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## Just Accepted



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# Structure-activity relationship and *in vitro* ADMET studies of N-aryl 3-trifluoromethyl pyrido[1,2-*a*]benzimidazoles that are efficacious in a mouse model of schistosomiasis.

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We have previously reported on the antischistosomal activity of pyrido [1,2-*a*] benzimidazole (PBI) derivatives. As a follow-up, we designed and prosecuted further structure-activity relationship (SAR) studies that incorporate N-aryl substitutions on the PBI scaffold. Investigations into the *in vitro* antischistosomal activity against newly transformed schistosomula (NTS) and adult worms revealed several leads with promising potency. Active compounds with a good cytotoxicity profile were tested *in vivo* whereby **6** and **44** induced noteworthy reduction (62-69%) in the worm load in the *Schistosoma mansoni* mouse model. Pharmacokinetic analysis on **44**

pointed to slow absorption, low volume of distribution and low plasma clearance indicating the potential of these compounds to achieve a long duration of action. Overall, our work demonstrates that PBI chemotype is a promising scaffold in the discovery of new antischistosomal leads.

KEYWORDS: *Schistosoma mansoni, Newly transformed schistosomula, Pyrido[1,2-a]benzimidazole, antischistosomal lead*

Flatworms of the genus *Schistosoma* cause schistosomiasis (also known as Bilharzia) with *S. haematobium*, *S. japonicum* and *S. mansoni* being largely responsible for most of the human cases of schistosomiasis. The latest estimates reveal that the disease is endemic in 78 countries where 780 million people are at risk and over 200 million infected with schistosmiasis.[1–4] Persistently, schistosomiasis has been rife in the tropical and sub-tropical regions of sub-Saharan Africa, Middle East, South East Asia, South America and the Caribbean and is associated with poor hygiene and sanitation that promotes completion of the parasite's life cycle and hence propagation of infection.[4,5] The chronic and insidious nature of the disease has been blamed for the under-estimation of its public health impact with the generalised non-specific symptoms, frequent in other disease states, being a common cause for either wrong or late diagnosis.[6,7]

Chemotherapy has played a pivotal role in the control of schistosomiasis. Praziquantel is currently the only drug of choice with pan-activity across the species whereas previously useful agents like oxamniquine and metrifonate that display activity singly against *S. mansoni* and *S. haematobium* respectively, are no longer in use.[7,8] Although praziquantel is effective, safe and affordable, its over-reliance especially in use during mass drug administration programs, so called preventive chemotherapy, has sparked concerns of resistance emerging with decreased clinical efficacy having been reported previously.[9] Additionally, praziquantel displays poor activity towards the immature stages of the worms, making repeated administration necessary.[10,11]

That a disease with public health significance of such magnitude should depend on a single drug is startling and raises discomforting concerns regarding emergence of resistance in the absence of effective alternatives. This scenario has provoked the scientific community into offering greater commitment to drug discovery and development towards schistosome infections. In contributing towards this goal, our research group has been interested in studying the antischistosomal activities of pyridobenzimidazole (PBI) analogues. We previously reported on the antischistosomal activity of a PBI series arising from SAR work that generated mainly aliphatic amine derivatives with substituted 3-phenyl groups on the PBI scaffold as exemplified by compound A in Figure 1.[12]Moreover, enhanced *in vitro* potency was noticed among a small subset of aromatic amine derivatives bearing a 3-trifluoromethyl substitution on the PBI core such as compound B. The aromatic analogues also displayed an improved cytotoxicity profile as suggested by higher selectivity indices with compound B. (Figure 1).

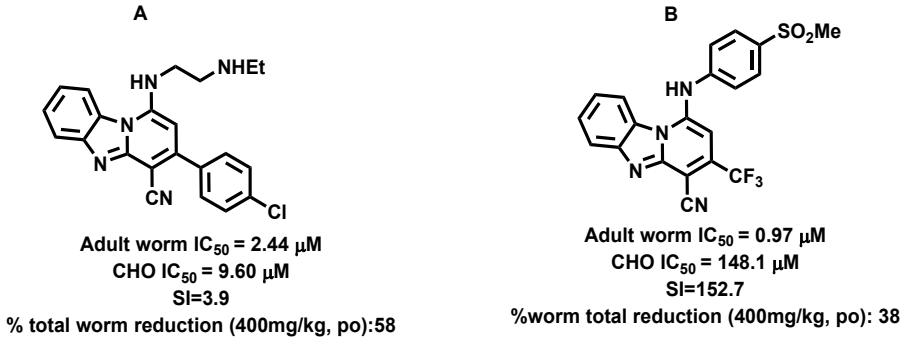



**Figure 1.** Exemplar compounds from previous SAR studies

As a follow-up, we set out an expanded SAR program involving modifications of the appendages to the PBI core as depicted in **Figure 2**. Presently, we report the *in vitro* potency, selectivity,

metabolic stability as well as *in vivo* efficacy and pharmacokinetics of compounds produced in the current work.

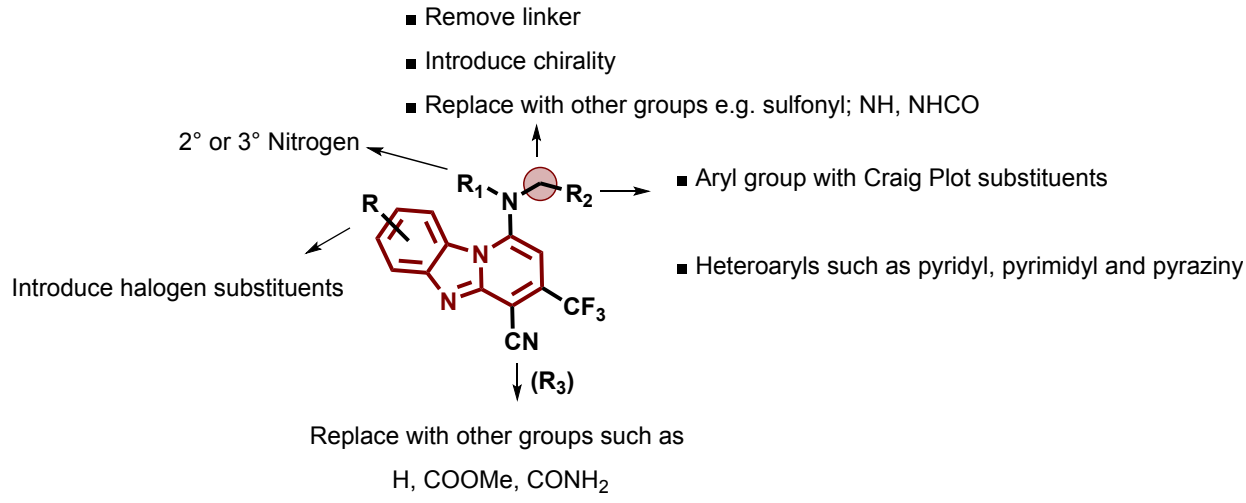
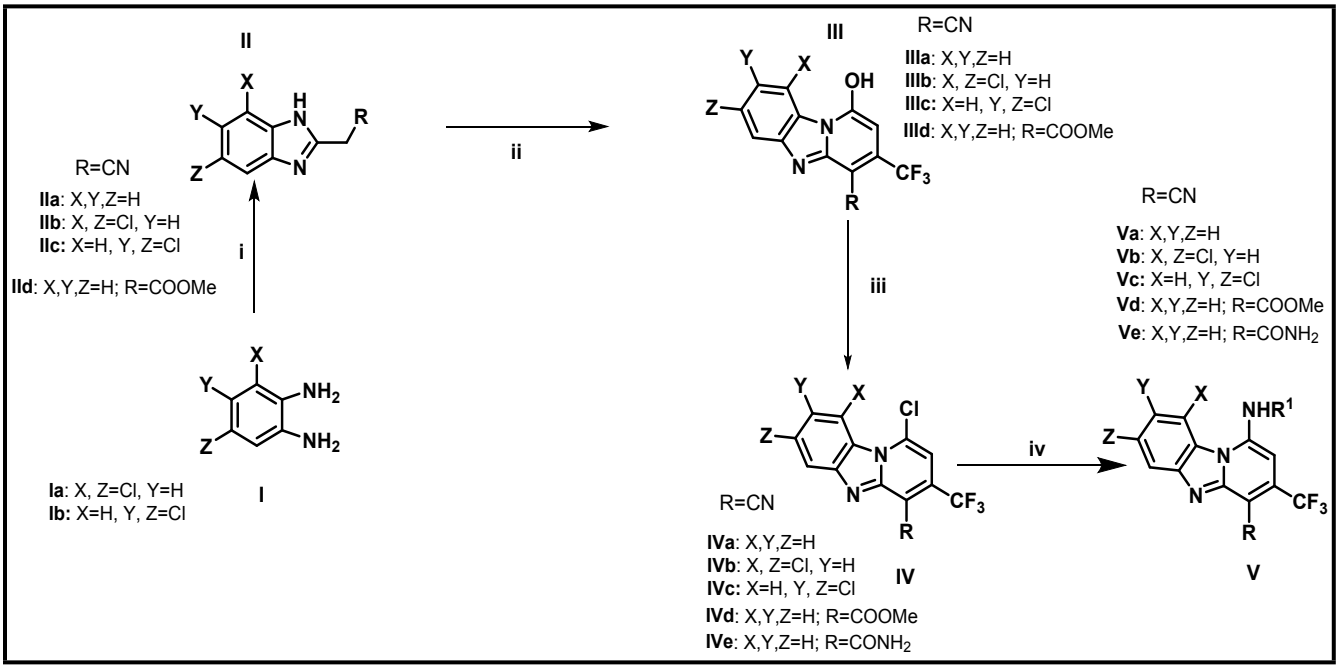



