

Development of Pyrazolopyrimidine Anti-*Wolbachia* Agents for the Treatment of Filariasis

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ABSTRACT: Anti-*Wolbachia* therapy has been identified as a viable treatment for combatting filarial diseases. Phenotypic screening revealed a series of pyrazolopyrimidine hits with potent anti-*Wolbachia* activity. This paper focuses on the exploration of the SAR for this chemotype, with improvement of metabolic stability and solubility profiles using medicinal chemistry approaches. Organic synthesis has enabled functionalization of the pyrazolopyrimidine core at multiple positions, generating a library of compounds of which many analogues possess nanomolar activity against *Wolbachia in vitro* with improved DMPK parameters. A lead compound, **15f** was selected for *in vivo* pharmacokinetics (PK) profiling in mice. The combination of potent anti-*Wolbachia* activity in two *in vitro* assessments plus the exceptional oral PK profiles in mice put this lead compound in a strong position for *in vivo* proof-of-concept pharmacodynamics studies and demonstrates the strong potential for further optimization and development of this series for treatment of filariasis in the future.

KEYWORDS: *Wolbachia*, Pyrazolopyrimidine, Onchocerciasis, Filariasis.

Filarial nematodes are the causative pathogens of the neglected tropical diseases lymphatic filariasis (LF) and onchocerciasis that affect tens of millions people throughout the tropics and contribute to serious public health and socio-economic problems. Onchocerciasis (the cause of river blindness) is the second leading infectious cause of blindness. These diseases combined are one of the leading causes of morbidity worldwide. The main causative agents for these conditions are the nematodes *Onchocerca volvulus* (onchocerciasis), *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (LF).¹ The latest recommended treatment for LF in areas which are not co-endemic for onchocerciasis or other filarial disease, loiasis is a triple combination of ivermectin, diethylcarbamazine plus albendazole.² Ivermectin is the recommended treatment for onchocerciasis, however it cannot be used in areas co-endemic for loiasis due to potentially fatal adverse effects. These direct acting anti-filarial drugs primarily target microfilariae, the immature worm-stage, thus can prevent transmission, but have little macrofilaricidal activity against the adult worms. Hence, these drugs require lengthy treatments that can be as long as 15 years.³⁻⁴ The association of current direct acting anti-filarial agents with undesired adverse effects, contraindicated patient groups combined with a growing concern of resistance devel-

opment is driving current research efforts to identify and generate safe therapeutic alternatives.⁵⁻⁶

The nematodes which are responsible for causing these two filarial diseases share an essential endosymbiotic relationship with the bacterium *Wolbachia*.⁷⁻⁸ Although the exact nature of this relationship is not yet fully understood, anti-*Wolbachia* therapy has been proven clinically by an existing antibiotic, doxycycline, which delivers safe macrofilaricidal activity with superior therapeutic outcomes compared to current anti-filarial drugs.⁹⁻¹¹

Pyrazolopyrimidine compounds have frequently appeared in the literature with a variety of different pharmacological activities such as; kinase inhibitors,¹²⁻¹³ anti-tuberculosis,¹⁴ antimalarial¹⁵ and antiviral¹⁶ agents, and antidepressants¹⁷. The original hit for this chemotype (**1**) which resulted from a phenotypic high-throughput screen (HTS) of a divergent chemical library donated by Medicines for Malaria Venture (MMV) is displayed in Figure 1 with measured *in vitro* activity (EC₅₀) against *Wolbachia* (wAlbB) infected insect cells (*Aedes albopictus*, C6/36) and drug metabolism/pharmacokinetic (DMPK) properties. According to the HTS data of other close

pyrazolopyrimidine analogues screened in the same campaign (data not shown), some preliminary indication of structure activity relationship (SAR) was observed and the areas of focus for SAR studies and optimization are highlighted in Figure 1. The aim of the work described here was to develop pyrazolopyrimidine leads with potent anti-*Wolbachia* activity and desired DMPK properties that could be further developed as oral drugs for the treatment of filariasis.

