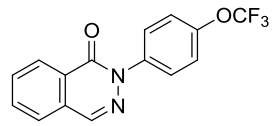
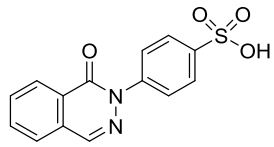
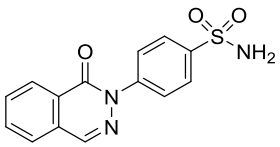
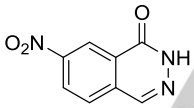
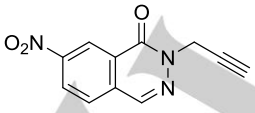
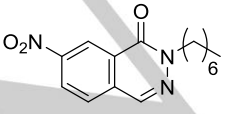
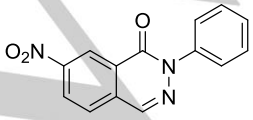
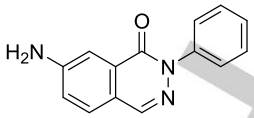
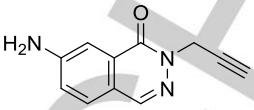
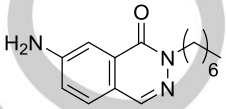
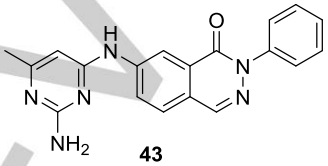
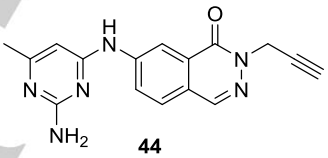
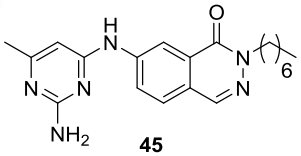
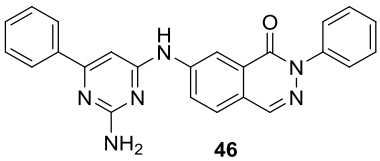
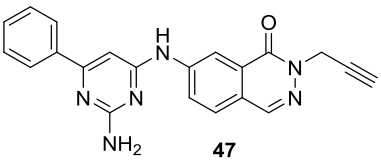
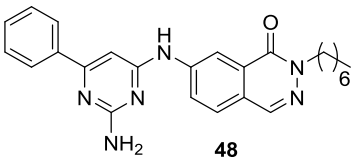
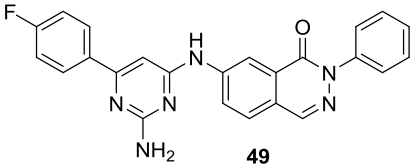
6	 **17**	50
7	 **18**	50
8	 **19**	12.5
9	 **20**	50
10	 **21**	50
11	 **22**	50
12	 **28**	3
13	 **29**	6
14	 **31**	100
15	 **26**	100
16	 **27**	100
17	 **30**	50
18	 **32**	100
19	 **43**	50
20	 **44**	100
21	 **45**	50
22	 **46**	100
23	 **47**	12.5
24	 **48**	100
25	 **49**	12.5

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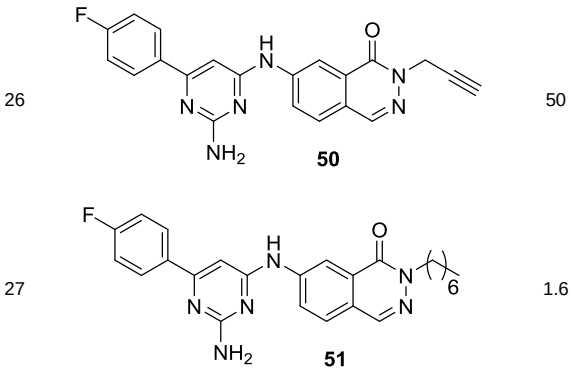

[a] Minimal inhibitory concentrations (MIC) were determined using a microtitre plate assay. *M. tb* mc^{2}6230 cultures were incubated with the compounds for 5 days before resazurin was added and MICs determined.

[b] isoniazid (INH) was used as a positive control (MIC = 0.2 µM).

