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Thiophene derivatives activity against the protozoan parasite *Leishmania infantum*

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ABSTRACT

Treatments against leishmaniasis are limited and the development of new molecules is crucial. One class of developmental drug that has shown activity against the parasite *Leishmania* are thiophene derivatives. Here we synthesized thirty-eight novel thiophene compounds and characterized their activity and potential for resistance against *L. infantum*. Half of the molecules had an EC₅₀ in the low micromolar range, the piperidine derivatives being more potent than the tetramethylpyran derivatives. Resistance was challenging to select for, and resistant cells could only be raised against one (GC1-19) of the four most active compounds. Using chemogenomic screens we show that a gene conversion event at the *ABCG2* locus as well as the overexpression of a trypanothione peroxidase are responsible for a weak but significant resistance to the GC1-19 drug candidate. Together, our results suggest that thiophene is a scaffold of interest for further drug development against leishmaniasis.

1. Introduction

The protozoan parasite *Leishmania* infects several millions of people per year and depending on the infecting species is responsible for a spectrum of clinical manifestations. The most severe form consists in visceral leishmaniasis (VL) and kills an estimated 20 000 people each year (Alvar et al., 2012). There are no human vaccines and the chemotherapeutic arsenal against VL is limited with only 4 licensed drugs (antimonials, amphotericin B, miltefosine, paramomycin) each with substantial shortcomings related to price, toxicity, mode of administration, or drug resistance (Ponte-Sucré et al., 2017). There is an urgent need for novel drugs, and a number of efforts are ongoing to develop novel molecules against *Leishmania* (De Rycker et al., 2014; Pena et al., 2015; Ortiz et al., 2017; Van Boclaer et al., 2019; Van den Kerkhof et al., 2021).

Thiophene is an important versatile sulphur containing building block in the generation of pharmaceutical derivatives with anti-infective properties (Keri et al., 2017). A number of thiophene derivatives (e.g. amino-thiophene, benzothiophenes, thiophene carboxaldehydes) were

shown to be active against various *Leishmania* species (Savornin et al., 1991; Rodrigues et al., 2015; Navin et al., 2017; Rodriguez et al., 2018; Borsari et al., 2019; Pacheco et al., 2021). However, the mode of action of thiophene derivatives is not clearly defined and no specific cellular target has yet been identified (Borsari et al., 2019). Some thiophene derivatives used against *Leishmania* were shown to produce reactive oxygen species (ROS) (Rodriguez et al., 2018) but other derivatives did not (Pacheco et al., 2021). Therefore, further work is warranted to decipher how thiophene derivatives are active against *Leishmania*.

Recently we synthesized a series of 2,2,6,6 tetramethylpiperidine thiophene derivatives that were active against *Leishmania major* (Rodriguez et al., 2018). We expanded our search for active thiophenes by synthesizing and testing against *Leishmania infantum*, a parasite responsible for VL, 16 different 2,2,6,6 tetramethylpiperidine thiophene derivatives and 22 distinct 2,2,6,6 tetramethylpyran thiophene derivatives using structure activity relationship studies. Through a series of chemogenomic screens and by selecting for resistance against a subset of the most active derivatives we investigated the potential routes of action and resistance mechanisms to thiophene derivatives.

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Table 1Activity and toxicity of thiophene derivatives against *L. infantum* promastigotes, intracellular amastigotes and THP-1 macrophages.