**Figure 2**. SAR approach to target compounds

## RESULTS AND DISCUSSION

**Chemistry.** Target compounds were synthesised using methods adapted from literature[13] and as shown in **Scheme 1**.

**Scheme 1. General reaction scheme for synthesis of pyridobenzimidazoles**.




*Reagents and conditions*: (i) Ethyl cyanoacetate, DMF, 160˚C, 2 h, 61-76%; (ii) Ethyl 4,4,4-trifluoro-3-oxobutanoate, $NH_4OAc$, 145˚C, 2h, 27-66%; (iii) $POCl_3$, (20eq), 130˚C, 3 h, 63-94%; (iv) Relevant amine, $Et_3N$, 80˚C, 150 W, 20-60 min (9-86%) or appropriate amine, $Pd_2(dba)_3$, BINAP or Brettphos, $K_2CO_3$ or $Cs_2CO_3$, toluene or 1,4-Dioxane or tert-butanol, 100-120˚C, 12-16 h, 6-54% . X, Y and Z are as described in Table 1.

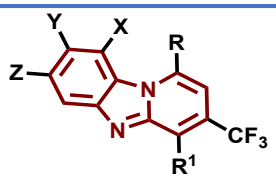
Compounds with no substitution on the left-hand side (LHS) of the PBI scaffold (X, Y, Z = H; **Table 1**) were synthesised from the commercially available 2-benzimidazole acetonitrile **IIa**. For other targets, the appropriately substituted diamino benzene starting material **I** was subjected to a condensation reaction with ethyl-2-cyanoacetate to form the appropriately substituted benzimidazole acetonitrile derivative **II**.

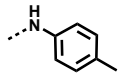
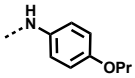
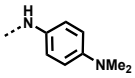
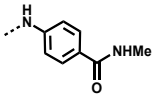
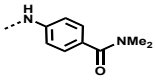
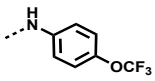
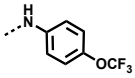
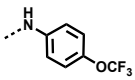
Thereafter, the condensation of the benzimidazole acetonitrile and ethyl 4,4,4-trifluoro-3-oxobutanoate occurs in the presence of ammonium acetate to give the tricyclic hydroxyl intermediate **III**, which is converted to the penultimate intermediate **IV** in a reaction involving substitution of the hydroxyl with a chloro, a process mediated by phosphoryl chloride ($POCl_3$).

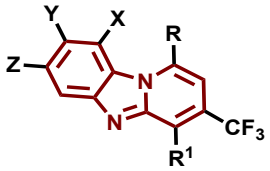
Finally, amination via nucleophilic aromatic substitution of **IV** using the relevant amine in the presence of triethylamine in a microwave reactor or the palladium -catalysed Buchwald-Hartwig reaction in the presence of a suitable ligand and base were employed to give the final compounds **1 - 4**, **6, 7, 9 - 13**, **17 - 25**, **27 - 35**, **37 - 45**, **48**, **49**, **53 - 55** and **5**, **8**, **14 - 16**, **26**, **36**, **46**, **47**, **50 - 52** respectively. To obtain **8**, the benzimidazole methyl ester **IId** was used as the starting material. For other nitrile replacement analogues **53 – 55**, intermediate **IVa** was converted to the amide **IVe** using concentrated sulphuric acid before amination as in step **iv**. The final compound **7** was accessed via ester decarboxylation of **8**.

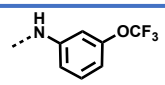
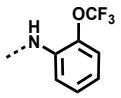
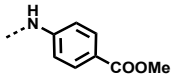
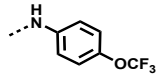
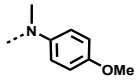
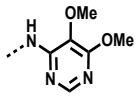
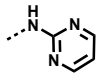
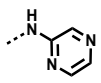
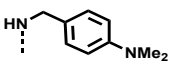
***In vitro*** **antischistosomal activity against NTS and adult *S. mansoni.*** With the aim of prioritising compounds capable of targeting newly transformed schistosomula (NTS), compounds were first screened for activity against these immature worms at an initial concentration of 10 μM. Thereafter, compounds that displayed significant activity against NTS were subsequently exposed to adult *S. mansoni* at the same concentration. Compounds with significant activity (≥70%) in the adult worms at 10 μM were progressed for further dose-response experiments from which $IC_{50}$ values were determined. The activity progression criteria adopted was intended to guide the progression of compounds with dual life cycle stage activity as this profile is a requisite for both the treatment and prevention of re-infection with schistosomiasis. Ensuing SAR trends were analysed based on the observed activities as presented in **Table 1**.

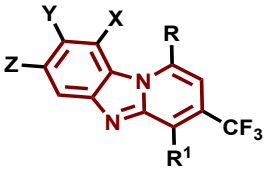
**Table 1**. ***In vitro*** **activity against** ***S. mansoni*** **NTS and adult worms.**

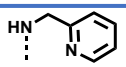
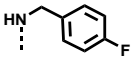
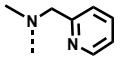
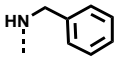
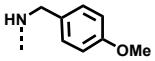
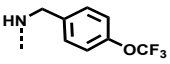
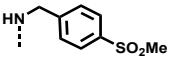
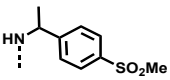
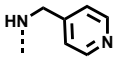
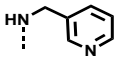
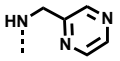


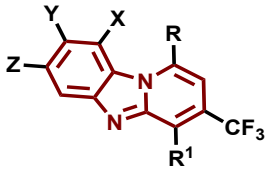

| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **1** | H | H | H | CN |  | 74 | 33 | |
| **2** | H | H | H | CN |  | 100 | 50 | |
| **3** | H | H | H | CN |  | 43 | | |
| **4** | H | H | H | CN |  | 30 | | |
| **5** | H | H | H | CN |  | 30 | | |
| **6** | H | H | H | CN |  | 100 | 100 | 0.210 |
| **7** | H | H | H | H |  | 100 | 77 | 3.37 |
| **8** | H | H | H |  |  | 67 | 56 | |

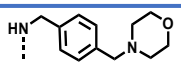
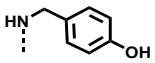
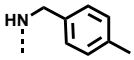
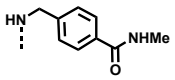
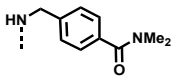
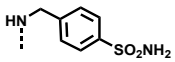
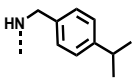
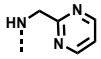
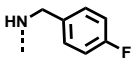
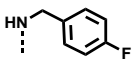


