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Research paper

Primaquine hybrids as promising antimycobacterial and antimalarial agents

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ABSTRACT

Four series of primaquine (PQ) derivatives were screened for antitubercular and antiplasmodial activity: amides **1a-k**, ureas **2a-s**, semicarbazides **3a-c** and bis-ureas **4a-u**. Antimycobacterial activity of PQ derivatives against *Mycobacterium tuberculosis* (MTB), *M. avium* complex (MAC) and *M. avium* subsp. *paratuberculosis* (MAP) were evaluated *in vitro* and compared with PQ and the standard antitubercular drugs. In general, the PQ derivatives showed higher potency than the parent compound. Most of the compounds of series **1** and **2** showed high activity against MAP, comparable or even higher than the relevant drug ciprofloxacin, and weak or no activity against MTB and MAC. bis-Trifluoromethylated cinnamide **1k** showed low cytotoxicity and high activity against all three *Mycobacterium* species and their activities were comparable or slightly higher than those of the reference drugs. PQ urea derivatives with hydroxyl, halogen and trifluoromethyl substituents on benzene ring **2f-p** exerted very strong antimycobacterial activity towards all tested mycobacteria, stronger than PQ and the relevant standard drug(s). Unfortunately, these compounds had relatively high cytotoxicity, except bromo **2l** and trifluoromethyl **2m**, **2n** derivatives. In general, *meta*-substituted derivatives were more active than analogues *para*-derivatives. Phenyl ureas were also more active than cycloalkyl or hydroxyalkyl ureas. Semicarbazide **3a** showed similar activity as PQ, while the other two semicarbazides were inactive. Bis-urea derivatives **4** were generally less active than the urea derivatives sharing the same scaffold, differing only in the spacer type. Out of 21 evaluated bis-urea derivatives, only *p*-Cl/*m*-CF₃ phenyl derivative **4p**, benzhydryl derivatives **4t** and **4u** and bis-PQ derivative **4s** showed high activity, higher than all three reference drugs. After comparison of activity and cytotoxicity, urea **2m** and bis-urea **4u** could be considered as the most promising agents. Antimalarial potential of PQ derivatives *in vitro* against the liver stage of *P. berghei* was evaluated as well. 3-(4-Chlorophenyl)-1-[(4-[(6-methoxyquinolin-8-yl)amino]pentyl)carbamoyl]amino]urea (**4l**) was the most active compound (IC₅₀ = 42 nM; cytotoxicity/activity ratio >2000). Our results bring new insights into development of novel anti-TB and antimalarial compounds.

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Abbreviations: CPX, ciprofloxacin; FtsZ, an essential bacterial cytokinesis protein; HEK 293, human embryonic kidney cell line; hRBC, human red blood cells; HuH7, hepatocyte-derived carcinoma cell line; INH, isoniazid; L6, rat skeletal myoblasts; MAC, *M. avium* complex; MAP, *M. avium* subsp. *paratuberculosis*; MB, Middlebrook broth; MDR, multi-drug resistance; MIC, minimum inhibitory concentration; MTB, *M. tuberculosis*; OADC, Oleic-Albumin-Dextrose-Catalase supplement; POD, podophyllotoxin; PQ, primaquine; RIF, rifampicin; RMSD, root-mean-square deviation; TDR, totally drug-resistant; TB, tuberculosis; XDR, extensively drug-resistant.

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1. Introduction

Tuberculosis (TB) is still a major health concern worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease. According to the World Health Organization report, there were an estimated 10.4 million new TB cases in 2015, of which 1.0 million among children. People living with HIV accounted for 1.2 million (11%) of all new TB cases. There were an estimated 1.4 million TB deaths and an additional 0.4 million deaths resulting from TB disease among people co-infected with HIV [1]. Side-effects of current anti-TB drugs coupled with drug combination regimens and lengthy treatment durations often complicate the therapy [2]. Finally, the control of tuberculosis is challenged by drug resistance, which can range from resistance to one drug to multi-drug resistance (MDR), extensive drug-resistance (XDR) or total drug-resistance (TDR), highlighting the urgent need for new anti-TB drugs.

Much effort has been dedicated to the discovery and development of new anti-TB agents [3,4], in the past as well as today [5–8]. However, no new drugs have been introduced in anti-TB therapy for a very long time, with the exception of bedaquiline (TMC-207), a new drug to treat MDR-TB approved in 2012, while the bedaquiline-pretomanid-pyrazinamide combination is still under clinical evaluation [9]. Bedaquiline is a diarylquinoline active against MDR and XDR strains, which displays no cross-resistance with any first-line anti-TB drugs. The identification of bedaquiline as the first drug in a new class of antimycobacterial drugs attracted the attention of medicinal chemists to explore quinoline as a potential scaffold for the development of antimycobacterial drugs [10]. Quinoline related antibacterial drugs – fluoroquinolones (ciprofloxacin, ofloxacin, gatifloxacin, moxifloxacin) have been used effectively as second-line drugs for the treatment of MDR-TB. Therefore, a number of quinoline and quinolone derivatives were evaluated as potential anti-TB agents [11–14]. A recently published review by Chetty et al. provides an overview of the drugs commonly used for the treatment of TB and progress in anti-TB drug discovery [2]. In addition, it covers identification of anti-TB targets and discusses anti-TB drug candidates in different phases of the drug discovery and development.