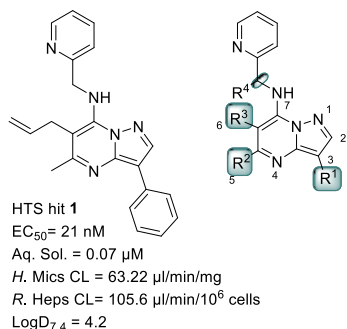


Figure 1. Structure of the HTS hit **1** and the pyrazolopyrimidine scaffold with the areas for SAR studies highlighted.

The cascade depicted in Figure 2 was followed for the optimization of the compounds described in this work. *In vitro* anti-*Wolbachia* potency was assessed in parallel with *in vitro* DMPK screening.¹⁸ Compounds showed a good balance of potency and metabolic stability were then selected to test their anti-*Wolbachia* activity against *Brugia malayi* microfilariae (mf) *in vitro* and for *in vivo* PK profiling in mice.²⁵ This secondary *in vitro* assay was developed to provide a link between the *in vitro* insect cell-based assay and the *in vivo* *B. malayi* SCID mouse model. Although the throughput of the mf assay is limited due to the availability of the *B. malayi* mf, it enables the assessment of the anti-*Wolbachia* activity in one of the targeted human parasites, providing important evidence of translation between different species of *Wolbachia* and hosts. Finally, front runners identified from the two above tests would be selected for the *in vivo* PD study where SCID mice are infected with the human parasite *B. malayi*.¹⁹

Despite the high potency ($EC_{50} = 21 \text{ nM}$) of the original hit **1**, DMPK assessments highlighted poor metabolic stability and low aqueous solubility (Fig.1) The phenyl ring, allyl substitution and methylene linker were some of the positions within the hit molecule that were predicted to be susceptible to oxidative CYP metabolism. Hence the early hit to lead optimisation was focused on enhancing the metabolic stability whilst maintaining potency.

The initial synthetic route (Scheme 1) was developed to allow for the synthesis of analogues with modifications at the R^2 , R^3 and 7-positions of the pyrazolopyrimidine ring. Whilst many necessary 5-amino pyrazoles (**1a**, **5d** $R^1 = \text{Ph}$, H) are commercially available, it was also possible to synthesise them in a two-step reaction from the corresponding nitrile (**4a** $R^1 = 4\text{-F-Ph}$) using base (NaOEt or LDA) and ethyl formate followed by hydrazine mediated cyclisation. This was followed by an acid-catalysed pyrimidine ring formation using the pyrazoles **1a**, **5a,d** and beta-keto esters **1b**, **6a-f**.²⁰

When the 5-position of the pyrazolopyrimidine is unsubstituted ($R^2 = \text{H}$), acid catalysed cyclisation is not suitable for the initial imine formation step. For these analogues the pyrimidine ring was formed by reaction of the appropriate pyrazole and aldehyde to give the imine intermediate which cyclises upon addition of KO^tBu. Chlorination of the 7-hydroxy pyrimidine intermediates **1c**, **7a-f** was achieved using phosphoryl chloride and subsequent substitution of intermediates **1d**, **8a-f** was achieved by aromatic substitution with the appropriate amines, **9a-b** to produce the pyrazolopyrimidines, **1**, **3** and **10a-d**.