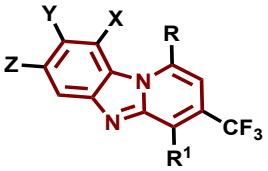

| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **9** | H | H | H | CN |  | 100 | 100 | 1.05 |
| **10** | H | H | H | CN |  | 100 | 100 | 1.65 |
| **11** | H | H | H | CN |  | 96 | 63 | |
| **12** | H | Cl | Cl | CN |  | 100 | 100 | 1.49 |
| **13** | H | H | H | CN |  | 26 | | |
| **14** | H | H | H | CN |  | 100 | 100 | 1.38 |
| **15** | H | H | H | CN |  | 30 | | |
| **16** | H | H | H | CN |  | 91 | 92 | 2.01 |
| **17** | H | H | H | CN |  | 17 | | |

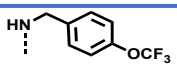
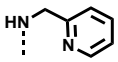
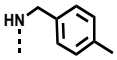
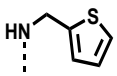
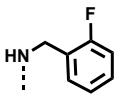
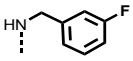
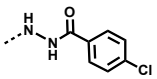
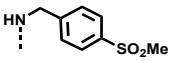


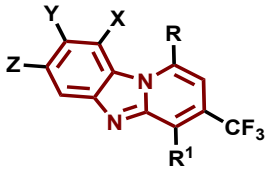

| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **18** | H | H | H | CN |  | 22 | | |
| **19** | H | H | H | CN |  | 4 | | |
| **20** | H | H | H | CN |  | 9 | 11 | |
| **21** | H | H | H | CN |  | 0 | 0 | |
| **22** | H | H | H | CN |  | 4 | | |
| **23** | H | H | H | CN |  | 9 | | |
| **24** | H | H | H | CN |  | 13 | | |
| **25** | H | H | H | CN |  | 48 | | |
| **26** | H | H | H | CN |  | 30 | | |
| **27** | H | H | H | CN |  | 4 | | |
| **28** | H | H | H | CN |  | 30 | | |

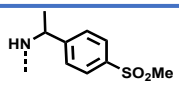
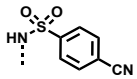
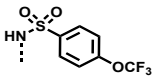
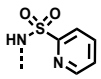
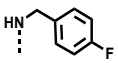
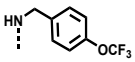
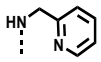



| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **29** | H | H | H | CN |  | 9 | | |
| **30** | H | H | H | CN |  | 4 | | |
| **31** | H | H | H | CN |  | 0 | 0 | |
| **32** | H | H | H | CN |  | 17 | | |
| **33** | H | H | H | CN |  | 26 | | |
| **34** | H | H | H | CN |  | 22 | | |
| **35** | H | H | H | CN |  | 26 | | |
| **36** | H | H | H | CN |  | 26 | | |
| **37** | Cl | H | Cl | CN |  | 100 | 100 | 0.770 |
| **38** | H | Cl | Cl | CN |  | 100 | 98 | 1.69 |




| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **39** | H | Cl | Cl | CN |  | 100 | 100 | 1.58 |
| **40** | H | Cl | Cl | CN |  | 100 | 99 | 1.74 |
| **41** | H | Cl | Cl | CN |  | 100 | 100 | 1.17 |
| **42** | H | Cl | Cl | CN |  | 100 | 100 | 0.470 |
| **43** | H | Cl | Cl | CN |  | 100 | 100 | 0.430 |
| **44** | H | Cl | Cl | CN |  | 100 | 100 | 0.380 |
| **45** | H | Cl | Cl | CN |  | 100 | 100 | 0.400 |
| **46** | H | H | H | CN |  | 89 | 29 | |
| **47** | H | H | H | CN |  | 9 | | |
| **48** | H | Cl | Cl | CN |  | 23 | 66 | |




| Compound Identity | X | Y | Z | $R^1$ | R | % *S. mansoni* mortality at 10 μM | | Adult *S. mansoni* |
|---|---|---|---|---|---|---|---|---|
| | | | | | | NTS | Adult | $IC_{50}$ (μM) |
| **49** | H | Cl | Cl | CN |  | 100 | 100 | 0.970 |
| **50** | H | Cl | Cl | CN |  | 44 | 84 | 1.11 |
| **51** | H | Cl | Cl | CN |  | 93 | 87 | 1.09 |
| **52** | H | Cl | Cl | CN |  | 9 | | |
| **53** | H | H | H |  |  | 4 | 34 | |
| **54** | H | H | H |  |  | 21 | 53 | |
| **55** | H | H | H |  |  | 8 | 15 | |
| Praziquantel | | | | | | | | 0.100 |

Values are a mean of n≥2 determinations. Generally, $IC_{50}$ values from individual experiments differed less than 2-fold; values that differed more than 2-fold were excluded from analysis.

**SAR plan and analysis**. The SAR program pursued resulted in compounds varying in the type of linkage between the side group and the PBI core by either being connected directly or via a

methylene group (or its isosteres) as depicted in **Figure 2**. Other alterations on the side group included the introduction of chirality at the methylene linker via installation of methyl substitutions. Similar substitutions were introduced on the nitrogen attached to the PBI core ($\mathbf{R_1}$, **Figure 2**) to make some analogues with a tertiary nitrogen. Additionally, small lipophilic halogen groups (**R**, **Figure 2**) were introduced on the left-hand side (LHS) of the PBI core and other small groups were introduced to replace the nitrile at $\mathbf{R_3}$ (**Figure 2**).

Overall, 19 compounds produced over >70% activity against NTS and adult worms at a concentration of 10 μM. Out of these, 18 compounds achieved an $IC_{50}$ of ≈2 μM and below (as low as 0.21 μM), similar to praziquantel, characterized by an $IC_{50}$ of 0.10 μM against adult worms. In the sub-series where the aromatic side group is directly linked to the PBI core by a nitrogen and with no changes on neither the LHS nor $\mathbf{R_1}$ (compounds **1 - 16**), the analogues **6**, **9**, **10**, **12**, **14** and **16** were most active with **6** ($IC_{50}$ = 0.21 μM), bearing a lipophilic electron withdrawing trifluoromethoxy (-$OCF_3$) group, being the most potent compound in this category. On the contrary, compounds with hydrophilic groups on the aromatic side group (**R**, **Table 1**) abrogated activity as seen, for example, with **3**, **4** and **5** which showed low activity against NTS at 10 μM. Additional compounds with good antischistosomal activity were identified among compounds incorporating heteroaromatic side groups instead of a phenyl moiety as exemplified by pyrimidyl and pyrazinyl groups in **14** ($IC_{50}$ = 1.38 μM) and **16** ($IC_{50}$ = 2.01 μM), respectively. The positioning of the nitrogen atoms in the aromatic side group seem crucial for antischistosomal activity since **15**, in which the nitrogen atoms have a 2,6 relationship, showed poor activity towards NTS at 10 μM compared to its regio-isomer **16** (NTS, 91% at 10 μM; adult worm $IC_{50}$=2.01 μM) where the nitrogen atoms occur in a 2,5 relationship.

The next SAR analysis concerns a series of analogues comprising the aromatic side group attached to the PBI core via a methylamine bridge (**17 - 45, 48** and **49**, **Table 1**). In the absence of modifications elsewhere in the scaffold, these compounds displayed poor antischistosomal activity. Most of these compounds were weakly active against NTS at the screening concentration of 10 μM rendering them ineligible for additional screening in adult worms according to the workflow adopted in the assay. For example, the potent activity observed with **6** ($IC_{50}$ = 0.21 μM) is lost in its methylene-linked analogue **23**. A similar pattern of inactivity is noticed with **28**, the analogue of the active aniline-type series compound **16** ($IC_{50}$ = 2.01 μM).

Notably, when the methylene linker in **21** (0% NTS and adult; 10 μM) was substituted by an amino group to produce **46**, better activity in both the immature and adult worms was observed. Other side groups such as the more polar hydrazide found in **47** were detrimental to activity (9% NTS, 10 μM). Subsequent SAR iterations involved introducing changes on the LHS of the PBI core through installation of chloro- substitutions. Interestingly, for target compounds in the benzylamine series which were previously inactive, this modification seemed to restore their activity. For example, **19** has poor activity with only 4% reduction against the larval stage at 10 μM. In contrast, the analogues **37** and **38** (**Table 1**) with a dichloro substitution pattern on the phenyl portion of the PBI scaffold have potent activity at 10 μM against both juvenile and adult worms (98-100% mortality) with promising adult worm $IC_{50}$ values of 0.77 μM and 1.69 μM, respectively. Consistent observations are seen with **31** vs **41**; **23** vs **39** and **18** vs **40** in which enhanced mortality is observed with the di-chlorinated analogues, in both NTS and adult worms at 10 μM.