In 2010, Laugheed and collaborators performed a medium-throughput assay using the AlamarBlue reagent to identify novel inhibitors of *M. tuberculosis* (MTB) from a library of 1514 known drugs and reported anti-TB activity of primaquine (PQ) as a novel finding [15]. PQ is an 8-aminoquinoline drug active against the liver and transmission stages of *Plasmodium* parasites, the causative agents of malaria [16]. It differs in structure from the known anti-TB drugs, raising the possibility that it could represent a novel scaffold with possibly a novel mechanism of action against MTB. PQ's ability to inhibit growth of *M. tuberculosis* in an intracellular macrophage model of infection was tested and it was found that a 5 μM concentration of PQ yielded complete inhibition of mycobacterial growth [15]. Other authors reported PQ MIC₉₉ value against replicating *M. tuberculosis* H37Rv of 80 μM [17]. Having all the above-mentioned facts in mind and possessing a huge number of home-made novel PQ derivatives designed and prepared by our research group, we wanted to explore their efficacy as potential anti-TB agents. During the last few years we have developed almost one hundred hybrid compounds of general formula PQ-spacer-R, in which R varies from hydroxyalkyl, cycloalkyl, aryl, substituted aryl to a cinnamoyl residue, while the spacer contains oxygen and nitrogen atoms determining the hybrid type as amide, urea, hydroxyurea, bis-urea, carbamate, semicarbazide or acylsemicarbazide. In several papers, we have shown that many of these PQ derivatives are efficient antiproliferative agents towards a number of tumour cell lines or highly selective against the breast

adenocarcinoma cell line MCF-7 [18–24]. In addition, many of our compounds exerted significant antimalarial activity on erythrocyte-stage of *P. falciparum* [25]. These findings motivated us to further evaluate their activity against the liver stage of the rodent malaria parasite *P. berghei*. *M. tuberculosis* and malaria parasites share some similarities in cell biology and biological processes [4] and evaluation of both anti-TB and antimalarial activity is often reported together [17,26–28]. Thus, screening for antimycobacterial and antimalarial potential across four series of PQ hybrids has been undertaken and presented in this paper.

2. Materials and methods

2.1. Chemistry

2.1.1. General information

Melting points were measured on a Stuart Melting Point (SMP3) apparatus (Barloworld Scientific, UK) in open capillaries with uncorrected values. IR spectra were recorded on FTIR Perkin Elmer Paragon 500 and UV-Vis spectra on Lambda 20 double beam spectrophotometer (Perkin-Elmer, UK). ¹H and ¹³C NMR spectra were recorded at 25 °C on NMR Avance 600 spectrometer (Bruker, Germany) at 300 and 150 MHz for ¹H and ¹³C nuclei, respectively. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates using the following solvent systems: petroleum ether/ethyl acetate/methanol 30:10:5, dichloromethane/methanol 97:3 and 95:5. Spots were visualized by short-wave UV light and iodine vapour. Column chromatography was performed on silica gel 0.063–0.200 mm (Kemika, Croatia) and 0.040–0.063 mm (Merck, Germany), with the same eluents used in TLC. All chemicals and solvents were purchased from Sigma-Aldrich (USA) and were used without further purification.

2.1.2. Synthesis

The target compounds 1–4 were obtained in moderate to excellent yields following the procedures developed by our group [18–24]. Their analytical and spectral data were fully in agreement with previously published data.

2.2. Biological evaluation

2.2.1. In vitro antimycobacterial evaluation

Mycobacterium tuberculosis H37Ra ATCC 25,177 (MTB) and well characterized clinical isolates of *M. avium* complex CIT19/06 (MAC) and *M. avium* subsp. *paratuberculosis* CIT03 (MAP) were grown in Middlebrook broth (MB), supplemented with Oleic-Albumin-Dextrose-Catalase supplement (OADC, Becton Dickinson, UK) and mycobactin J (2 $\mu\text{g}/\text{ml}$). Identification of these isolates was performed using biochemical and molecular protocols. At log phase growth, a culture sample (10 ml) was centrifuged at 15,000 rpm/20 min using a bench top centrifuge (Model CR 4–12, Jouan Inc., UK). Following removal of the supernatant, the pellet was washed in fresh Middlebrook 7H9GC broth and re-suspended in fresh supplemented MB (10 ml). The turbidity was adjusted to match McFarland standard No. 1 (3×10^8 cfu) with MB broth. A further 1:20 dilution of the culture was then performed in MB broth. The antimicrobial susceptibility of all three mycobacterial species was investigated in a 96-well plate format. In these experiments, sterile deionised water (300 μl) was added to all outer-perimeter wells of the plates to minimize evaporation of the medium in the test wells during incubation. Each evaluated compound (100 μl) was incubated with each of the mycobacterial species (100 μl). Dilutions of each compound were prepared in duplicate. For all synthesized compounds, final concentrations ranged from 1 mg/ml to 2 $\mu\text{g}/\text{ml}$. All compounds were prepared in DMSO and subsequent dilutions