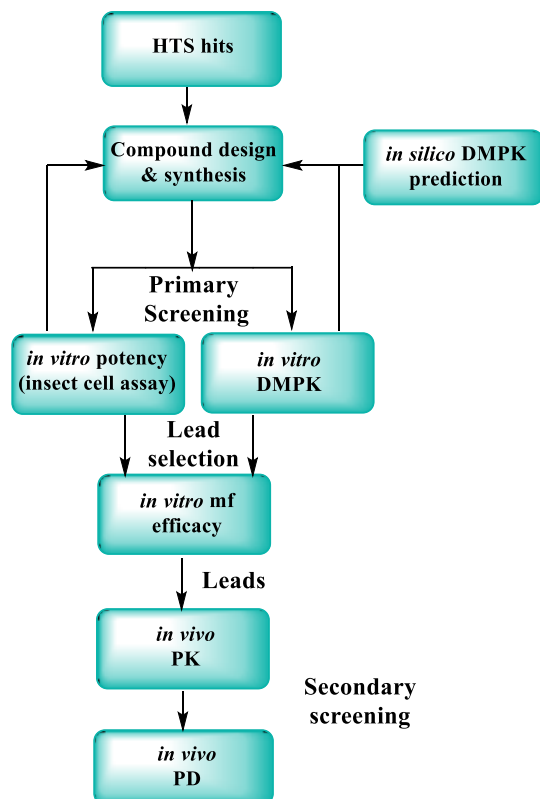
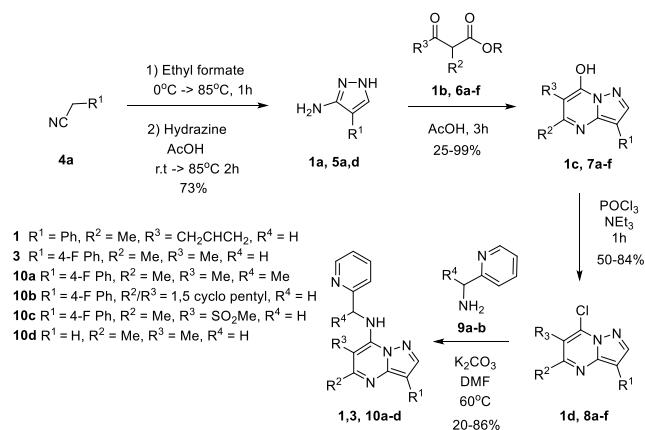


Figure 2. Screening cascade for the design, synthesis and testing of anti-*Wolbachia* compounds.

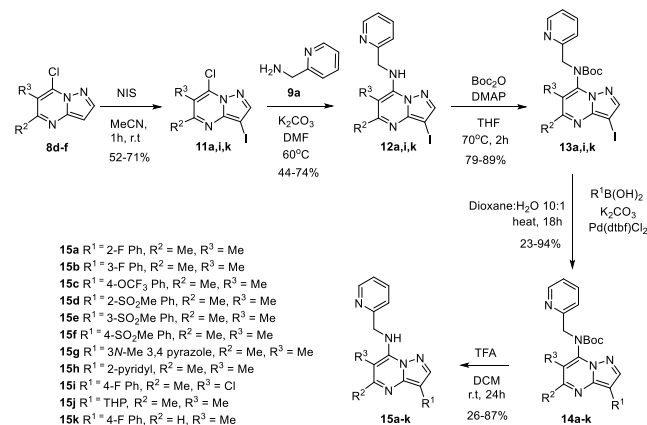
Scheme 1. Synthetic route for analogues in the pyrazolopyrimidine template.



While it is possible to use the appropriate nitriles as starting material for the synthesis of target compounds with different

groups at the R¹ position, a divergent orientated synthesis at this position was desirable for the production of a number of target analogues from a common intermediate. (Scheme 2) Functionalisation at the R¹ can also be achieved *via* iodination of the pyrazolopyrimidines **8d-f** (R¹ = H) using *N*-iodosuccinimide (NIS).² This pathway requires Boc-protection of the free amines **12** to allow for Suzuki-mediated coupling of intermediates **13** with the corresponding boronic acid. Finally TFA-mediated Boc-deprotection of compounds **14a-k** afforded the desired targets **15a-k**, which contained a variety of monosubstituted phenyl rings and nitrogen heterocycles.

Scheme 2. Optimised route for the synthesis of analogues with modified 3-positions

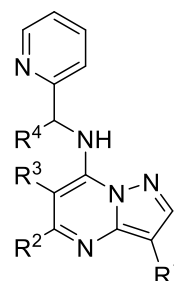


Note: THP = Tetrahydropyran

Substitutions at the R³ position: Replacement of the allyl group in this position in the original hit **1** with a methyl substituent resulted in excellent potency (compound **2**, EC₅₀ = 19 nM) and improved metabolic stability as shown by rat hepatocyte clearance of compound **2** (Table 2). Analogue with ring-fusion of a lipophilic cyclopentyl ring at R² and R³ (**10b**) showed good anti-*Wolbachia* activity (EC₅₀ = 79 nM) and demonstrated that modifications at both positions could be tolerated. This modification did not improve aqueous solubility, but by cyclopentyl ring connecting these two positions increased metabolic stability. Chlorine was also tolerated at the R³ position in terms of potency and has positive effect in metabolic stability in comparison to the corresponding 6-methyl analogues (**15i** vs **3**). On the other hand, SO₂Me group at the R³ position (**10c**) reduced anti-*Wolbachia* activity noticeably.