With regards to regio-isomerism in the benzylamine side group, **38** ($IC_{50}$ = 1.69 μM), was four times less potent compared to its *ortho*- [**44** ($IC_{50}$ = 0.38 μM)] and *meta*- [**45** ($IC_{50}$ = 0.40 μM)]

analogues. On the other hand, incorporating a methyl substitution at the benzylic position of **48** (23% NTS and 66% adult) resulted in the improvement of potency of the analogous compound **49** (100%, NTS and adult) at 10 μM. It may be postulated that the methyl group offers additional binding interactions or influences compound orientation for interactions with biological targets in the parasite.

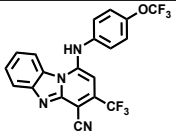
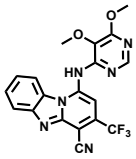
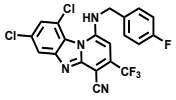
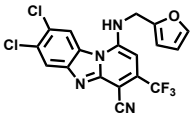
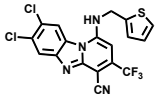
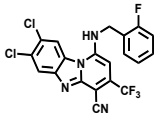
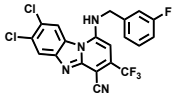
When the methylene linker in the side group **R** was replaced with a sulfonyl group, there were mixed observations since activity was lost with **52** (9% NTS, 10 μM), an analogue of **40** (100% NTS, 99% adult at 10 μM and adult worm $IC_{50}$ = 1.74 μM) whereas similar changes conserved the activity of **51** (93% NTS, 87% adult at 10 μM and adult $IC_{50}$ = 1.09 μM), the analogue of **39** (100% NTS and adult; 10 μM; $IC_{50}$ = 1.58 μM).

Towards interrogating the relevance of the nitrile substitution on the pyridyl portion of the PBI scaffold, some compounds were synthesised wherein this group was replaced by other small groups. Of note, this modification led to a decrease in antischistosomal activity as seen with **7** and **8**, the analogues of active compound **6** which contain a hydrogen and a methyl ester, respectively, instead of a nitrile. Other compounds (**53 - 55**) in which the nitrile was replaced with an amide did not produce potent activity (**Table 1)**. Taken together, these results suggest that the nitrile at this position is important for antischistosomal activity.

***In vitro* hepatic microsomal metabolic stability and cytotoxicity evaluation**. To gain insight into the anticipated pharmacokinetic behaviour, selective toxicity and to prioritise compounds for *in vivo* efficacy studies, *in vitro* metabolic stability and cytotoxicity experiments were conducted on prioritised compounds based on *in vitro* potency**.** Metabolic stability was carried out using mouse and human liver microsomes whereas cytotoxicity was evaluated using the Chinese Hamster Ovarian cell lines. The percentage of compound remaining after 30 minutes incubation

was determined and the projected half-life calculated as previously described.[14] The data from these assays is presented in **Table 2.**

**Table 2**. Physicochemical, ***in vitro*** **microsomal metabolic stability and cytotoxicity profiles.**

| Compound Code | Chemical Structure | Log D | Solubility | % rem. after 30 min ($t_{½\ min}$)[a] MLM | HLM | [a]CHO $IC_{50}$ (μM) | [b]Selectivity Index |
|---|---|---|---|---|---|---|---|
| **6** |  | 5.34 | <5 | >99 (>150) | >99 (>150) | 17.58 | 84 |
| **14** |  | 3.91 | 6.4 | 28 (17) | 69 (57) | 23.99 | 17 |
| **37** |  | 4.29 | <5 | 76 (75) | 82 (105) | 1.66 | 2 |
| **42** |  | 3.77 | <5 | 66 (50) | 100 (>150) | 4.84 | 10 |
| **43** |  | 4.13 | <5 | | | 8.48 | 20 |
| **44** |  | 4.16 | <5 | 84 (119) | 97 (>150) | 4.30 | 11 |
| **45** |  | 4.11 | 5 | 84 (119) | 100 (>150) | 7.03 | 18 |
| | Midazolam | | | 1.3 | 0.1 | | |
| | MMV390048 | | | 94 | 93 | | |
| | Emetine | | | | | 0.095 | |

LogD: logarithm of distribution co-efficient, computed using StarDrop™ version 4.0. Solubility was determined as previously described[15]. MLM: Mouse liver microsomes; HLM: Human liver microsomes; $t_{½}$: projected half-life in minutes; CHO: Chinese hamster ovarian cell lines. [a]Value is a mean of n≥2 independent determinations; [b]Selectivity Index being the ration of $IC_{50}$ CHO: $IC_{50}$ adult *S. mansoni.*

Overall, the most stable compounds were **6**, **44** and **45** which contain lipophilic electron-withdrawing substituents. For these compounds, over 80% of the compound was remaining after

incubating for 30 minutes with an estimated half-life of over 100 minutes in both MLM and HLM. Introducing heteroatoms in the side chain decreased metabolic stability as evident with **14**. Species variation in the metabolic stability of this series was evident. Metabolism was more efficient in MLM for **14**, **42, 44** and **45** whereas compound **6** displayed equal stability in both species.

Most of the compounds had a good cytotoxicity profile (SI>10) with only **37**, with a selectivity index of 2, showing high potential for causing cytotoxicity. Evidently, although the introduction of chloro substitutions on the LHS of the molecules improved their antischistosomal potency, this change also increased their risk for inducing cytotoxicity as reflected in their reduced selectivity indices of most compounds bearing the LHS modifications (**Table 2**).

***In vivo* oral efficacy.** Compounds were selected for *in vivo* proof-of-concept studies, using the oral route of administration, in a mouse model of schistosomiasis based on their *in vitro* potency and acceptable cytotoxicity profile. *In vivo* potency was measured based on the percent worm burden reduction achieved in infected and treated NMRI mice and comparing with infected but untreated mice which served as controls. The results are shown in **Table 3**.

**Table 3**. ***In vivo* antischistosomal oral efficacy for prioritised compounds.[a]**

| Compound tested | Number of mice cured/investigated | Mean number of worms (SD) | Per cent worm burden reduction |
|---|---|---|---|
| **Control 1** | 0/8 | 41.0 (16.2) | |
| **6** | 0/2* | 15.5 (0.7) | 62.2 |

| | | | |
|---|---|---|---|
| **14** | 0/4 | 52.3 (45.2) | 0 |
| **16** | 0/4 | 25.3 (3.3) | 38.8 |
| **Control 2** | 0/8 | 47.0 (36.2) | -- |
| **42** | 0/4 | 32.0 (19.8) | 31.9 |
| **43** | 0/5 | 27.6 (11.1) | 41.3 |
| **44** | 0/4 | 14.5 (2.5) | 69.1 |
| **45** | 0/5 | 15.4 (3.8) | 67.3 |
| **Praziquantel** | | | 94.1[16] |

[a]Compounds were dissolved in 7% Tween 80 and 3% ethanol in water (v/v/v) prior to dosing. SD: standard deviation *One mouse was not infected and excluded from analysis, and one mouse died prematurely.

Of the compounds tested, **14** failed to induce any *in vivo* effect, **16**, **42** and **43** produced slight (30-42%) reduction in worm burden while moderate (>50%) reduction (yet statistically not significant $p > 0.05$) in worms was realised with **6**, **44** and **45**. It is notable that compounds bearing heteroaromatic side chains (**14**, **16**, **42** and **43**) generally produced lower *in vivo* efficacy. Expectedly, there was a trend towards poor activity for compounds that had shown low metabolic stability and higher activity for those with better metabolic stability. Biotransformation of these compounds to metabolites with decreased or no antischistosomal activity is likely. Furthermore, it is also probable that physicochemical hurdles are responsible, at least in part, for the low or lack of *in vivo* activity of some compounds such as **14**, is likely poorly absorbed, a hypothesis supported by a negative human intestinal absorption (HIA) index score predicted by StarDrop™[17,18] software; all other tested compounds were predicted to have a positive HIA score (**Table S1**,

*Supporting Information*). Moreover, suboptimal bioavailability may be the reason for the lower efficacy of otherwise *in vitro* potent compounds with outstanding metabolic stability, such as **6**, **44** and **45**. Pharmacokinetic profiling was undertaken using compound **44** to understand its *in vivo* disposition.