Analogues	Drug ^a	EC50 promastigote (µM) ^b		EC50 amastigote (µM) ^c		Macrophage CC ₅₀ (µM) ^d	Selectivity index ^{c,e}	
		<i>L. infantum</i> 263	<i>L. infantum</i> JPCM5	<i>L. infantum</i> 263	<i>L. infantum</i> JPCM5		<i>L. infantum</i> 263	<i>L. infantum</i> JPCM5
–	Amphotericin B	0.062 ± 0.005	–	0.32	–	12.5	39.1	–
–	Miltefosine	8.2 ± 1.3	8.9 ± 3.0	0.37	–	–	–	–
Nitrogen	GP1-04	7.4 ± 0.4	4.6 ± 0.5	4.2	–	21.5	5.1	–
	RS2-13	0.29 ± 0.2	0.26 ± 0.1	0.7	–	8.3	11.9	–
	AV1-76	2.6 ± 0.8	3.5 ± 0.7	–	4	21.4	–	5.4
	AV1-77	1.5 ± 0.2	1.3 ± 0.3	2.7	–	11.2	4.1	–
	AV1-78	9.7 ± 0.5	14.7 ± 3.0	–	9.7	> 75	–	> 7.7
	AV1-79	10.4 ± 1.8	7.4 ± 1.2	9.3	–	> 75	> 8.1	–
	AV1-80	8.4 ± 1.3	6.0 ± 1.9	9.8	–	> 50	> 5.1	–
	AV1-81	6.4 ± 1.0	3.4 ± 1.4	–	–	11.3	–	–
	AV1-82	6.5 ± 1.1	3.2 ± 0.6	–	–	8.2	–	–
	AV1-83	> 25	> 25	–	–	–	–	–
	AV1-84	10.1 ± 1.7	8.4 ± 3.4	–	–	5.2	–	–
	AV1-85	7.9 ± 0.2	7.2 ± 2.6	9.5	–	> 70	> 7.4	–
	AV1-86	8.7 ± 0.4	5.8 ± 0.5	3.6	–	17.4	4.8	–
	GC1-30	17.2 ± 3.9	19.2 ± 5.4	10.4	–	26.4	2.5	–
	GC1-31	9.2 ± 2.8	5.6 ± 1.2	–	7.4	87.3	–	11.8
	GC1-32	12.3 ± 0.3	16.7 ± 4.0	–	–	12.5	–	–
Oxygen	AV1-54	> 25	> 25	–	–	–	–	–
	AV1-55	> 25	> 25	–	–	–	–	–
	AV1-56	> 25	21.1 ± 8.0	–	–	–	–	–
	AV1-57	> 25	> 25	–	–	–	–	–
	AV1-58	> 25	> 25	–	–	–	–	–
	CN1-64	> 25	> 25	–	–	–	–	–
	CN1-67	> 25	> 25	–	–	–	–	–
	CR1-50	> 25	> 25	–	–	–	–	–
	CR1-51	Insoluble	–	–	–	–	–	–
	GC1-18	> 25	> 25	–	–	–	–	–
	GC1-19	10.1 ± 4.2	4.2 ± 1.34	17.65	6	50	2.8	8.3
	MI 1-1	> 25	> 25	–	–	–	–	–
	MI 1-2	> 25	> 25	–	–	–	–	–
	MI 1-5	> 25	> 25	–	–	–	–	–
	RS2-24	18.1 ± 4.1	18.9 ± 4.7	5.5	–	46.2	8.4	–
	GC1-107	18 ± 3.3	11.6 ± 3.9	–	7.5	> 100	–	> 13.3
	AV1-134	> 25	> 25	–	–	–	–	–
	AV1-135	> 25	> 25	–	–	–	–	–
	AV1-142	> 25	> 25	–	–	–	–	–
	AV1-143	4.8 ± 1.5	7.1 ± 0.5	–	15	> 100	–	> 6.7
AV1-138A	> 25	> 25	–	–	–	–	–	
CR1-52	Insoluble	–	–	–	–	–	–	

^a Compounds in bold were studied further by genomic screens.^b Results are shown as the mean ± SD of at least three biological replicates.^c Results are shown as the average of three biological replicates.^d Results of 50% THP-1 macrophage survival are shown as the average of two biological replicates. The toxicity threshold is defined as the concentration causing <50% THP-1 macrophage survival.^e The selectivity index was calculated as the ratio of the threshold concentration for CC₅₀ values over the EC₅₀ against intracellular amastigotes.

2. Material and methods

2.1. Cell culture

L. infantum strain MHOM/MA/67/ITMAP-263 (Sereno et al., 2001) and JPCM5 (Peacock et al., 2007) were maintained as promastigotes at 25 °C in SDM-79 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 5 µg/mL hemin. *L. infantum* parasites were differentiated into amastigotes inside the macrophage cell line THP-1 as previously described (Sereno et al., 2001). Drug activity in the promastigote stage was determined by measuring the OD₆₀₀ after 72h of drug exposure in a multi-well scanning spectrophotometer (Thermo Labsystems Multiskan Spectrum UV/visible Microplate Reader) as described (Ouellette et al., 1990). For drug activity assays in the amastigote stage, THP-1 cells were plated in LabTek slides and infected with promastigotes at a macrophages to parasites ratio of 1:15 for 3h as described (Roy et al., 2000). After adding the drug, cells were incubated for 48h, washed and incubated for an additional 4 days. Slides were fixed 1 min with methanol and stained 30 min with 1/20 Giemsa. The parasitic index was determined from at least 200 macrophages per

well as described (Roy et al., 2000). The EC_{50s} correspond to the drug concentrations reducing the parasitic indexes by 50% when compared to the no drug control. THP-1 macrophage toxicity assays were performed with the resazurin substrate (BioRad) that was read at OD₅₇₀ and OD₆₀₀ in a multi-well scanning spectrophotometer (Thermo Labsystems Multiskan Spectrum UV/visible Microplate Reader). Resistant *L. infantum* JPCM5 mutants were selected against four thiophene derivatives by exposure to stepwise drug increments starting with the EC₅₀ of the drug. Cells were allowed to adapt between each drug increment. Standard practice is to perform 2-fold increase in drug concentration at each increment but since resistance was difficult to achieve drug concentration was increased by only 1.5-fold at each selection step. Depending on the drug studied, the selection process spanned 3–5 months with a minimum of 15 passages.

2.2. DNA constructs and transfections

Genes were amplified from *L. infantum* JPCM5 genomic DNA using primers listed in Table S1. PCR fragments were cloned into the pSP72α-puro-α or pSP72α-zeo-α expression vectors (Papadopoulos et al., 1992).

The integrity of each insert was confirmed by Sanger sequencing. For transfection, 10 µg of plasmid DNA were electroporated into *L. infantum* JPCM5 promastigotes in the logarithmic phase of growth using a Gene Pulser Xcell Electroporation System (Biorad) at 450 V, 500 µF, 2 mm and time constant range between 4 and 6 ms. Transfected cells were selected with 100 µg/ml puromycin or 400 µg/ml zeocin.