Substitutions at the R⁴ position: In an effort to reduce potential metabolism of the methylene linker, an analogue with methyl substitution at this position (**10a**) was investigated. Methylation of the linker imparts a 2-fold increase in human microsomal stability but this modification leads to a 4-fold loss in activity (compounds **10a** vs **3**). According to HTS results (data not shown here) we found that the SAR was restricted at the 2'-pyridyl side-chain. Any minor modifications to this ring significantly reduced compound potency. For this reason, we focused more on the R¹ position of the pyrazolopyrimidine core since the HTS data suggested that a wider range of modifications might be tolerated at this position.

Substitutions at the R¹ position: The R¹-aryl side-chain is not essential for anti-*Wolbachia* activity, however, removal of the aromatic ring resulted in some reduction in potency (**10d** EC₅₀ = 105nM) coupled with a significant increase in aqueous solubility. A range of small substitution at the *para*-position of the phenyl ring is generally tolerated, while a 4-fluoro substitution (**3**) appears to be optimal for activity (EC₅₀=17 nM). Substitution of the polar SO₂Me group at the *para*-position (**15f**) resulted in reducing LogD, increasing aqueous solubility and improving metabolic stability. However, similar substitution with the SO₂Me group at the *ortho*- and *meta*-positions of the phenyl ring is not tolerated, as shown by compounds **15d** & **15e**, (where substitution results in a significant reduction in potency). On the other hand, smaller substituents, such as fluorine are tolerated in the *ortho*-position although these small substitutions offered no significant improvements to DMPK properties. While compound **3** displayed improved *in vitro* metabolic stability comparing with HTS hit **1**, it still suffered from poor aqueous solubility.



	R ¹	R ²	R ³	R ⁴	*Anti- <i>Wolbachia</i> EC ₅₀ (nM)
1	Ph	Me	CH ₂ CH=CH ₂	H	21
2	Ph	Me	Me	H	19
3	4-F Ph	Me	Me	H	17
10a	4-F Ph	Me	Me	Me	93
10b	4-F Ph	1-cyclopentyl	5-cyclopentyl	H	79
10c	4-F Ph	Me	SO ₂ Me	H	704
10d	H	Me	Me	H	105
15a	2-F Ph	Me	Me	H	11
15b	3-F Ph	Me	Me	H	1000
15c	4-OCF ₃ Ph	Me	Me	H	664
15d	2-SO ₂ Me Ph	Me	Me	H	>2500
15e	3-SO ₂ Me Ph	Me	Me	H	>2500
15f	4-SO ₂ Me Ph	Me	Me	H	143
15g		Me	Me	H	119
15h		Me	Me	H	43
15i	4-F Ph	Me	Cl	H	51
15j		Me	Me	H	52
15k	4-F Ph	H	Me	H	38

Note: *All tested compounds showed no cytotoxicity against the insect cells at the top concentration (5 μM) in the assay.

Table 1. *In vitro* potency data of key analogues.

In an attempt to reduce LogD further and to improve aqueous solubility, analogues **15g** and **15h** containing the heterocyclic aromatic 1-methyl-1H-pyrazolyl and 2-pyridyl groups were synthesised as more polar/LogD reducing phenyl ring replacements. These modifications both greatly enhanced the aqueous solubility and lowered LogD with the 2-pyridyl analogue **15h** maintaining the majority of its potency.