***In vivo* pharmacokinetic studies**. The oral disposition of **44** was studied in mice to enable investigation of its PK profile. In this regard, C57B1/6 mice (n=3) were dosed orally at 20 mg/kg while in another set, the animals (n=2 mice) were dosed intra-venously at 2 mg/kg. Blood was sampled between 0-24 hours at the intervals shown in **Figure 3**. Corresponding PK parameters were calculated using non-compartmental analysis and are presented in **Table 4**.

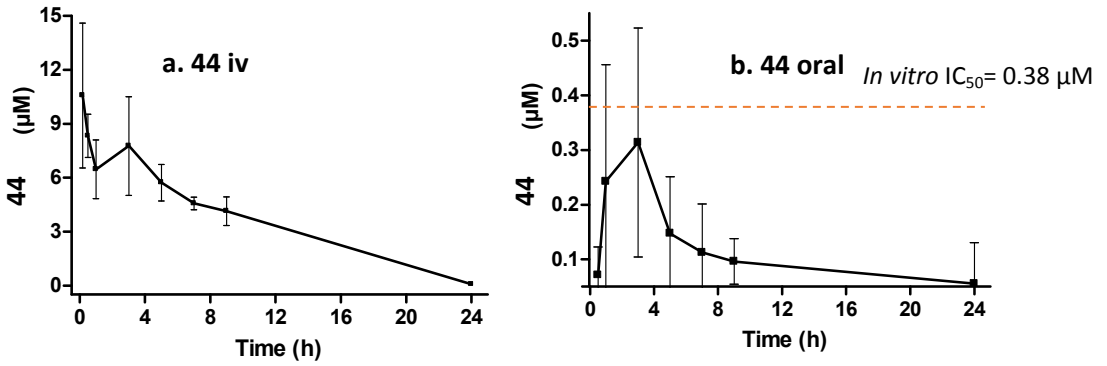
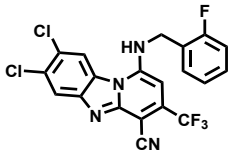



**Figure 3.** Pharmacokinetics profile for **44** after a) intravenous and b) oral administration. Each data point represents the mean from 2 (iv) and 3 (oral) mice with error bars indicating the standard deviation.

**Table 4. Pharmacokinetic parameters for 44 in mice.**



| Parameter | i.v. (2mg/kg) [a] | p.o. (20mg/kg) [b] |
|---|---|---|
| $C_{max}$ (μM) | - | 0.3 |
| $T_{max}$ (h) | - | 3 |
| $t_{1/2}$ (h) | 2.6 | 0.3 |
| $V_d$ (L/kg) | 0.18 | - |
| CL (mL/min/kg) | 0.81 | - |
| $AUC_{0-\infty}$ (μM/L.min) | 5426 | 138 |
| Oral bioavailability (%) | - | <1% |

[a]For intravenous dosing (n=2 mice), compounds were formulated in a solution of dimethylacetamide, polyethylene glycol and propylene glycol/ethanol mixture 4:1 at a ratio 1:3:6. [b]For oral dosing (n=3 mice), compounds were formulated as suspension in 100% HPMC.

Compound **44** attained peak plasma concentrations ($C_{max}$ = 0.3 μM) 3h post oral administration, displayed a short half-life ($t_{½}$ = 0.3h), slow clearance (CL = 0.81 mL/min/kg) and low volume of distribution (Vd = 0.18 L/kg) with low bioavailability (F < 1%). Solubility-limited absorption, as further suggested by the delayed peaking of plasma compound concentrations, is a potential phenomenon contributing to the suboptimal efficacy of these compounds. It is feasible that at the 400 mg/kg oral dose used in efficacy studies, saturation of absorption and compound precipitation occurs thereby limiting systemic exposure. Of note, the 400 mg/kg dose is the efficacious dose for praziquantel[16] and therefore routinely used in the *S. mansoni* mouse model, however it might be worthwhile to test lower doses of this compound series. On the other hand, improved bioavailability through modulation of solubility has the potential to enhance the *in vivo* efficacy of this series.

**Conclusion:** Antischistosomal structure-activity relationship studies have been conducted around a 3-trifluoromethyl PBI core using N-aryl appendages with SAR trends as summarised in **Figure 4.**

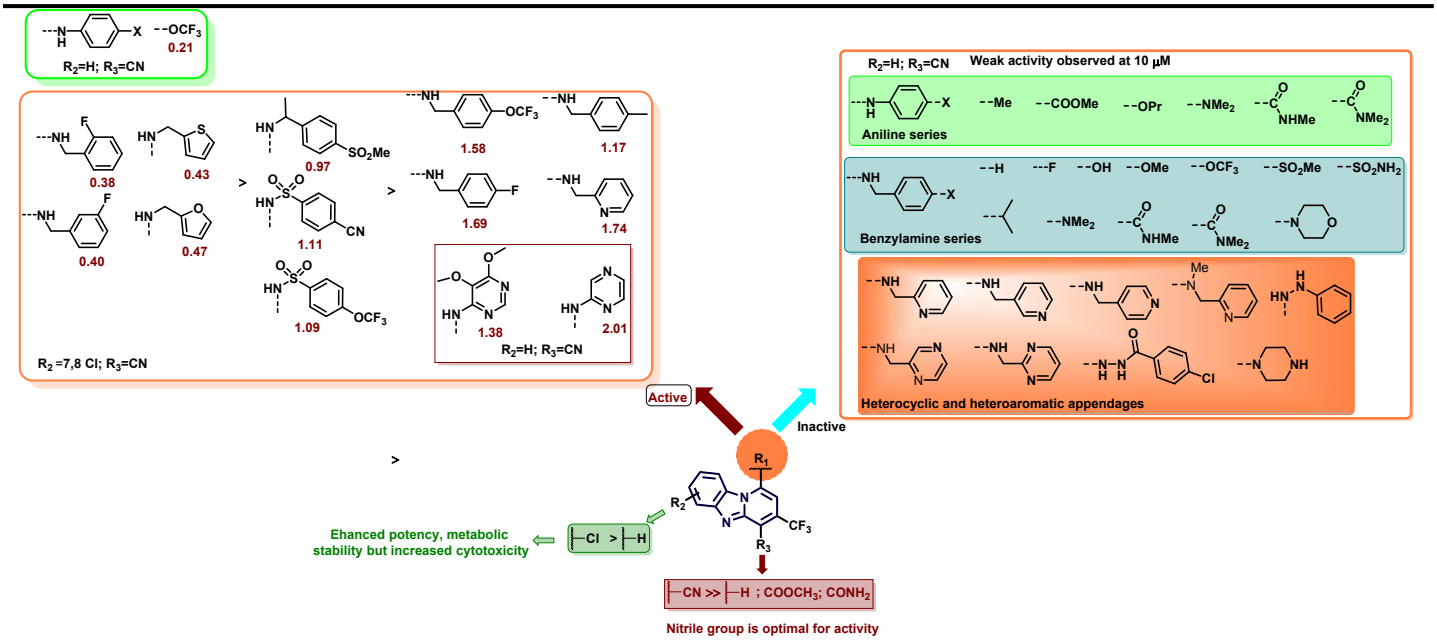



**Figure 4**: Observed antischistosomal SAR trends. Values are adult worm $IC_{50}$ in µM.

Generally, electron-withdrawing groups as substituents on the aromatic appendage favoured antiparasitic activity. The aniline series generally provided more active compounds in the absence of changes on the LHS, compared to the benzylamine series. It was notable that changes on the LHS of the PBI scaffold restored the activity of previously inactive benzylamine series analogues. The changes explored on the LHS, however, diminished the selectivity of these compounds while changing the nitrile on the pyridyl portion of the PBI core was detrimental to antischistosomal activity. Evaluation of the *in vitro* microsomal metabolic stability indicated that heteroatoms in the structures of target compounds rendered them more susceptible to metabolism. *In vivo* efficacy

studies conducted on selected compounds produced good antischistosomal activities with **6**, **44** and **45** which effected 62-69% worm burden reduction. Pharmacokinetics hurdles, especially solubility-limited absorption was identified as likely a contributing factor for the sub-optimal potency achieved by the lead compounds as was microsomal metabolic instability. Overall, this work shows that, when duly optimized, N-aryl 3-trifluoromethyl PBI analogues are promising leads for antischistosomal drug discovery.