2.3. CRISPR-Cas9 based genome editing

For CRISPR-Cas9 based genome editing, a plasmid containing the CRISPR associated protein 9 (Cas9) nuclease, pLPhygCAS9 (plasmid #63555, addgene) (Zhang and Matlashewski, 2015) was transfected into *L. infantum* JPCM5 promastigotes. Transfectant cells were selected with 300 µg/ml of hygromycin. A guide RNA (gRNA) targeting *ABCG2* (LinJ060090_1642 ATCTTGATTCCGCTGGCTCT) annealed with tracrRNA was co-transfected in *L. infantum* parasites (harboring the pLPhygCAS9 episome) along with a 165 bp repair cassette covering the *ABCG1/2* gene conversion derived from the GC1-19.12.1b mutant using an Amaxa Nucleofector (Lonza) with the U-014 program. Cells were incubated for 48h at 25 °C with shaking. Transfectant cells were cloned on SDM agar plates and then grown independently. The presence of the gene conversion was confirmed by PCR amplification of *ABCG2* followed by Sanger sequencing.

2.4. ROS measurement

Promastigotes (5×10^5 per mL) were grown in SDM medium for 48h at 25 °C in the presence or absence of thiophene derivatives at their EC₅₀ and EC₉₀ concentrations. Cells were washed in Hepes-NaCl (21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄·7H₂O, 6 mM glucose, pH 7.4) and incubated in the presence of 20 µg/ml H₂DCFDA (Invitrogen) for 30 min at 25 °C in the dark, as described (Moreira et al., 2011). ROS accumulation in relative fluorescence units (RFU) was measured using a Victor fluorometer (PerkinElmer, Turku, Finland) at 485 nm excitation and 535 nm emission wavelengths. Fluorescence was normalized according to the number of cells.

2.5. Chemogenomic screens and whole genome sequencing and analysis

Genomic libraries were prepared from the genome of thiophene-resistant mutants using the Illumina DNA prep kit and these were sequenced on a NovaSeq6000 instrument (Illumina). Sequencing reads have been deposited to the Sequence Reads Archive under Bioproject accession PRJNA872125. Sequencing reads were trimmed according to their base quality using the trimmomatic software and aligned to the *L. infantum* JPCM5 (TriTrypDB release 45) reference genome using the BWA software (Li and Durbin, 2009). Read duplicates were flagged using Picard and GATK (McKenna et al., 2010; DePristo et al., 2011) was used to identify SNPs and Indels. The Cos-Seq screen was carried out essentially as described in great details elsewhere (Potvin et al., 2019). The cosmid library was generated as described (Potvin et al., 2019). To ensure proper genome coverage, seven transfections were pooled and grown under hygromycin drug pressure to select for transfectants. Cosmids were extracted from parasites at the first passage and a NGS library was prepared and sequenced to monitor baseline cosmid levels (Fig. S1). Cosmids were also extracted from cells at each drug concentration during the Cos-Seq screen and sequenced. NGS reads were aligned to the *L. infantum* JPCM5 reference genome with the BWA software. Gene abundance in FPKM (fragments per kilobase of transcript per million fragments mapped) was determined using the kallisto software. Gene enrichment analysis was performed using edgeR.

2.6. Statistical analysis

The statistical analyses were performed in GraphPad Prism 5.1 software using two-tailed unpaired *t*-test.

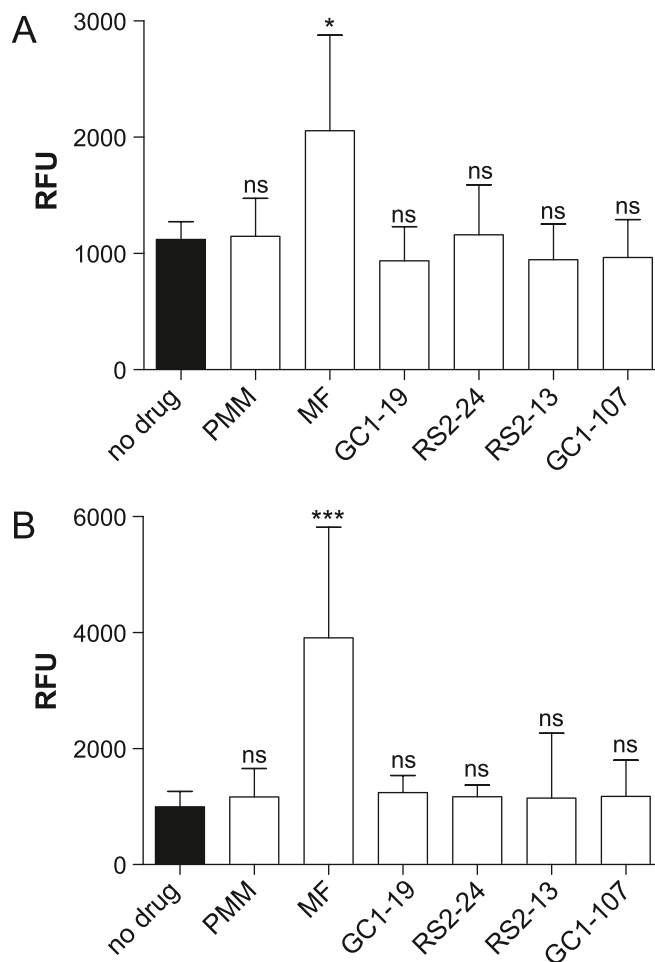


Fig. 1. Measurement of ROS, expressed in relative fluorescence units (RFU), in *L. infantum* JPCM5 after 48h of exposure to the EC₅₀ (A) and the EC₉₀ (B) of thiophene derivatives. RFU was normalized according to the number of cells. Paromomycin (PMM) and miltefosine (MF) were used as negative and positive controls for drug-induced ROS production, respectively. Results are shown as the mean \pm SD of at least three biological replicates. RFU levels in drug-treated cells were compared to the no drug control using unpaired two-tailed *t*-test. **P* \leq 0.05; ****P* \leq 0.001.