were made in supplemented MB. The plates were sealed with parafilm and incubated at 37 °C, for 5 days in the case of *M. avium* complex, 7 days in the case of *M. tuberculosis*, and 11 days in the case of *M. avium* subsp. *paratuberculosis*. Following incubation, a 10% addition of AlamarBlue (AbD Serotec, Kidlington, UK) was mixed into each well and readings at 570 nm and 600 nm were taken, initially for background subtraction and subsequently after 24 h reincubation. The background subtraction was necessary for strongly coloured compounds, where the colour may have interfered with the interpretation of any colour change. For non-interfering compounds, a blue colour in the well was interpreted as an absence of growth and a pink colour was scored as growth. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound at which no visible bacterial growth was observed, i.e. the MIC is the lowest concentration that prevented a visual colour change from blue to pink. The MIC value is routinely and widely used in bacterial assays and is a standard detection limit according to the Clinical and Laboratory Standards Institute (CLSI) [29]. Isoniazid (INH), rifampicin (RIF) and ciprofloxacin (CPX) (Sigma-Aldrich) were used as the standards, as they are clinically used antimycobacterial drugs.

2.2.2. Evaluation of *in vitro* activity against *P. berghei* hepatic stages

Compound effect on *P. berghei* infection of a human hepatoma cell line was assessed employing a luminescence-based method, as previously described [30]. Briefly, hepatic infection was determined by measuring the luminescence intensity of lysates of HuH7 cells infected with a firefly luciferase-expressing *P. berghei* line. HuH7 cells (1.0×10^4 per well) were seeded in 96-well plates the day before infection. One hour prior to infection the medium was replaced by medium containing the appropriate drug concentrations. Addition of 1.0×10^4 sporozoites was followed by centrifugation at $1800 \times g$ for 5 min and parasite infection load was measured 48 h after parasite addition by a bioluminescence assay (Biotium, USA) using a multi-plate reader Infinite M200 (Tecan, Switzerland). The effect of the different treatments on the viability of HuH7 cells was assessed by the CellTiter-Blue assay (Promega, USA) according to the manufacturer's protocol. Nonlinear regression analysis was employed to fit the normalized results of the dose-response curves, and IC_{50} values were determined using GraphPad Prism V 5.0.

2.2.3. *In vitro* cytotoxicity on L6 cells

Assays were performed in 96-well microtiter plates, each well containing 100 μ l of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% foetal bovine serum, and 4000 L-6 cells (a primary cell line derived from rat skeletal myoblasts) [31,32]. Serial drug dilutions of eleven 3-fold dilution steps covering a range from 100 to 0.002 μ g/ml were prepared. After 70 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. 10 μ l of Alamar Blue was then added to each well and the plates incubated for another 2 h. Then the plates were read with a microplate fluorometer using an excitation wave length of 536 nm and an emission wave length of 588 nm. The IC_{50} values were calculated by linear regression from the sigmoid dose inhibition curves using SoftmaxPro software (Molecular Devices Cooperation, USA) [33]. Podophyllotoxin (POD) was used as a control.

2.2.4. Docking study – examination of putative binding site in FtsZ

To examine binding of eight selected ligands (**1a**, **1k**, **2h**, **2m**, **2p**, **4p**, **4s**, **4t**) to FtsZ of *Mycobacterium tuberculosis* (Mtb-FtsZ), a docking study was conducted [8]. FtsZ is an essential protein for bacterial cell division and a promising therapeutic target [34].

Screening experiments were conducted on protein crystal structures of head-to-head lateral dimers of Mtb-FtsZ with GTP γ S (1RLU) and GDP (1RQ7), which are available in the RCSB Protein Data Bank [35], and AutoDock 4.2.6. (The Scripps Research Institute, USA) [36]. Before docking, hydrogen atoms were added to the proteins. All Lys, Arg, His, and Cys side-chains were protonated, all Asp and Glu side-chains were deprotonated, and the amino and carboxy termini were charged. The three-dimensional forms of the ligands were drawn and their initial geometries were optimized in HyperChem 8.0 (Hypercube, Inc., Gainesville, FL, USA). Their charge was set to represent the most abundant species at pH 7.4, calculated at chemicalize.com. Afterward, ligands were read in the AutoDock software in a compatible file format and partial charges were set according to Ionescu et al. [37]. For all ligands and both proteins, a grid map of size $126 \times 126 \times 126$ Å was generated with 0.375 Å spacing centered on the coordinates of the GTP γ S or the GDP molecule by the AutoGrid program [36] and Lamarckian genetic algorithm (LGA) [38] was applied. The receptor molecule was regarded as rigid while all ligand single bonds were allowed to rotate freely during the Monte Carlo simulated annealing procedure. Ligand flexible docking simulations were performed with 100 runs, population size of 150, 25,000,000 energy evaluations, 27,000 numbers of generations, rate of gene mutation of 0.02, and rate of crossover 0.08. Root-mean-square deviation (RMSD) of 2.0 Å was used as a criterion for cluster analysis of the docking results.