**Experimental procedures**. All commercially available chemicals were purchased from either Sigma-Aldrich or Combi-Blocks. All solvents were dried by appropriate techniques. Unless otherwise stated, all solvents used were anhydrous. $^{1}$H NMR spectra were recorded at 300 or 400 MHz and $^{13}$C NMR spectra at 100 or 151 MHz, on a Brucker Spectrometer. Analytical thin-layer chromatography (TLC) was performed on aluminium-backed silica-gel 60 $F_{254}$ (70-230 mesh) plates. Column chromatography was performed with Merck silica-gel 60 (70-230 mesh). Chemical shifts (δ) are given in ppm downfield from TMS as the internal standard. Coupling constants, *J*, are recorded in Hertz (Hz). Purity was determined by HPLC and all compounds were confirmed to have > 95% purity. The data that is not shown below is supplied in the Supporting Information (Section A).

**General procedures for the synthesis of compounds 1-4, 6, 7, 9 - 13, 17 - 25, 27 - 35, 37 – 45, 48, 49, 53 - 55: Method A**. The appropriate amine (1.2 to 2 equiv.) was added to a stirred mixture of the relevant chloro intermediate **IV** (1.015mmol, 1 equiv.), triethylamine (2.03mmol, 2 equiv.) and tetrahydrofuran (THF) (4mL) and subjected to microwave irradiation at 150W, 80°C for 20-40 minutes. The cooled reaction mixture was transferred to a round bottom flask and concentrated. A minimum amount of either acetone or ethanol was added to precipitate the final product which

was filtered off and dried. Occasionally, recrystallization in ethanol or column chromatography was performed to improve purity.

*1-((4-(trifluoromethoxy)phenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **6***. Obtained from **IVa** (0.3g, 1.015mmol, 1 equiv.) using 4- (trifluoromethoxy)aniline (0.36g, 2.03mmol, 2 equiv.). Yield: 0.071 g, 16%, as a light green fluffy powder. $^{1}$H NMR (400 MHz, DMSO) δ 8.82 (d, *J* = 8.1 Hz, 1H), 7.69 (d, *J* = 8.0 Hz, 1H), 7.64 – 7.54 (m, 1H), 7.46 – 7.42 (m, 1H), 7.39 (d, *J* = 8.3 Hz, 2H), 7.20 (d, *J* = 8.4 Hz, 2H), 6.24 (s, 1H). $^{13}$C NMR (151 MHz, DMSO) δ 148.53, 144.88, 135.23, 133.74, 128.91, 127.21, 125.36, 123.71, 122.92, 122.71 (2C), 121.81, 121.58, 120.05, 119.68, 117.77 (2C), 115.20, 113.38, 95.93. LC-MS APCI+ *m/z* calcd for $C_{20}H_{10}F_6N_4O$:436.08; found, 437.10 $[M+H]^+$. ). HPLC purity: 99%

*1-((3-(trifluoromethoxy)phenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **9.*** Obtained from **IVa** (0.1g, 0.338 mmol, 1 equiv.) using 3-(trifluoromethoxy) aniline (0.072g, 0.406 mmol, 1.2 equiv.). Yield: 0.072g, 10%, as a yellow solid. $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ 8.81 (d, J = 8.3 Hz, 1H), 7.58 (m, 3H), 7.42 (t, J = 7.8 Hz, 1H), 7.19 – 6.96 (m, 3H), 6.23 (s, 1H). $^{13}$C NMR (101 MHz, DMSO) δ 149.82, 148.61, 148.38, 135.72, 133.54, 131.74, 128.58, 127.31, 124.01, 123.11, 121.86, 121.27 (2C), 119.32, 117.88, 115.96, 115.17, 114.61, 112.64, 96.61. MS (EI+) m/z calcd for $C_{20}H_{10}F_6N_4O$:436.08; found, 437.10 (M + 1). HPLC purity: 98%.

*1-((2-(trifluoromethoxy)phenyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile **10***. Obtained from **IVa** (0.1g, 0.338 mmol, 1 equiv.) using 2-(trifluoromethoxy) aniline (0.072g, 0.406 mmol, 1.2 equiv.). Yield: 0.043 g, 29%, as a yellow solid.$^{1}$H NMR (300 MHz, DMSO-$d_6$) δ 8.87 (d, J = 8.3 Hz, 1H), 7.75 – 7.52 (m, 2H), 7.52 – 7.34 (m, 3H), 7.22 (m, 2H), 6.10 (s, 1H). $^{13}$C NMR (101 MHz, DMSO) δ 148.65, 141.74, 140.85, 135.03, 134.72, 133.29,

129.07, 128.59, 127.43, 124.84, 123.90, 123.24, 122.01, 121.27, 119.47, 117.73, 115.18, 112.16, 100.00, 97.15. MS (EI+) m/z calcd for $C_{20}H_{10}F_6N_4O$:436.08; found, 437.10 (M + 1). HPLC purity: >99%.

*7,9-dichloro-1-((4-fluorobenzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***37***. Obtained from **IVb** (0.1g, 0.274mmol, 1 equiv.) and (4-fluorophenyl)methanamine (0.041g, 0.329mmol, 1.2 equiv.). Yield: 0.107 g, 86%, as a yellow solid. $^1$H NMR (300 MHz, DMSO-d6) δ 8.82 (d, J = 4.1 Hz, 1H), 8.70 (s, 1H), 7.81 (d, J = 6.8 Hz, 1H), 7.56 (dd, J = 8.5, 5.5 Hz, 2H), 7.20 (t, J = 8.9 Hz, 2H), 6.32 (s, 1H), 4.89 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 163.13, 160.71, 150.15 (2C), 141.56, 137.93, 133.95, 129.83, 129.50 (2C), 126.22, 125.36, 123.06, 121.27, 115.87 (2C), 114.78, 112.78, 88.01, 46.85. MS (EI+) m/z calcd for $C_{20}H_{10}C_{l2}F_4N_4$:452.02; found, 453.00 (M + 1). HPLC purity: 99%.

*7,8-dichloro-1-((4-fluorobenzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***38***. Obtained from **IVc** (0.163g, 0.448mmol, 1 equiv.) and (4-fluorophenyl)methanamine (0.0618g, 0.493mmol, 1.2 equiv.). Yield: 0.045 g, 22%, as a yellow solid. $^1$H NMR (300 MHz, DMSO-d6) δ 9.03 (s, 1H), 7.69 (s, 1H), 7.52 (dd, *J* = 8.6, 5.6 Hz, 2H), 7.43 (dd, *J* = 8.4, 5.6 Hz, 2H), 6.00 (s, 1H), 4.59 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 162.97, 160.18, 154.24, 150.99, 145.67, 139.02, 135.82, 134.57, 130.64, 129.58, 125.88, 119.90, 117.50, 116.55, 115.63, 115.57, 115.18, 100.00, 88.62, 43.67. MS (EI+) m/z calcd for $C_{20}H_{10}Cl_2F_4N_4$:452.02; found, 453.00 (M + 1). HPLC purity: 97%.

*7,8-dichloro-1-((4-(trifluoromethoxy)benzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***39***. Obtained from **IVc** (0.15g, 0.411mmol, 1 equiv.) and (4-(trifluoromethoxy)phenyl)methanamine (0.094g, 0.493mmol, 1.2 equiv.). Yield: 0.053 g, 25%, as a brown solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 9.04 (s, 1H), 7.70 (s, 1H), 7.59 (dd, *J* = 14.0, 8.6

Hz, 2H), 7.38 (t, *J* = 8.8 Hz, 2H), 6.01 (s, 1H), 4.64 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 151.42, 148.67, 148.48, 137.28, 134.58, 132.48, 130.43, 129.51, 128.51, 127.00 (2C), 124.13, 121.48 (2C), 119.22, 116.56, 115.75, 114.49, 112.94, 86.73, 51.67. MS (EI+) m/z calcd for $C_{21}H_{10}Cl_2F_6N_4O$:518.01; found, 519.00 (M + 1). HPLC purity: 99%.