Table 2. *In vitro* DMPK data of pyrazolopyrimidine analogues

	LogD _{7.4}	Aq. Sol. (μM)	H. Mics. CL. (μl/min/mg)	R. Hep. CL. (μl/min/10 ⁶ cells)	H. PPB (%)
1	4.2	0.07	63.22	105.60	99.9
2	4.0	0.40	ND	59.03	99.80
3	4.3	0.90	49.19	67.66	99.8
10a	4.3	0.30	24.49	75.43	ND
10b	3.3	0.50	48.86	41.25	99.0
10c	3.7	0.01	8.76	46.48	99.7
10d	2.0	561	261.80	109.60	77.0
15a	4.0	0.60	83.97	113.20	99.9
15b	4.1	1.00	59.33	86.16	99.9
15c	4.5	5.00	8.76	156.60	97.9
15d	2.3	11.0	74.15	>300.00	88.0
15e	3.0	2.00	12.08	147.80	96.6
15f	3.3	2.00	21.78	27.58	99.8
15g	2.7	43.0	44.83	10.31	91.8
15h	2.3	30.0	243.60	144.90	97.4
15i	4.3	2.0	44.62	26.37	99.5
15j	2.4	21.0	23.65	71.84	ND
15k	4.0	6.0	37.48	45.53	99.6

Notes: Aq. Sol. = aqueous solubility in pH7.4 PBS; H. Mics. CL. = intrinsic human microsomal clearance measured *in vitro*; R. Hep. CL. = intrinsic Rat hepatocyte clearance measured *in vitro*; ND = not determined.

Compound **15g** proved to be reasonably stable metabolically however, a near 10-fold drop in activity supported further investigation into other areas of the scaffold to improve overall properties. Exploration of saturated heterocyclic ring systems led to the synthesis of compound **15j** containing the tetrahydropyran (THP) moiety. The THP side-chain is well tolerated

for potency (EC₅₀ = 52 nM) whilst improving DMPK properties comparing with HTS hit **1**.

Substitutions at the R² position: Substitution at this position is not essential for anti-*Wolbachia* activity. It was demonstrated by the removal of the methyl substituent from the R²-position (**15k**) was tolerated in terms of potency and resulted in some improvement in DMPK properties when compared to compound **3**. This result suggests the R²-position was another area that could be further explored for optimisation of potency and DMPK in future work.

Table 3. *In vitro* potency of key analogues in the *B. malayi* mf assay

Molecule	Anti- <i>Wolbachia</i> potency from cell assay EC ₅₀ (nM)	Anti- <i>Wolbachia</i> potency from mf assay, (6 days at 5 μM % <i>Wolbachia</i> reduction in wsp:gst*)
DOX	17	86.5
1	21	75.60
3	17	83.20
10d	105	77.10
15f	143	80.40
15i	51	Toxic**
15j	52	85

*wsp - *Wolbachia* surface protein copy number, median % reduction cf. vehicle (DMSO) control, gst - GST copy number (single copy gene, worm size biomarker); **significantly reduced motility of the mf after 6 days incubation comparing with vehicle (DMSO) control.

Anti-*Wolbachia* activity assessment in *B. malayi* mf assay:

The mf assay is an orthogonal *in vitro* assay that uses *B. malayi* microfilariae to confirm the anti-*Wolbachia* activity of tested compounds against the human parasitic nematode.²¹⁻²² After being screened for potency and DMPK properties *in vitro*, a number of selected analogues were tested at 5 μM alongside the gold-standard doxycycline for comparison of anti-*Wolbachia* activity in the mf assay. The majority of tested compounds demonstrated good activity, comparable to doxycycline, in this assay except for **15i** (Table 3). The secondary readout of the mf assay is the motility of the mf by the tested compounds comparing with vehicle control. For anti-*Wolbachia* drugs, such as doxycycline, they should not affect the motility of the parasitic worm since this indicates off-target effects. For this reason the chloro-substituted analogue **15i** was considered unsuitable for further development as an anti-*Wolbachia* drug as it had significant effects on worm motility in the assay.

***In vivo* pharmacokinetic (PK) profiling in mice:** Taking all the *in vitro* results into consideration, compounds **15f** and **15j** possessed a suitable balance of high potency, good DMPK properties and acceptable preliminary safety profiles (e.g. cytotoxicity and hERG inhibition²³), and **15f** was chosen for *in*

in vivo PK evaluation. Compound **15f** was dosed orally to SCID mice at 50 mg/kg and 100 mg/kg using a standard suspended vehicle (SSV); results from the study are shown in Chart 1 and Table 4. Despite limited aqueous solubility, this compound demonstrates good tolerability, excellent *in vivo* PK profiles with high exposure, reasonable half-life and dose-proportional AUC. Based on this data **15f** has been selected as a lead for an *in vivo* proof-of-concept pharmacodynamics study and further optimisation.