3. Results and discussion

3.1. Chemistry

PQ hybrids **1–4** share the common pattern and consist of three building blocks: PQ-spacer-variable aromatic/aliphatic region. According to the functional group present in the spacer area one could classify them as amides **1**, ureas **2**, semicarbazides **3** and bis-ureas **4**. Their general structures are revealed in [Table 1](#).

Synthetic routes for the preparation of the PQ hybrids **1–4** have been previously described by our group and vary from simple condensation reactions (preparation of cinnamamides **1**) to more complex, multi-step reactions leading to derivatives **2–4**.

3.2. Biological evaluation

PQ derivatives **1–4** were evaluated *in vitro* for their activity against three different *Mycobacterium* species and liver-stages of *P. berghei*. The corresponding assays carried out are described in detail in the Experimental Section. Antimycobacterial evaluation was performed against *M. tuberculosis* H37Ra ATCC 25,177 (MTB), well characterized clinical isolates of *M. avium* complex CIT19/06 (MAC) and *M. avium* subsp. *paratuberculosis* CIT03 (MAP). PQ diphosphate and three of the main frontline anti-TB drugs INH, RIF and CPX were used as the standard drugs. The results are summarized in [Table 2](#).

In order to better estimate the potential of the selected PQ derivatives as antitubercular and antiplasmodial agents, the compounds' cytotoxicity was assessed against a mammalian cell line derived from rat skeletal myoblasts (L6). Cytotoxicity data are presented in [Table 2](#). The numbers displayed are the average values obtained from two replicate experiments. Cytotoxicity data of PQ derivatives **2i**, **5c**, **5q** and **5s** on L6 cells [22] and compounds **2f**, **2l**, **2n** and **2o** on human embryonic kidney (HEK) 293 cell line and their haemolytic activity on fresh human red blood cells (hRbc) were previously reported [23]. The highest haemolysis was observed for compound **2n** (7.85% at 100 μ g/ml concentration), while other compounds showed very low haemolytic potential. The experiments performed on HEK cells showed low toxicity (**2f** and

Table 1
Structures of the tested compounds: amides **1a-k**, ureas **2a-s**, semicarbazides **3a-c** and bis-ureas **4a-u**.

R	
-NH ₂	
-PQ-residue	

1a-k

2a-s

3a-c

4a-u

2n) or no toxicity (**2l**). However, **2o** showed strong cytotoxicity, starting from concentration 3.125 µg/ml. Cytotoxicity of the tested PQ hybrids towards a number of cancer cell lines was also previously described [18–24].

In general, the PQ derivatives investigated here displayed higher antimycobacterial potency than the parent compound. As can be expected for compounds which do not necessarily attain their antimycobacterial effect through interaction with the same target sites, structural features important for activity differ between the respective series of compounds. Most of the compounds of series **1** and **2** showed high activity against MAP, were found to be comparable or even higher than the relevant drug CPX, and displayed weak or no activity against the other two mycobacteria. Compounds of series **1** are PQ-cinnamic acid hybrids. The covalent attachment of cinnamoyl moiety induced a significant increase in potency compared to the parent drug. This is not surprising, since antimicrobial activity of cinnamic acid derivatives is well documented [40]. In addition, some cinnamic acid derivatives have shown antitubercular activity in low micromolar scale and good safety profiles [41–44]. Methoxy derivatives **1c–e** showed the lowest cytotoxicity, followed by trifluoromethyl derivatives **1j** and **1k**.

Cinnamamide **1a** and bis-trifluoromethylated cinnamamide **1k** were active against all three *Mycobacterium* species in comparable (MAC), slightly higher (MTB) or lower concentrations (MAP) than the reference drugs. However, compound **1k** showed much lower cytotoxicity towards murine L6 cell line than cinnamamide **1a**. PQ-urea derivatives with hydroxyl, halogen and trifluoromethyl substituted benzene ring **2f–p** exerted very strong antimycobacterial activity towards all three tested bacteria, stronger than PQ and the reference drugs. Unfortunately, these compounds had relatively high cytotoxicity, except bromo **2l** and trifluoromethyl derivatives **2m** and **2n**. Comparison of *meta*-substituted derivatives with their *para*-substituted partners (**1i** vs. **1j**, **2f** vs. **2g**, **2h** vs. **2i**, **2j** vs. **2k**, **2m** vs. **2n**) revealed a clear preference for *meta*-position over the *para*. The benzene substituted ureas were also more active than the urea derivatives with cycloalkyl or hydroxyalkyl moieties. Lipophilicity is an important requirement for antimalarial, but more so antimycobacterial agents because of the unusual lipid-rich cell wall of mycobacteria [40]. Log *P* of the most active urea derivatives **2f–n** ranged from 3.07 to 4.25. The more lipophilic derivatives with bulky lipophilic substituents **2q** and **2r** (log *P* 5.08 and 5.46, respectively) were inactive (and less toxic), suggesting that such substituents are not preferred for antimycobacterial activity. However, Cl/CF₃-derivative **2p** (log *P* 4.85) was active and its activity was similar to activity of CF₃-cinnamamide **1k**. Semicarbazide **3a** showed similar activity as PQ,