*7,8-dichloro-1-((pyridin-2-ylmethyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4- carbonitrile* ***40***. Obtained from **IVc** (0.15g, 0.411mmol, 1 equiv.) and (pyridin-2-ylmethanamine (0.0534g, 0.493mmol, 1.2 equiv.). Yield: 0.070 g, 39%, as a brown solid. $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ 9.01 (s, 1H), 8.66 – 8.52 (m, 1H), 8.10 (s, 1H), 7.83 (td, *J* = 7.6, 1.8 Hz, 1H), 7.58 (d, *J* = 7.9 Hz), 7.42 – 7.26 (m, 1H), 6.39 (s, 1H), 4.94 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 150.22, 149.36 (2C), 145.21, 137.52 (2C), 129.15, 128.31, 127.64, 123.18, 122.26 (2C), 119.39, 118.56, 117.09, 116.96, 115.08, 88.03, 49.95. MS (EI+) m/z calcd for $C_{19}H_{10}Cl_2F_3N_5$:435.03; found, 436.00 (M + 1). HPLC purity: 97%.

*7,8-dichloro-1-((4-methylbenzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***41***. Obtained from **IVc** (0.15g, 0.411mmol, 1 equiv.) and *p*-tolylmethanamine (0.058g, 0.494mmol, 1.2 equiv.). Yield: 0.092 g, 50%, as a brown powder. $^{1}$H NMR (300 MHz, DMSO-$d_6$) δ 9.06 (s, 1H), 7.74 (s, 1H), 7.35 (d, *J* = 8.1 Hz, 2H), 7.20 (d, *J* = 7.9 Hz, 2H), 6.03 (s, 1H), 4.59 (s, 2H), 2.31 (s, 3H). $^{13}$C NMR (101 MHz, DMSO) δ 160.73, 159.00, 155.06, 146.31, 135.62, 134.93, 129.58, 129.21 (2C), 127.62 (2C), 126.38, 121.69, 119.39, 117.52, 116.80, 115.61, 114.83, 88.39, 42.88, 21.08. MS (EI+) m/z calcd for $C_{21}H_{13}Cl_2F_3N_4$:448.05; found, 449.00 (M + 1). HPLC purity: 99%.

*7,8-dichloro-1-((furan-2-ylmethyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4- carbonitrile* ***42***. Obtained from **IVc** (0.15g, 0.411mmol, 1 equiv.) and furan-2- ylmethanamine (0.048g, 0.494mmol, 1.2 equiv.). Yield: 0.098 g, 56%, as a brown solid. $^{1}$H NMR (300 MHz,

DMSO-$d_6$) δ 9.00 (s, 1H), 7.90 (s, 1H), 7.63 (dd, $J$ = 1.8, 0.9 Hz, 1H), 6.43 (m, 2H), 6.27 (s, 1H), 4.72 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 155.27, 153.52, 150.99, 145.30, 142.96, 142.30, 134.97, 130.77, 128.78, 123.76, 117.67, 117.45, 114.20, 110.89, 107.15, 94.33, 93.02, 43.95. MS (EI+) m/z calcd for $C_{18}H_9Cl_2F_3N_4$:424.01; found, 425.00 (M + 1). HPLC purity: 99%.

*7,8-dichloro-1-((thiophen-2-ylmethyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine- 4-carbonitrile* ***43***. Obtained from **IVc** (0.15g, 0.411mmol, 1 equiv.) and thiophen-2-ylmethanamine (0.056g, 0.494mmol, 1.2 equiv.). Yield: 0.051 g, 28%, as a brown solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 9.16 (s, 1H), 7.77 (s, 1H), 7.42 (dd, $J$ = 5.0, 1.3 Hz, 1H), 7.11 – 7.05 (m, 1H), 7.02 (dd, $J$ = 5.0, 3.4 Hz, 1H), 6.05 (s, 1H), 4.80 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 150.91, 145.53, 138.51, 130.23, 129.21, 128.19, 127.02 (2C), 124.34 (2C), 123.52, 123.23, 117.75 (2C), 116.95 (2C), 88.34, 47.38. MS (EI+) m/z calcd for $C_{18}H_9Cl_2F_3N_4S$:439.99; found, 441.00 (M + 1). HPLC purity: 98%.

*7,8-dichloro-1-((2-fluorobenzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***44***. Obtained from **IVc** (0.1g, 0.274mmol, 1 equiv.) and (2-fluorophenyl)methanamine (0.069g, 0.548mmol, 2 equiv.). Yield: 0.072 g, 58%, as an orange solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 9.00 (s, 1H), 7.94 (s, 1H), 7.59 (t, $J$ = 7.5 Hz), 7.40 – 7.12 (m, 3H), 6.22 (s, 1H), 4.79 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 161.91, 159.49, 152.40, 150.75, 145.34, 136.58, 132.84, 131.42, 129.92, 129.22, 126.94, 127.87, 124.84, 121.78, 118.14, 117.37 (2C), 116.51, 115.46, 87.88. MS (EI+) m/z calcd for $C_{20}H_{10}Cl_2F_4N_4$:452.02; found, 453.00 (M + 1). HPLC purity: 98%.

*7,8-dichloro-1-((3-fluorobenzyl)amino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***45***. Obtained from **IVc** (0.1g, 0.274mmol, 1 equiv.) and (3-fluorophenyl)methanamine (0.069g, 0.548mmol, 2 equiv.). Yield: 0.046 g, 37%, as a yellow solid.

$^1$H NMR (300 MHz, DMSO-$d_6$) δ 9.05 (s, 1H), 8.69 (bs, 1H), 8.20 (s, 1H), 7.51 – 7.29 (m, 3H), 7.18 – 7.06 (m, 1H), 6.32 (s, 1H), 4.96 (s, 2H). $^{13}$C NMR (101 MHz, DMSO) δ 161.91, 159.49, 152.40, 150.75, 145.34, 136.58, 131.42, 129.96, 129.22, 127.87, 125.10, 124.70, 121.78, 118.14, 117.37(2C), 116.51, 115.65, 115.46, 87.83. MS (EI+) m/z calcd for $C_{20}H_{10}Cl_2F_4N_4$:452.02; found, 453.00 (M + 1). HPLC purity: 97%.

**General procedures for the synthesis of compounds 5**, **8**, **14 - 16, 26, 36, 46, 47, 50 - 52**: **Method B**. A mixture of the relevant chloro intermediate **IV**, the appropriate amine (1.2 equiv.), Tris(dibenzylideneacetone) dipalladium(0), $Pd_2(dba)_3$ (0.1 equiv.), 2,2'-Bis(diphenylphosphino)-1,1'-binaphthyl (BINAP) (0.06 equiv.) or BrettPhos (0.04 equiv.) or RuPhos (0.1 equiv.), Potassium carbonate, $K_2CO_3$ or Caesium Carbonate, $Cs_2CO_3$ (3 equiv.) and toluene:tert-butanol (1:1; 5ml) or 1,4-dioxane (5mL) were stirred in a sealed tube at 100-120˚C for 4-17 hours. The cooled reaction mixture was stirred in ethylacetate (50ml) and water (100ml) for 10-20 minutes. The separated organic layer was washed with water (2X50ml), followed by saturated NaCl (2X50ml), dried over magnesium sulphate and filtered over celite. The organic fraction was concentrated under reduced pressure and ethanol used to precipitate the crude product which was filtered to furnish the final product. In some cases, compounds were purified by recrystallization from ethanol or by column chromatography.