Chart 1. Mean plasma concentration of compound 15f following dosing to SCID mice with SSV.

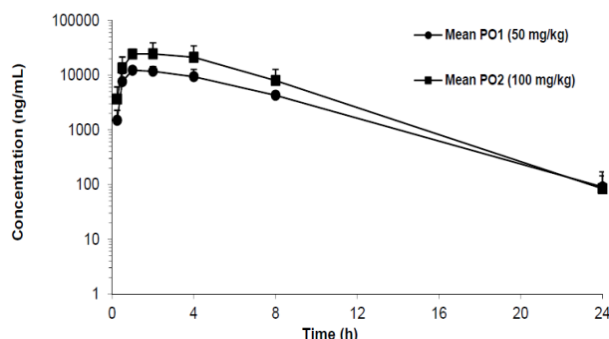


Table 4. *In vivo* PK profile of compound 15f, dosing to SCID mice using SSV.

Dosage (Oral)	50 mg/kg	100 mg/kg
T _{1/2} (h)	2.9	2.4
C _{max} (µg/L)	13,067	27,800
T _{max} (h)	1.33	1.67
AUC _{0-t} (µg·h/L)	82,209	162,617

In summary, the initial potent pyrazolopyrimidine hit **1**, which has poor metabolic stability and inadequate aqueous solubility parameters, has been optimised to provide a number of highly potent compounds with enhanced DMPK properties as represented by lead molecules **15f** and **15j**. A summary of the key SAR is highlighted in Figure 3.

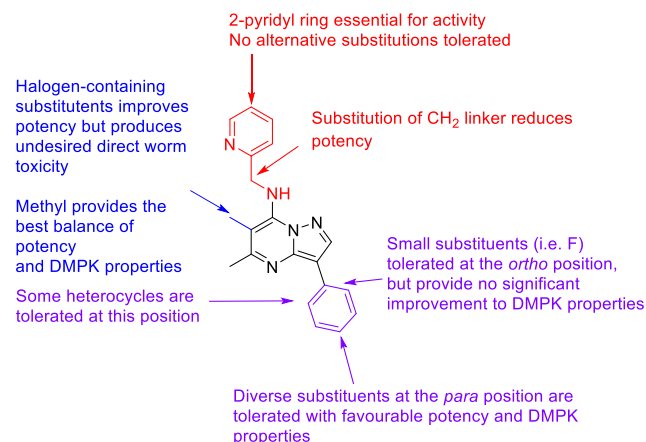


Figure 3. Summary of the SAR of anti-*Wolbachia* pyrazolopyrimidines.

More explorations of the R¹-position and potential functionalisation of the 2-position of the pyrazolopyrimidine core could further improve the anti-*Wolbachia* potency and the overall DMPK properties. These future directions will be determined by the results of *in vivo* efficacy studies of **15f** which will be reported in due course. In addition, the *in vivo* PK and PD studies of the other lead, **15j** will be triggered if the proof-of-concept *in vivo* efficacy study of **15f** is positive.

ASSOCIATED CONTENT

Supporting Information

Supporting information is provided on the *in vitro* biological testing methods and the experimental procedure of the synthesis and characterization of the reported compounds.

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Author Contributions

The manuscript was written through contributions of all authors. W.D.H., M.J.T., S.A.W. and P.M.O. designed research; P.McG. performed synthesis; P.J.H.W., M.C.W. S.K. performed *in silico* and *in vitro* DMPK and safety studies; A.C., R.H.C., D.A.C., K.L.J. performed parasitology studies; W.D.H., N.G.B. G.L.N., L.F., K.L.J., and P.M.O. analyzed data and P. McG., N.G.B, W.D.H., G.L.N. and P.M.O. wrote the paper.

Notes

The authors declare no competing financial interests.

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ABBREVIATIONS

LF, lymphatic filariasis; DEC, diethylcarbamazine; HTS, high-throughput screening; DMPK, Drug Metabolism Pharmacokinetics

ics; DOX, Doxycycline; PD, Pharmacodynamics; PK, Pharmacokinetics; SAR, Structure-activity relationships; *B.malayi*, *Brugia malayi*; *mf*, microfilariae, SCID, Severe combined immunodeficient

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