while the other two semicarbazides were inactive. In general, bis-urea derivatives **4a–n** were less active than the analogous urea derivatives sharing the same substituents, differing only in the spacer type. Bis-urea derivatives have a double number of heteroatoms than the urea analogues and consequently higher distance from the PQ and alkyl/aryl substituents on the opposite wings of the molecules. In addition, more oxygen and nitrogen atoms in the spacer, reduced log *P* values for 0.92 (**2f/2g** vs. **4g/4h**, **2h/2i** vs. **4i/4j**, **2j/2k** vs. **4k/4l**, **2l** vs. **4m**, **2m/2n** vs. **4n/4o**). Out of 21 evaluated bis-urea derivatives only *p*-Cl/*m*-CF₃ phenyl derivative **4p**, benzhydryl derivatives **4t** and **4u** and bis-PQ derivative **4s** showed high activity, higher than all three reference drugs. All these compounds bear very lipophilic residue, which compensates the hydrophilic central part and elevates log *P* values up to 3.93. Among them, **4u** had the lowest cytotoxicity.

Antimalarial screening of PQ derivatives was performed *in vitro* against *P. berghei* liver-stages. Two concentrations of the tested compounds were evaluated: 1 and 10 µM. For comparison, PQ at the same concentrations was included in the assay as a positive control and DMSO as a negative control (Fig. 1).

As shown in Fig. 1, the majority of the tested compounds were more active than the parent drug and their activities were concentration depended. For two selected compounds from series **1** and five compounds from series **4**, IC₅₀ were determined. IC₅₀ values ranged from 42 nM to 2.4 µM. The results are summarized in Table 3.

Screening of compound activity against *P. berghei* hepatic stages showed that bis-urea derivatives **4** were superior to other candidates. Chloro-derivative **4l** was the most active compound (IC₅₀ = 42 nM), followed by trifluoromethyl **4o** (IC₅₀ = 74.9 nM) and bromo-derivative **4m** (IC₅₀ = 0.18 µM). All three derivatives share the same spacer in the central part of the molecules, PQ and *para*-substituted benzene moieties on the wings, and vary only in substituent type. The activity decreases from chloro-, to CF₃- and bromo-derivative. Analogues fluoro-derivative **4j** showed approximately 45-fold decrease in activity compared to the chloro-derivative (IC₅₀ = 1.87 µM). The results obtained for the liver-stages do not match the results reported for the erythrocyte-stage antimalarial screening [25]. Such results could be expected since these two activities do not involve the same mechanisms and different drugs are being used to stop the various stages of *Plasmodium* life-circle. IC₅₀ values for erythrocyte-stage were from 25- to 425-fold higher, confirming that our compounds were significantly more active towards the liver-stage of infection (Table 3). However, we found an interesting correlation with the previously reported antiproliferative screening [23]. All three compounds showed a high and selective activity towards MCF-7 cancer cell line

Table 2
Antimycobacterial screening of primaquine derivatives *in vitro* against *M. tuberculosis*, *M. avium* complex and *M. avium* subsp. *paratuberculosis* and cytotoxicity against L6 cells.

Compd.	Structural formula	log P ^a	MIC (µg/ml)			Cytotox. µg/ml	Ref. ^b
			MTB	MAC	MAP		
1a		3.82	16	32	16	7.2	24
1b		4.22	–	–	–	>100	24
1c		3.66	64	128	64	>100	24
1d		3.51	128	256	128	>100	24
1e		3.35	256	256	256	>100	24
1f		3.44	64	64	64	12.58	24
1g		4.43	64	128	64	7.1	24
1h		3.96	32	128	64	1.5	24
1i		4.70	32	128	32	7.4	24
1j		4.70	32	128	64	73.1	24
1k		5.58	16	64	16	83.1	24
2a		0.66	32	64	64	–	19
2b		0.72	64	64	64	–	19
2c		2.71	16	32	16	–	18

(continued on next page)

Table 2 (continued)

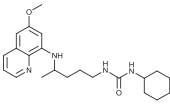
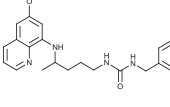
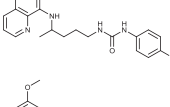
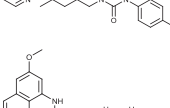
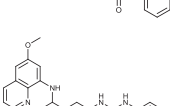
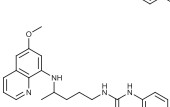
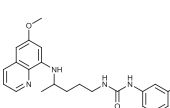
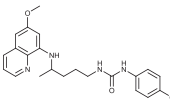
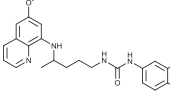
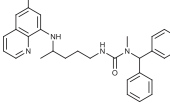