3. Results

The 2,2,6,6-tetramethyl piperidine derivatives were synthesized in two steps synthesis as we described previously (Rodriguez et al., 2018). We expanded our synthesis design and created the synthesis of the 2,2,6,6-tetramethyl-tetrahydro-pyran derivatives. The synthesis of these derivatives is described in Supplementary File 1. The identity and purity of all derivatives were assessed by ¹H-NMR, ¹³C-NMR, and mass spectrometry. The structures of the 38 thiophene derivatives can be found in Fig. S2.

The activity of these derivatives was first tested in the promastigote stage of two different *L. infantum* strains. Nineteen derivatives had EC_{50s} ranging from 0.3 to 18 µM, 17 had EC_{50s} >25 µM (16 of which were pyran derivatives) and two were insoluble (Table 1). The activity of the compounds was similar (less than two-fold difference) against the two strains with the possible exception of GC1-19 (Table 1). We followed up with compounds that showed activity below the 20 µM level. The toxicity of these active thiophene derivatives was also assessed against THP-1 macrophages and all compounds tested were more active against *Leishmania* than the macrophages (Table 1). Additional modifications would be required for several compounds however in order to further increase the selectivity index (Table 1). The efficacy of the 15 less toxic

Table 2Selection of five independent *L. infantum* JPCM5 lines resistant to GC1-19.

Mutants ^a	EC ₅₀ (μM) ^c
WT	6.3 ± 2.3
GC1-19-12.1	26.4 ± 3.0 (4.2 ***)
GC1-19-12.1a	14.2 ± 2.6 (2.3 ***)
GC1-19-12.1b	16.9 ± 5.1 (2.7 ***)
GC1-19-12.1c	21.8 ± 4.2 (3.5 ***)
GC1-19-12X1c-rev ^b	12.9 ± 4.0 (2.1 **)
GC1-19-10.2	14.2 ± 5.1 (2.3***)
GC1-19-10.3	18.8 ± 5.4 (3.0 ***)
GC1-19-10.3a	9.1 ± 1.6 (1.5)
GC1-19-10.3b	10.7 ± 5.6 (1.7 *)
GC1-19-10.3c	15.4 ± 10 ⁶ (2.5 **)
GC1-19-10.4	12.3 ± 4.8 (2.0 **)
GC1-19-10.5	15.3 ± 6.8 (2.4 **)
GC1-19-12.1b	4.9 ± 2.0 (0.8) (RS2-24) ^d

^a Mutants with a letter in their names are clones derived from the resistant lines.

^b Clone GC1-19-12X1c-rev was cultured without GC1-19 for 10 passages.

^c Results are shown as the mean ± SD of at least three biological replicates. Fold resistance increase compared to the wild-type is indicated within parentheses. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; by unpaired two-tailed *t*-test.

^d For clone GC1-19-12.1b we also measured cross-resistance to RS2-24, another thiophene derivative.

and more active derivatives were also tested against intracellular *L. infantum* infecting THP-1 monocytes differentiated into macrophages (Sereno et al., 2001). The EC₅₀s found with the promastigote stage were mostly paralleled with what observed with intracellular parasites (Table 1) and the most active compound RS2-13 in promastigotes was also the most active against intracellular parasites. The only two exceptions were RS2-24 which was 3-times more active against intracellular parasites and AV1-143 which was more active against promastigotes (Table 1).

Four compounds were chosen for further analysis: RS2-13, the most active compound; RS2-24, the most active against the intracellular stage; GC1-107, which showed no measurable toxicity against the host cells; and GC1-19, for its strain specificity, being more active against *L. infantum* JPCM5 (p-value ≤0.05; two-tailed unpaired *t*-test). None of these four thiophene derivatives induced ROS (Fig. 1). The mode of action and resistance mechanisms for these four compounds were studied by using two genomic screens coupled to next-generation sequencing (NGS). One consisted in sequencing the genome of parasites in which resistance was induced experimentally (Leprohon et al., 2015) and the second consisted in Cos-Seq, a gain of function screen based on episomal rescue (Gazanion et al., 2016; Fernandez-Prada et al., 2018).