Next, the inhibitory activity of phthalazinones containing electron-withdrawing and electron-donating groups at the 7-positions were investigated. Here, the incorporation of a nitro group at the 7-position led to a remarkable increase in anti-tubercular activity for phthalazinone **28** (MIC = 3 µM, entry 12) compared to the unfunctionalised phthalazinone **14** (MIC = 50 µM, entry 1) and a two-fold improvement in activity for nitro-phthalazinone **29** (MIC = 6 µM, entry 13) compared to the corresponding propargyl-phthalazinone **24** (MIC = 12.5 µM, entry 4). In addition, it is noteworthy that phthalazinones **28** and **15** have appreciable anti-tubercular activities despite their low lipophilicities (cLogP = -0.102 and 1.848, respectively). For heptyl-phthalazinone **31** however, incorporation of the 6-nitro group led to a loss in anti-tubercular activity (MIC = 100 µM, entry 14 *vs*. 6 µM for **23**, entry 3), while no change in inhibitory activity was observed between phenyl-phthalazinones **26** and **16** (entries 15 and 5, respectively). Amino-phthalazinones **27**, **30**, and **32** (entries 16-18) resulted in a loss of *M. tuberculosis* growth inhibition when compared to the equivalent unsubstituted phthalazinones **16**, **24**, and **23**, respectively (entries 3-5), thus demonstrating that the instalment of an electron-donating amino group appears to lead to a reduction in potency of the drugs. This may be due to the ability of the amine groups in the amino-phthalazinones to form hydrogen bonds with amino acid residues away from the putative NDH-II Q-site, *e.g.* glutamic acid E324, or the relatively high polarity of the amino-phthalazinones could lower their binding affinity for the hydrophobic NDH-II Q-site.

Finally, the ability of pyrimidinyl-phthalazinones **43**-**51** to inhibit *M. tuberculosis* growth were determined (entries 19-27). In general, compounds bearing a 6-methyl-pyrimidine moiety were poor *M. tuberculosis* inhibitors, with phthalazinones **43**-**45** having high MIC values (50 – 100 µM, entries 19-21). Similarly, while phenyl-pyrimidinyl-alkyne **47** elicited good anti-tubercular activity (MIC = 12.5 µM, entry 23), 6-phenyl-pyrimidines **46** and **48** had low potency against *M. tuberculosis* with an MIC value of 100 µM (entries 22 and 24). Aside from propargyl-phthalazinone **50** (entry 26), the incorporation of the more electron-withdrawing fluoro-phenyl-pyrimidine led to an increase in anti-tubercular activity in two of the pyrimidinyl-phthalazinones, as evidenced by the low MIC values of fluorophenyl-phthalazinones **49** (MIC = 12.5 µM, entry 25) and **51** (MIC = 1.6 µM, entry 27), with phthalazinone **51** exhibiting the highest anti-tubercular activity of the series. Thus, while the incorporation of a pyrimidine group did not significantly increase anti-tubercular activity of all phthalazinones, the lead *M. tuberculosis* inhibitor did contain this functional group. Taken together, these studies demonstrate that the inclusion of electron-withdrawing substituents and lipophilic group at the 2-position can increase the anti-tubercular activity of phthalazinones, however, the size and position of functional groups on the phthalazinone scaffold also influences inhibitory activity.

In an attempt to better explain the anti-tubercular activity of the phthalazinones, investigations into the ability of the compounds to affect respiratory NDH-II-dependent NADH oxidation were undertaken. Here, the rates of NADH oxidation in the absence and presence of inhibitors were measured using inverted membrane vesicles (IMVs) from *M. smegmatis*.[43,44] The addition of several phthalazinones to IMVs oxidising NADH showed a trend whereby the rates of NADH oxidation were enhanced compared to the basal rate (Figure 5), particularly pyrimidinyl-phthalazinones **46**, **49** and **50**. These data suggest that these