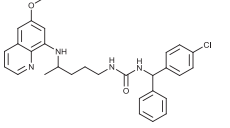
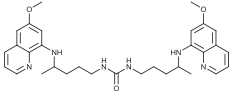
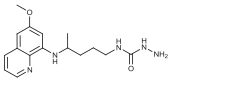
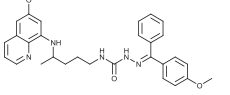
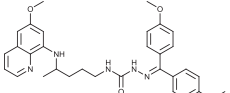
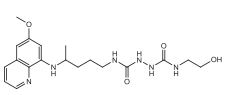
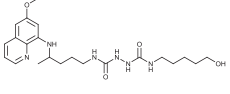
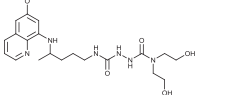
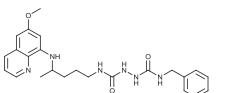
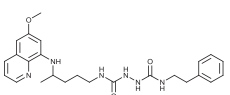
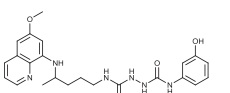
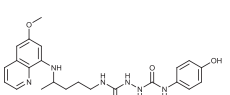
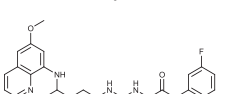
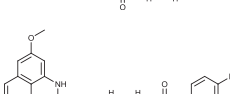
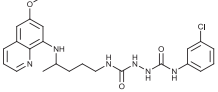
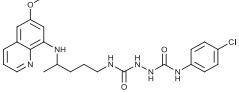
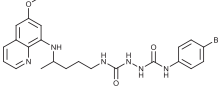
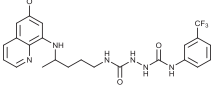
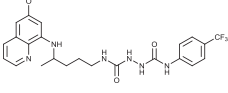
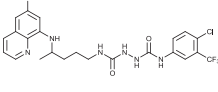
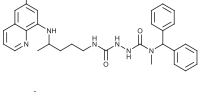
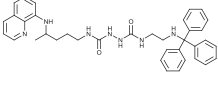
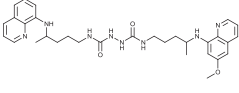
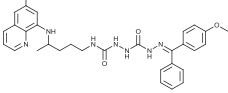
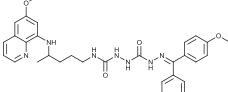
Compd.	Structural formula	log P ^a	MIC (µg/ml)			Cytotox. µg/ml	Ref. ^b
			MTB	MAC	MAP		
2d		3.15	16	32	16	—	18
2e		1.86	32	32	32	—	18
2f		3.07	8	16	16	38	23
2g		3.07	128	256	128	14.8	23
2h		3.51	2	8	4	9.18	23
2i		3.51	8	16	16	9.30	23
2j		3.97	16	16	16	10.0	23
2k		3.97	16	64	32	—	23
2l		4.14	8	16	16	200 ^c	23
2m		4.25	2	2	2	150	23
2n		4.25	4	16	8	200 ^c	23
2o		5.13	—	—	—	7.67 ^c	23
2p		4.85	16	16	16	—	21
2q		5.08	128	128	128	80.5	21

Table 2 (continued)

Compd.	Structural formula	log P^a	MIC ($\mu\text{g/ml}$)			Cytotox. $\mu\text{g/ml}$	Ref. ^b
			MTB	MAC	MAP		
2r		5.46	128	128	128	70.2	21, 22
2s		3.62	128	256	128	>100	21
3a		0.83	32	32	32	—	21
3b		4.86	128	256	128	>100	21
3c		4.70	128	256	128	>100	21
4a		-0.26	—	—	—	—	21
4b		-0.20	256	256	256	—	21
4c		0.76	256	256	256	—	21
4d		-0.73	256	256	256	79.9	21, 22
4e		2.15	128	256	128	—	20
4f		2.44	128	256	128	—	20
4g		2.14	128	256	128	—	23
4h		2.14	128	256	128	—	23
4i		2.59	128	256	128	—	23
4j		2.59	—	—	—	—	23

(continued on next page)

Table 2 (continued)

Compd.	Structural formula	log P ^a	MIC (µg/ml)			Cytotox. µg/ml	Ref. ^b
			MTB	MAC	MAP		
4k		3.05	128	256	128	27.6	23
4l		3.05	128	256	128	43.3	23
4m		3.22	128	256	128	20.0	23
4n		3.32	128	128	128	16.2	23
4o		3.32	128	128	128	7.5	23
4p		3.93	2	2	2	30.0	21
4q		4.16	64	64	64	5.6	21
4r		5.22	128	128	128	>65	21
4s		2.70	2	16	4	46.2	21, 22
4t		3.93	2	16	4	9.1	21
4u		3.78	2	16	4	81.6	21
PQ diphosphat			32	32	32	27.1	
INH			5.0	>256	>256	–	
RIF			8.0	30	90	–	
CPX			16	60	60	–	
POD			–	–	–	0.007	22

MIC – minimum inhibitory concentration, a concentration that inhibits visible growth of the bacteria; MTB – *M. tuberculosis* H37Ra ATCC 25,177; MAC – *M. avium* complex CIT19/06; MAP – *M. avium* subsp. *paratuberculosis* CIT03; INH – isoniazid; RIF – rifampicin; CPX – ciprofloxacin; POD – podophyllotoxin.

^a calculated with [Chemicalize.org](https://www.chemicalize.org) program [39].