While we and others have succeeded in selecting *Leishmania* parasites for resistance to a plethora of bioactive molecules, it was challenging to select parasites for resistance to the four thiophene drug candidates. For RS2-13 and RS2-24, we passaged cells from 5 independent populations for up to 4 months (>15 passages), starting at 0.5 × EC₅₀ with several attempts in making small drug concentration increments, but could not establish resistant lines. For GC1-107, we were able to select resistance from a single population but after fifteen passages this also failed in generating resistant parasites. On the other hand, we were able to select 5 populations of *L. infantum* parasites that were more resistant to GC1-19 than wild-type cells (Table 2). The most resistant line (GC1-19-12.1) was 4.2-fold more resistant than the wild-type. This mutant population was cloned and resistance was maintained in the three clones analyzed (a to c), albeit at slightly lower levels compared to the parent line (Table 2). One of those clones was grown in absence of GC1-19 for 10 passages and these cells (GC1-19.12.1c-rev) were still two-fold more resistant to the drug (Table 2). The resistant line

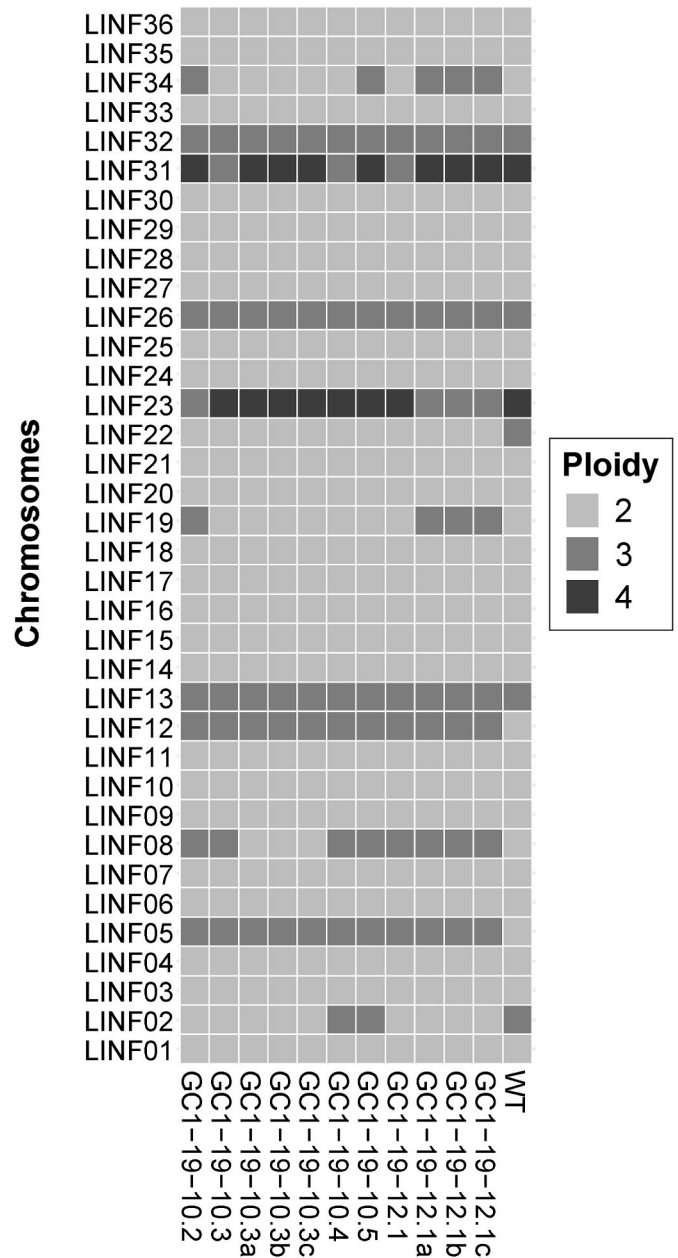


Fig. 2. Chromosome ploidy inferred from NGS coverage for *L. infantum* wild-type and mutants resistant to GC1-19.

GC1-19-12.1 was not cross resistant to RS2-24, another 2,2,6,6 tetra-methylpyran thiophene derivative (Table 2).

Whole genome sequencing of resistant pathogens is a useful strategy in studying mode of action, target identification and resistance mechanisms (Ouellette and Bhattacharya, 2020). We sequenced the five GC1-19 resistant lines as well as the three clones derived from mutants 1 and 3 (Table 2). *Leishmania* is a diploid organism but with occasional changes in ploidy for specific chromosomes. Our wild-type cell was polyploid for chromosomes 2, 13, 22, 23, 26, 31 and 32 (Fig. 2). *L. infantum* cells selected for resistance to GC1-19 had a similar ploidy but with some differences (Fig. 2). Interestingly, chromosomes 5 and 12 became polyploid and chromosome 22 reverted to the diploid state in all resistant lines and clones (Fig. 2). While these recurrent changes in ploidy correlated with resistance, changes in ploidy are frequently observed with *Leishmania* and it remains to be seen whether any of these contribute to resistance. As a complement we also analyzed the genomes of resistant parasites for single nucleotide polymorphisms (SNPs) and