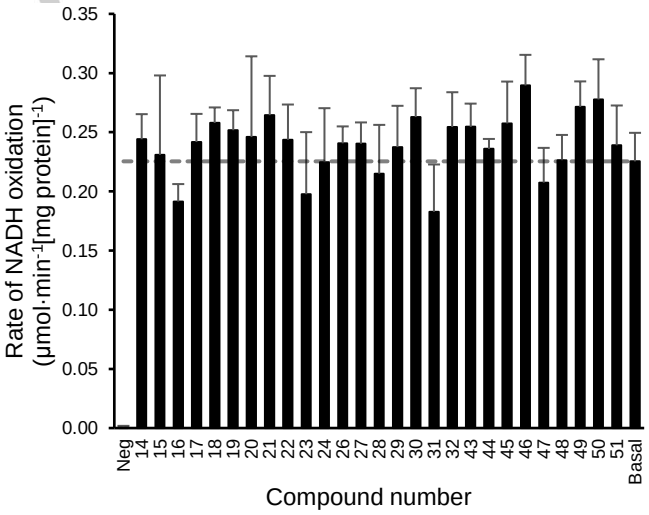

Figure 5: Rates of NADH oxidation when IMVs are treated with phthalazinones **14**-**24**, **26**-**32**, and pyrimidinyl-phthalazinones **43**-**51**. Negative control is vehicle only, whereby IMV was not treated with compound or NADH. The basal rate is the rate of NADH oxidation by IMVs without the addition of drug. Data are expressed as average ± standard deviation of three independent experiments.

compounds might activate NDH-II activity probably via a redox cycling mechanism.[21,27] Conversely, many phthalazinones appeared to cause a lower rate of NADH oxidation, notably phenyl-phthalazinone **16**, heptyl-phthalazinone **23**, and nitro-heptyl-phthalazinone **31**, which could be a result of NDH-II inhibition (direct or indirect). Accordingly, certain phthalazinones and pyrimidinyl-phthalazinones appear to bind NDH-II, although with different modes of action. Whereas representative

pyrimidinyl-phthalazinones activate NDH-II, which could lead to the formation of lethal levels of ROS,[21,27] some phthalazinones may exert their anti-tubercular activity through the inhibition of NDH-II and/or acting as an uncoupler of oxidative phosphorylation in mycobacteria.[15] On the other hand, a lack of correlation was observed between the MIC values of alkyne bearing compounds and their ability to affect NDH-II. For example, the rates of NADH oxidation upon treatment with alkyne-substituted phthalazinones **24** and **29** (MIC = 12.5 μM and 6 μM, respectively) were comparable to the basal rate of NADH oxidation, while alkynes **30** and **50**, with comparatively high MIC values (50 μM for both), increased the rate of NADH oxidation in IMVs. As alkynes can undergo covalent interactions with non-target enzymes, it is possible that the observed anti-tubercular activities of propargyl-phthalazinones **24** and **29** resulted from off-target effects. Similarly, the mode of action of the lead phthalazinones (*e.g.* **15** and **28**) and pyrimidinyl-phthalazinones (*e.g.* **51**) appears to be independent of NDH-II.

Conclusions

In conclusion, we determined that phthalazinones are a promising class of TB inhibitors. In the present study, computational modelling using a representative phthalazinone scaffold indicated that these derivatives could be accommodated in the putative NDH-II Q-site. Accordingly, a library of phthalazinones functionalised at the 2- and 7-positions were synthesised and tested for their ability to inhibit the growth of *M. tuberculosis*. In general, the presence of electron-withdrawing or lipophilic groups on the phthalazinone or pyrimidinyl-phthalazinone scaffolds increased the anti-tubercular activity of the compounds, with several promising TB inhibitors, including fluorophenyl-pyrimidinyl-heptyl-phthalazinone **51** with an MIC = 1.6 μM, being identified. The compounds were then screened for their ability to affect NADH oxidation in IMVs, which demonstrated that certain pyrimidinyl-phthalazinones increased the rate of NADH oxidation, while some phthalazinones decreased the rate of NADH oxidation. Other derivatives, such as heptyl-phthalazinone **51**, did not greatly affect NADH oxidation, thus suggesting a different mode of action. Taken together, these studies are the first to demonstrate the potential of phthalazinones to act as anti-tubercular inhibitors. Further studies into optimising the anti-tubercular activity of these compounds and to better understand their mode of action are underway.

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Keywords: Nitrogen Heterocycles • Medicinal Chemistry • Phthalazinones • Tuberculosis

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