^b reference for synthesis.

^c cytotoxicity tested on HEK 293 cell line [23].

(IC₅₀ = 0.10 µM) and very weak or negligible activity (chloro- and bromo-derivatives) or medium activity (CF₃-derivative) towards the other tested cancer cell lines. This observation suggests that antiproliferative activity against MCF-7 and antimalarial activity might share the same/similar mechanism.

PQ-cinnamamides **1a-k** exerted significant activities in low micromolar range, similar in liver-stage and erythrocyte-stage antimalarial screening [25]. Dual antimalarial activities of the related cinnamic acid hybrids derived from PQ or other quinoline antimalarial drug against both erythrocyte and liver-stages of

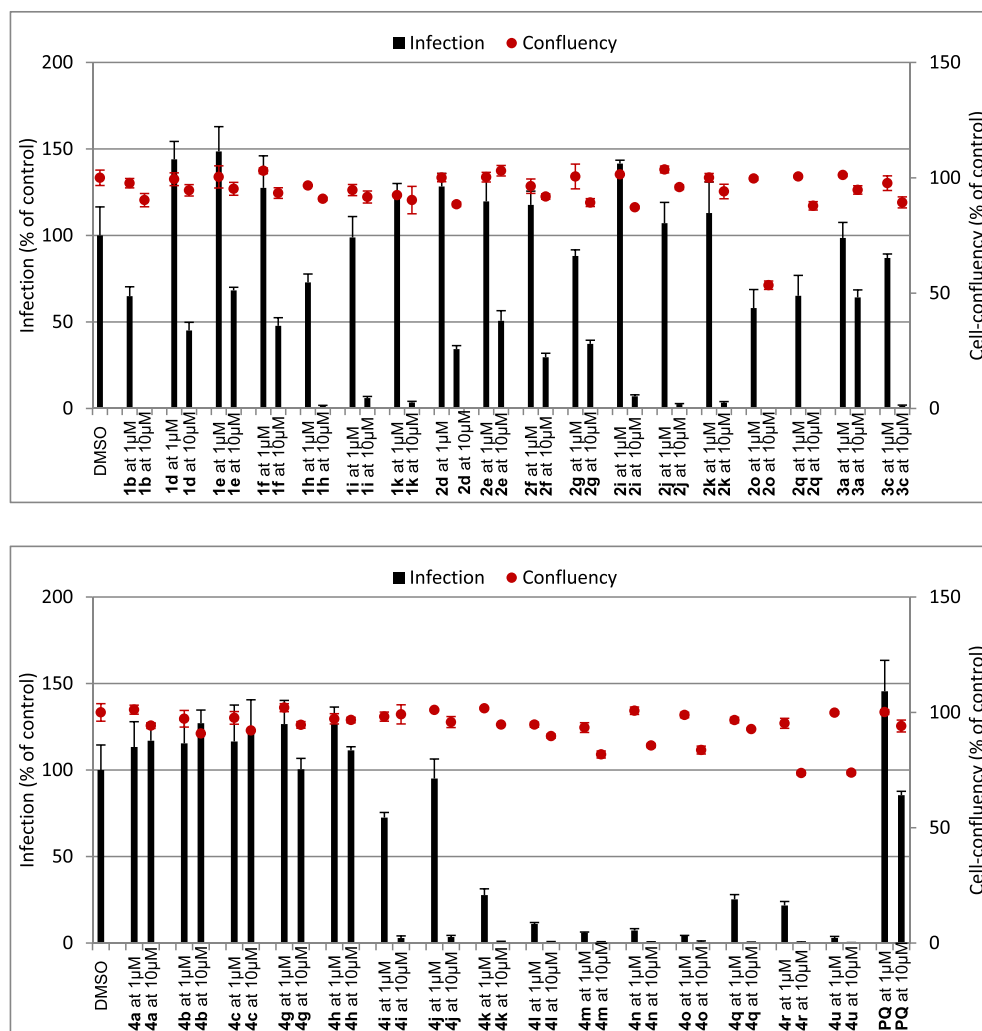


Fig. 1. Activity of compounds 1–4 and PQ against *P. berghei* liver-stages at 1 and 10 μM concentrations. Anti-infective activity (infections scale, bars) are shown.

Plasmodium parasite have been reported by other authors [45–49]. Our results confirmed these findings.