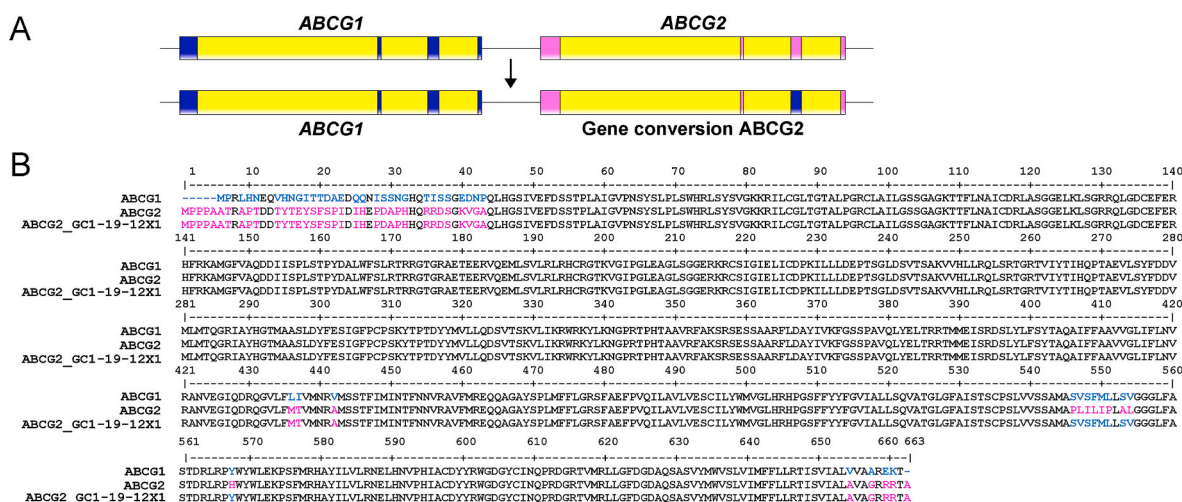


Fig. 3. Gene conversion between *ABCG1* and *ABCG2*. (A) Representation of the *ABCG1* and *ABCG2* locus on chromosome 6. Regions highlighted in yellow are identical between the two gene paralogs. (B) Protein sequence alignment for the wild-type and recombinant *ABCG2*. Sequences derived from *ABCG1* is highlighted in blue and those from *ABCG2* in pink.

small insertions and deletions (Indels) in the coding sequences. As with many other screens (Bhattacharya et al., 2019) we concentrated on the genes mutated in more than one mutant as this recurrence increases the likelihood of a mutation being phenotypic. A list of the genes mutated in a recurrent fashion among mutants is provided in Table S2. Based on our previous experience and to further limit the number of SNPs and Indels to be experimentally validated, we excluded mutations that were identical in many mutants (these likely represent natural polymorphism rather than a resistance selected mutation). Of the remaining candidate genes, we focussed on LINP_06000590 as it encodes for an ABC transporter of the G subfamily, *ABCG2* (Leprohon et al., 2006), a protein involved in drug resistance in *Leishmania* (Perea et al., 2016). Moreover, the mammalian *ABCG2* was shown to produce resistance to a thiophene derivative (Wu et al., 2017).

Our initial sequence analysis of *ABCG2* identified SNPs in three resistant lines and their clones. A range of mutation profiles were observed between the mutants or clones at five amino acids between residues 546 to 568 (Table S2). To validate the mutations, we amplified a gene fragment covering this region in clone 12.1b and its parent line 12.1. However, instead of the P546S, L554V and H568Y substitutions identified by NGS in 12.1b we found a stretch of 9 amino acids (residues 546–554) that were substituted in 12.1b (and also in the parent line 12.1) (Table S3). This stretch of amino acids is identical to a part of *ABCG1*, a paralog of *ABCG2* whose gene is located immediately upstream on chromosome 6 (Fig. 3A). The sequence identity of *ABCG1* and *ABCG2* is high both at the level of amino acids (Fig. 3B) and nucleotides (Fig. S3) and the chimeric gene observed in 12.1b is most likely a product of gene conversion. Both alleles of chromosome 6 in mutant 12.1b had this gene conversion changing a short stretch of 9 amino acids (residues 546–554) in addition to the H568Y substitution at residue 568 (Table S3). While the NGS data had revealed punctuated SNPs in the same *ABCG2* region in several lines (and clones) resistant to GC1-19, the former data prompted us to validate these by Sanger in all the clones and mutants that we had generated. It came out that the gene conversion described above also occurred in a homozygous fashion in the 10.3 resistant line and its three clones, and in a heterozygous fashion in the resistant line 10.5 (Table S3). The discrepancy between NGS and Sanger sequencing is likely due to the high sequence identity between *ABCG1* and *ABCG2* (Fig. S3) which made the alignment of the short NGS reads derived from the *ABCG1-ABCG2* hybrid more difficult. This was validated by manual inspection of the alignment which showed that 34% of SNPs found by Sanger sequencing weren't covered by NGS reads and that 58% of the locus was covered by less than 10 reads.