*1-((5,6-dimethoxypyrimidin-4-yl)amino)-3-(trifluoromethyl) benzo[4,5] imidazo[1,2-a]pyridine-4- carbonitrile **14***. Obtained from **IVa** (0.3g, 1.015mmol, 1 equiv.) using 5,6-dimethoxypyrimidin-4-amine (0.189g, 1.218mmol, 1.2 equiv.). Yield: 0.071 g, 17%, as a yellow fluffy powder.$^1$H NMR (300 MHz, DMSO) δ 9.08 (d, *J* = 10.1 Hz, 1H), 8.36 (s, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 7.67 – 7.59 (m, 1H), 7.52 – 7.43 (m, 1H), 7.40 (s, 1H), 4.00 (s, 3H), 3.80 (s, 3H). $^{13}$C NMR (101 MHz, DMSO) δ 163.29, 162.70, 151.38, 147.54, 141.49, 135.42, 135.09, 128.73,

127.71(2C), 124.15, 123.12 (2C), 121.42, 118.52, 114.91, 100.05, 60.64, 54.00. LC-MS APCI+: found *m/z* = 415.10 $[M+H]^+$, (calculated for: $C_{19}H_{13}F_3N_6O_2$:414.11). HPLC purity: 98%

*1-(pyrazin-2-ylamino)-3-(trifluoromethyl)benzo[4,5]imidazo[1,2-a]pyridine-4-carbonitrile* ***16***. Obtained from **IVa** (0.15g, 0.51mmol, 1 equiv.) using pyrazin-2-amine (0.058g, 0.61mmol, 1.2 equiv.). Yield: 0.041 g, 23%, as an orange powder.$^1$H NMR (400 MHz, DMSO) δ 9.08 (d, *J* = 8.4 Hz, 1H), 8.54 (d, *J* = 1.4 Hz, 1H), 8.41 (dd, *J* = 2.7, 1.4 Hz, 1H), 8.25 (d, *J* = 2.7 Hz, 1H), 7.69 (ddd, *J* = 8.1, 1.2, 0.7 Hz, 1H), 7.63 (ddd, *J* = 8.2, 7.3, 1.1 Hz, 1H), 7.57 (s, 1H), 7.47 (ddd, *J* = 8.5, 7.3, 1.3 Hz, 1H). $^{13}$C NMR (101 MHz, DMSO) δ 161.20, 156.43, 149.86, 147.99, 147.30, 142.50 (2C), 138.59, 135.58, 128.83, 127.54, 123.16, 118.91, 118.58, 114.80, 112.86, 99.37. LC-MS APCI+: found *m/z* = 355.10 $[M+H]^+$, (calculated for: $C_{17}H_9F_3N_6$:354.08). HPLC purity:98%

## MATERIALS AND METHODS

***In vitro*** **antischistosomal assays.** ***Screening on Newly Transformed Schistosomula***. *S. mansoni* cercariae were mechanically trans formed to newly transformed schistosomula as described elsewhere.[19] Briefly, snails were placed under light to stimulate cercarial shedding, and cercarial suspension was collected. The tails were separated from the heads by rinsing three times with cold HBSS. NTS were then incubated overnight in culture medium and used the next day. Test compounds and controls were dissolved in DMSO (Fluka, Buchs, Switzerland) to a concentration of 10 mM. One hundred NTS were then incubated in each well of a 96-well plate with culture medium and the test compound for a final well volume of 250 µL. Culture medium was composed of Medium 199 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Lucerne, Switzerland) and 1% penicillin/ streptomycin mixture (Lucerne, Switzerland). Compounds were tested at 10 µM in triplicate with NTS incubated in no more than 1% DMSO serving as control. NTS were kept in an incubator at 37 °C and 5% $CO_2$ for up to 72 h, after which

the condition of the NTS was microscopically evaluated using a scale from 3 (normal activity and morphological alteration) to 0 (dead).

***Adult S. mansoni Worms***. To obtain adult schistosomes, mice were infected subcutaneously with 80−100 cercariae. The mice were then euthanized after 7−8 weeks with $CO_2$ and the worms collected from the hepatic portal and mesenteric veins. Two pairs of adult worms were incubated in each well of a 24- well plate with 2 mL culture medium and the test compound. Culture medium was composed of RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Lucerne, Switzerland) and 1% penicillin/streptomycin mixture (Lucerne, Switzerland). Compounds with >70% activity against NTS were evaluated against adult worms at 10 μM with incubation at <1% DMSO used as control. Worms were kept in an incubator at 37 °C and 5% $CO_2$ for up to 72 h, after which their condition was microscopically evaluated using a scale from 3 (normal activity and no morphological alterations) to 0 (dead). $IC_{50}$ determination was conducted for compounds with >60% activity. For the *in vitro* drug sensitivity assays, all viability scores were averaged across replicates and normalized to the average viability scores of the control wells. $IC_{50}$ values were calculated using CompuSyn2 (ComboSyn Inc., Paramus, NJ).

**Cytotoxicity Testing**. Compounds were screened for *in vitro* cytotoxicity against CHO mammalian cell lines, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The reference standard, emetine, was prepared to 2 mg/mL in distilled water while stock solutions of test compounds were prepared to 20 mg/mL in 100% DMSO with the highest concentration of solvent to which the cells were exposed having no measurable effect on the cell viability. The initial concentration of the drugs and control was 100 μg/mL, which was serially diluted in complete medium with 10-fold dilutions to give six concentrations, the lowest being 0.001 μg/ mL. Plates were incubated for 48 h with 100 μL of drug and 100 μL of cell suspension

in each well and developed afterward by adding 25 μL of sterile MTT (Thermo Fisher Scientific) to each well and followed by 4 h incubation in the dark. The plates were then centrifuged, medium aspirated off, and 100 μLof DMSO added to dissolve crystals before reading absorbance at 540 nm. $IC_{50}$ values were then obtained from dose−response curves, using a nonlinear dose−response curve fitting analysis via GraphPad Prism v.4.0 software (La Jolla, USA). The assay was conducted in triplicate and conducted on two separate occasions.

**Single point *In vitro* metabolic stability assay**. The metabolic stability assay was performed in duplicate in a 96-well microtiter plate. The test compounds (0.1 μM) were incubated individually at 37 °C in a solution containing 0.35 mg/mL mouse liver microsomes (male Mouse BALB/c, Xenotech) and metabolic reactions initiated by the addition of NADPH (1 mM) in phosphate buffer (100 mM, pH 7.4) and incubated for 30 min. Reactions were quenched by adding 300 μL of ice-cold acetonitrile containing internal standard (carbamazepine, 0.0236 μg/mL). Test compounds in the supernatant were centrifuged, filtered, and analyzed by means of LC-MS/MS (Agilent Rapid Resolution HPLC, AB SCIEX 4000 QTRAP MS). The incubations of compounds and controls were performed in triplicate. The relative disappearance of parent compound over the course of the incubation was monitored by LC-MS/MS, and results were reported as % remaining after 30 min incubation. Metabolite searches were not conducted during the metabolic stability assay.

***In vivo* Studies in *S. mansoni*-Infected Mice**. Groups of 4 NMRI mice harbouring chronic *S. mansoni* infection were treated 49 days post-infection with single oral doses of 400 mg/ kg of candidate compounds dissolved in 7% Tween 80 and 3% ethanol in water (v/v/v) while untreated mice served as controls. The drug vehicle (7% Tween 80 and 3% ethanol in water) has no antischistosomal properties *in vitro* and *in vivo* (unpublished observation). At 21 days post-treatment, animals were euthanized by the $CO_2$ method and dissected. Surviving schistosomes

residing in the mesenteric veins and the liver were counted and sexed as previously described elsewhere.[20] Activity of test compounds was determined by comparing the worm reduction in the treated animals relative to the worm burden in the infected but untreated control groups. The difference was considered statistically significant if $p<0.05$ using the non-parametric Kruskal Wallis test.

**Mouse Pharmacokinetic Analysis**. Compound **44** was weighed out and prepared freshly immediately prior to dosing. The compound was prepared in dimethylacetamide, poly- ethylene glycol, and propylene glycol/ethanol mixture 4:1 at a ratio of 1:3:6 for intravenous dosing (n = 3) and administered in a volume of 50 μL via the tail vein after anesthetizing the animals. For the orally dosed group (n = 3), the compound was formulated as suspension in 100% hydroxypropyl methylcellulose and the dose administered by gavage. Approximately 20 μL of blood was drawn from the tail of each animal at a series of predetermined time-points and placed into a microcentrifuge tube coated with lithium heparin S12 to prevent clotting. Samples were kept on ice and transferred to storage at −80 °C within 1 h. Quantitation of the levels of the compound was carried out using high-performance liquid chromatography and mass spectrometry (LC-MS/MS) method developed in-house.

Animals are housed in temperature-controlled rooms and fed a standard diet with water provided *ad libitum*. The study was conducted using three animals per dosing route; all animals were killed by inhalation of isoflurane to knock them out followed by cervical dislocation. Authorization for the study was obtained from the University of Cape Town Research Ethics Committee (approval number 017/026).

ASSOCIATED CONTENT

**Supporting information**

The Supporting Information containing additional details of synthesis, structures as well as tabulated biological activity data of all compounds evaluated is available free of charge on the ACS Publications website (PDF).

Compounds SMILES format (XLSX).

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Notes

The authors declare no competing financial interests

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