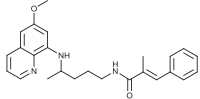
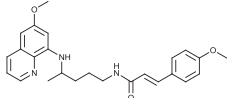
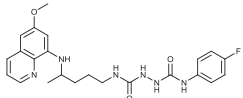
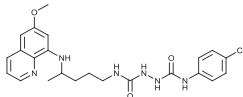
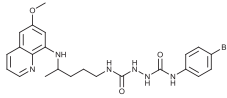
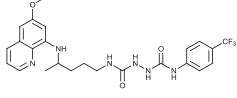
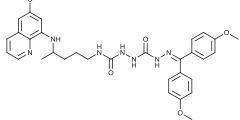
Quinolines, particularly hydroxy- and/or aminoquinolines, show a wide range of biological effects [50,51]. It was found that hydroxy- and aminoquinolines display activity against replicating MTB and non-replicating MTB, and similar activity against both susceptible and mono-MDR-TB strains, which suggests that their mechanism of action might differ from the mechanism of known antimycobacterial drugs [15,52,53]. Although it is known that these compounds are able to chelate iron, copper, manganese, magnesium, zinc and other metal ions [54,55], recent investigations suggested that the mode of action of these compounds is more complex than the deprivation of metals essential to the bacteria. It was found that anti-TB active ring-substituted quinolines exhibit various mechanisms of action, including the inhibition of mycobacterial gyrase, ATP synthase, FtsZ protein, glutathione S-transferase, enoyl-ACP reductase, decaprenylphosphoryl- β -D-ribose-2'-epimerase (DprE1) or FadD32 [14,52,56–63]. As such, it might be expected that the PQ derivatives described here, like many other quinoline-based compounds, may constitute multitarget agents [10]. The fact that structural features important for activity differ between the four series of compounds tested suggests that their antimycobacterial action is not a consequence of interaction with the same target sites. Although the identification of the underlying

mechanism is beyond the scope of the current manuscript, *in silico* binding of the most potent PQ derivatives (1a, 1k, 2h, 2m, 2p, 4p, 4s, 4t) to Mtb-FtsZ, an essential protein for bacterial cell division, was studied. The docking studies indicate that the selected ligands do not interfere with FtsZ, as no conclusive binding sites for selected ligands have been identified. Even though binding energies of up to -7.5 kcal/mol were estimated, in most cases no significant clustering was found (1–2 molecules), preventing the determination of the putative binding site. These results can also be explained by a very high flexibility of examined ligands with a large number of single bonds which bind unselectively to different proteins, thus making them very promiscuous. Additionally, the problem may also lie in the treatment of the protein molecule as rigid, as opposed to flexible ligands, or the possibility that the ligands tested do not bind to the proposed proteins.

4. Conclusions

Antimycobacterial and antiplasmodial properties of four series of compounds that contain the key PQ pharmacophore were evaluated: amides 1, ureas 2, semicarbazides 3 and bis-ureas 4. In general, the PQ derivatives investigated had higher antimycobacterial potency than the parent compound. Urea derivatives with hydroxyl, halogen and particularly trifluoromethyl substituted

Table 3
Comparison of IC₅₀ of PQ-derivatives in antimalarial screening *in vitro* against liver-stage of *P. berghei* and previously reported erythrocyte-stage of *P. falciparum* [25].

Compd.	Structural formula	Liver-stage	Erythrocyte-stage	Erythrocyte-stage/liver-stage IC ₅₀ ratio
		IC ₅₀ (nM)	IC ₅₀ (μM)	
1b		723.8	>24.8	34.3
1k		2393.3	12.4	5.1
4j		1873.0	–	–
4l		42.0	17.8	423
4m		181.7	17.8	98
4o		74.9	10.3	137.5
4u		82.8	2.2	26.6

benzene ring **2f–p** exerted very strong antimycobacterial activity towards all three tested mycobacteria, while amides from series **1** showed selectivity against MAP, but only weak or negligible activity against the other two mycobacteria. Semicarbazide **3a** showed similar activity as PQ. In the bis-urea series only four derivatives showed strong activity (**4p**, **4s**, **4t** and **4u**). *N*¹-(4-chloro-3-trifluoromethylphenyl)-*N*²-(4-((6-methoxyquinolin-8-yl)amino)pentyl)hydrazine-1,2-dicarboxamide (**4p**), 2-(2-(bis(4-methoxyphenyl)methylene)hydrazinecarbonyl)-*N*-(4-((6-methoxyquinolin-8-yl)amino)pentyl)hydrazinecarboxamide (**4u**) and 1-(4-[(6-methoxyquinolin-8-yl)amino]pentyl)-3-[3-(trifluoromethyl)phenyl]urea (**2m**) were the most active compounds among 54 compounds tested. The fact that two of them possess trifluoromethyl group makes them good candidates for further drug development. Fluorine is recognized as the second favourite heteroatom after nitrogen in the current drug design since the introduction of fluorine atom or organofluorine groups in biologically active compounds often induces beneficial pharmacological properties [8,42]. In addition, compounds **2m** and **4p** are fully in agreement with the Lipinski's and Gelovani's rules for prospective small molecular drugs [23,24], while **4u** shows minimal aberrations. Taking into account cytotoxicity data as well, PQ derivatives **2m** and **4u** may be considered as the leading compounds for the development of new anti-TB drugs.

Further, we confirmed that most of the PQ derivatives were active against liver stage *P. berghei* parasites in low micromolar or nanomolar scale, with 3-(4-chlorophenyl)-1-[(4-[(6-methoxyquinolin-8-yl)amino]pentyl)carbonyl]amino]urea (**4l**)

displaying the highest activity (IC₅₀ = 42 nM). Cytotoxicity on L6 cells observed for **4l** was 43.3 μg/ml (91.9 μM), indicating very high cytotoxicity/activity ratio (>2000). Our results bring new insights into development of dual action antimalarial agents.

Acknowledgments

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