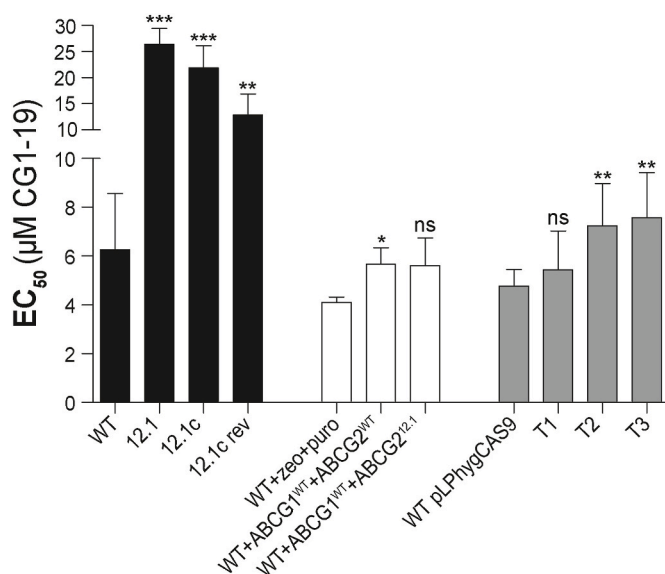


Fig. 4. Inhibition assays with GC1-19. The EC₅₀ to GC1-19 for *L. infantum* wild-type, the GC1-19-12X.1 and GC1-19-12X.1c mutants, and the GC1-19-12X.1c-rev line passed without GC1-19 for 10 passages are shown in black. The EC₅₀ to GC1-19 for *L. infantum* WT overexpressing *ABCG2* (WT or mutated versions) and *ABCG1* (WT version) as part of pSP72α-puro-α and pSP72α-zeo-α episomes are shown in white. The EC₅₀ to GC1-19 for *L. infantum* wild-type edited by CRISPR-Cas9 for *ABCG2* are shown in gray. T1, heterozygote for the *ABCG2* mutated version coding for the 9 amino acid substitutions at residues 546 to 554; T2 homozygote for the same *ABCG2* version as in T1; T3, same as T2 but also heterozygous for the mutation responsible for the H568Y substitution in *ABCG2*. Results are shown as the mean ± SD of at least three biological replicates. The mean expression levels between the control and mutants (or transfected parasites) were compared using unpaired two-tailed *t*-test. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

The role of the gene conversion in GC1-19 resistance was first tested by gene overexpression experiments. Since *ABCGs* often function as heterodimers (Graf et al., 2003; Sun et al., 2021) we co-transfected *L. infantum* wild-type cells with both the *ABCG1* and *ABCG2* (wild-type and mutated versions) genes cloned into *Leishmania* expression vectors (Papadopoulou et al., 1994). Resistance was low and surprisingly only the co-transfection of the wild-type version of *ABCG2*, and not

Table 3
Genomic loci enriched in a Cos-Seq screen performed with *L. infantum* promastigotes against the drug GC1-19.

Cosmid	Maximum fold-enrichment (selection pressure) ^a	Cosmid insert start ^b	Cosmid insert stop ^c	Gene of interest ^d	Function	EC ₅₀ (μM) ^e
Shuttle	–	–	–	–	–	3.6 ± 0.7
LINF_01	85 (8 X EC ₅₀)	–	–	LINF_010006000	monothiol glutaredoxin	3.5 ± 0.4 (1.0)
LINF_15 (a)	237 (8 X EC ₅₀)	467640	503146	LINF_150018600	tryparedoxin peroxidase	4.8 ± 0.6 (1.4 *)
LINF_15 (b)	321 (2 X EC ₅₀)	479800	519324	na	–	–
LINF_15 (c)	151 (2 X EC ₅₀)	554543	590691	na	–	–
LINF_18	102 (16 X EC ₅₀)	48186	519597	na	–	–
LINF_19	1443 (8 X EC ₅₀)	–	–	LINF_190013760	ATG8/AUT7/APG8/PAZ2 - putative	3.8 ± 2.3 (1.1)
LINF_29	391 (2 X EC ₅₀)	1218103	1252145	na	–	–

Results are shown as the mean ± SD of at least three biological replicates. * $P \leq 0.05$ by unpaired two-tailed *t*-test.

^a Maximum fold enrichment for the cosmids. The drug pressure, expressed in fold EC₅₀, at which maximum enrichment occurred is indicated within parentheses.

^b Start position (in nucleotides on the chromosome for the cosmid's insert).

^c End position (in nucleotides) on the chromosome for the cosmid's insert.

^d The genes on cosmids whose function is the most interesting in terms of drug resistance. na, all genes on cosmids code for hypothetical proteins.

^e EC₅₀ values for parasites expressing the gene of interest as part of a pSP72α-puro-α episome. Fold resistance compared to mock-transfected parasites is shown within parentheses.

the mutated one, reached significance ($p < 0.05$) (Fig. 4). To obtain evidence that the gene conversion had a role in resistance to GC1-19 we also used CRISPR-Cas9 mediated gene editing for reconstructing the gene conversion event in an otherwise *L. infantum* wild-type background. We obtained three independent transfectants (T1 to T3). The edited genome of T1 had only one allele mimicking the gene conversion event between amino acids 546–554, but with the wild-type histidine at position 568 (Table S3). T2 had both alleles edited between amino acids 546–554 but kept the wild-type H568 (Table S3). Finally, T3 had both alleles edited between amino acids 546–554, with one H568Y allele (Table S3). Both T2 and T3, but not T1, showed weak (1.5-fold) but significant ($p < 0.01$) resistance to GC1-19 (Fig. 4). The T3 edited line was not cross-resistant to trivalent antimony with an EC₅₀ of 29.3 ± 8.92 not significantly different than control cells (26.5 ± 8.01).

We further studied the mode of action and resistance mechanisms of the thiophene derivative GC1-19 using a Cos-Seq screen. We generated a new cosmid library derived from the genome of *L. infantum* LEM1317 (see Material and Methods) and transfected it into *L. infantum* JPCM5, the *L. infantum* strain the most sensitive to GC1-19 (Table 1). These transfected cells were exposed to stepwise increments of GC1-19 ($1 \times$, $2 \times$, $4 \times$, $8 \times$ and $16 \times$ the EC₅₀). Cosmids were extracted from each selection step for their characterization by NGS. The fold-enrichment of cosmids was relatively low and the maximal enrichment occurred for a cosmid containing a DNA fragment derived from chromosome 19 (Table 3). This cosmid, as well as two other cosmids derived from chromosomes 1 and 15, had genes with functions that could be remotely related to a resistance phenotype. The tryparedoxin peroxidase gene from cosmid LINF_15a, but not the other two, when cloned into a *Leishmania* expression vector and transfected into *L. infantum* produced a modest level of resistance to GC1-19 (1.4 fold; $p < 0.05$) (Table 3).

4. Discussion

Leishmania is the etiological agent of leishmaniasis, a series of neglected tropical diseases that urgently requires novel drug treatment. Indeed, the majority of ongoing phase 2 clinical trials concerns the current four licensed anti-leishmanials while varying dosing, timing, or by combining these drugs (Bush et al., 2017). Thiophene derivatives have shown experimental efficacy against various *Leishmania* species (Savornin et al., 1991; Rodrigues et al., 2015; Navin et al., 2017; Rodriguez et al., 2018; Borsari et al., 2019; Pacheco et al., 2021). In this study we further our preliminary analysis (Rodriguez et al., 2018) by testing additional piperidine derivatives and adding pyran derivatives as

well. The 2,2,6,6-tetramethyl-tetrahydro-pyran derivatives were in general less active and the presence of a protonatable nitrogen group in the 2,2,6,6-tetramethyl-piperidine derivatives probably increases basicity and solubility and thus their activity.

Our screens revealed a number of features that could help in the optimization of thiophene derivatives against *Leishmania*. First, it proved very difficult to select for drug resistant mutants against the 4 active thiophene compounds we have investigated in more details. Indeed, we could get resistance only with GC1-19 and even in this case resistance was low. This difficulty in selecting for resistance to thiophene derivatives in *Leishmania* has also been reported previously (Borsari et al., 2019). This suggests thiophene derivatives may fare well in the context of drug resistance emergence. Alternatively, it may also indicate the absence of a specific target to this class of drug, or that mutation or alteration in expression of the putative target is incompatible with life. The unlikelihood of thiophenes having a specific target gathered further support from our Cos-Seq screen with GC1-19 that did not lead to cosmids producing high levels of resistance. A similar outcome was also observed with a different thiophene derivative in *Leishmania* (Borsari et al., 2019). Our published (Gazanion et al., 2016; Bhattacharya et al., 2021) and ongoing work suggest that when a specific protein target exists for a drug, Cos-Seq will usually allow isolating a cosmid overexpressing that target. The only gene tested that produced a minimal amount of resistance was a tryparedoxin peroxidase (TryP), a key protein for protection of *Leishmania* against oxidative damage (Levick et al., 1998; Flohe et al., 2002) previously shown to also produce resistance to antimonials, the chemotherapeutic mainstay against *Leishmania* (Wyllie et al., 2010; Andrade and Murta, 2014). The weak but significant contribution of TryP in GC1-19 resistance is unlikely due to its antioxidant property however since none of the thiophene derivatives tested produced ROS. It is worth mentioning that there is no consensus on whether thiophene derivatives produce ROS (Rodriguez et al., 2018; Borsari et al., 2019; Pacheco et al., 2021), and that this is likely to depend on the actual functional groups connected to the thiophene moiety. It is salient to mention that through *in silico* docking studies, thiophene derivatives were predicted to bind to trypanothione reductase (Patterson et al., 2009; Baquedano et al., 2016; Jacomini et al., 2016; Rodriguez et al., 2018). Since TryP is an abundant protein detected in several proteomic screens (Brotherton et al., 2013, 2014), it may have the ability to bind some of the thiophene derivatives, hence reducing its bioavailability and ultimately allowing the cells to survive while sufficient amount of TryP free of thiophenes would remain to perform its natural function.

Selection of GC1-19 resistant mutants was followed by a gene conversion event between *ABCG1* and *ABCG2* in independent mutants. Resistance in *Leishmania* is usually due to gene amplification or point mutation (Leprohon et al., 2015), but gene conversion has been described previously (Ouameur et al., 2008). By gene editing we achieved in mimicking this gene conversion and proved that it contributes in a significant manner to resistance to GC1-19. The fold resistance is low but for example the clone 12.1c rev, whose genome contains the *ABCG1/2* gene conversion and many other SNPs, is only 2.1-fold resistant to GC1-19, a resistance level close to the one observed in cells edited for having the gene conversion but in an otherwise wild-type background (Fig. 4). Thus, theoretically in the genesis of thiophene derivatives against *Leishmania* one should consider the possible role of efflux pumps that may reduce efficacy. This is relevant as several reports have linked *ABCG2* SNPs to resistance to a diversity of thiophene molecules in mammalian cells (Kondo et al., 2005; Keskitalo et al., 2009; Wu et al., 2017).

5. Conclusion

In summary, we have provided further support for the activity of thiophene derivatives against *Leishmania*. We have shown that resistance selection to this class of molecules is not easy and this probably means that there is no specific protein target to thiophenes in *Leishmania*. A gene conversion event detected by a genomic analysis demonstrated the potential role of *ABCG* proteins in the susceptibilities of cells to thiophene derivatives.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2022.11.004>.

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