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Investigating the antiplasmodial activity of primary sulfonamide compounds identified in open source malaria data

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ABSTRACT

In the past decade there has been a significant reduction in deaths due to malaria, in part due to the success of the gold standard antimalarial treatment - artemisinin combination therapies (ACTs). However the potential threat of ACT failure and the lack of a broadly effective malaria vaccine are driving efforts to discover new chemical entities (NCEs) to target this disease. The primary sulfonamide (PS) moiety is a component of several clinical drugs, including those for treatment of kidney disease, glaucoma and epilepsy, however this chemotype has not yet been exploited for malaria. In this study 31 PS compounds sourced from the GlaxoSmithKline (GSK) Tres Cantos antimalarial set (TCAMS) were investigated for their ability to selectively inhibit the *in vitro* growth of *Plasmodium falciparum* asexual stage malaria parasites. Of these, 14 compounds were found to have submicromolar activity (IC_{50} 0.16–0.89 μM) and a modest selectivity index (SI) for the parasite versus human cells (SI > 12 to >43). As the PS moiety is known to inhibit carbonic anhydrase (CA) enzymes from many organisms, the PS compounds were assessed for recombinant *P. falciparum* CA (*Pf*CA) mediated inhibition of CO_2 hydration. The *Pf*CA inhibition activity did not correlate with antiplasmodial potency. Furthermore, no significant difference in IC_{50} was observed for *P. falciparum* versus *P. knowlesi* (P > 0.05), a *Plasmodium* species that is not known to contain an annotated *Pf*CA gene. Together these data suggest that the asexual intraerythrocytic stage antiplasmodial activity of the PS compounds examined in this study is likely unrelated to *Pf*CA inhibition.

1. Introduction

Malaria remains one of the world's most important infectious diseases, causing approximately 438,000 deaths in 2015, mainly African children under the age of five (WHO, 2015). While it is possible that the first generation RTS,S malaria vaccine will be employed in some regions in the future, the World Health Organization (WHO) remains cautious and recommends that other malaria prevention and treatment strategies continue, including the development of new drugs (WHO, 2016). This recommendation is driven by the threat of malaria parasite resistance or reduced clinical efficacy emerging to all current antimalarial drugs including the gold standard artemisinin combination therapies (ACTs) (WHO, 2015; Fairhurst and Dondorp, 2016). Added to this, the majority of agents in the current antimalarial drug development portfolio are based on known antimalarial pharmacophores (Wells et al., 2015), which may compromise their widespread use due to potential issues of cross resistance. With few antimalarial chemical classes (e.g. spiroindoline, imidazolepiperazine and triazolopyrimidine chemotypes) presently under advanced development (MMV, 2016) there is an urgent need to ensure that the antimalarial drug discovery pipeline is primed with new chemical entities, ideally those with novel modes of action to avoid cross-resistance to existing drugs. The primary sulfonamide (PS) chemotype is not currently

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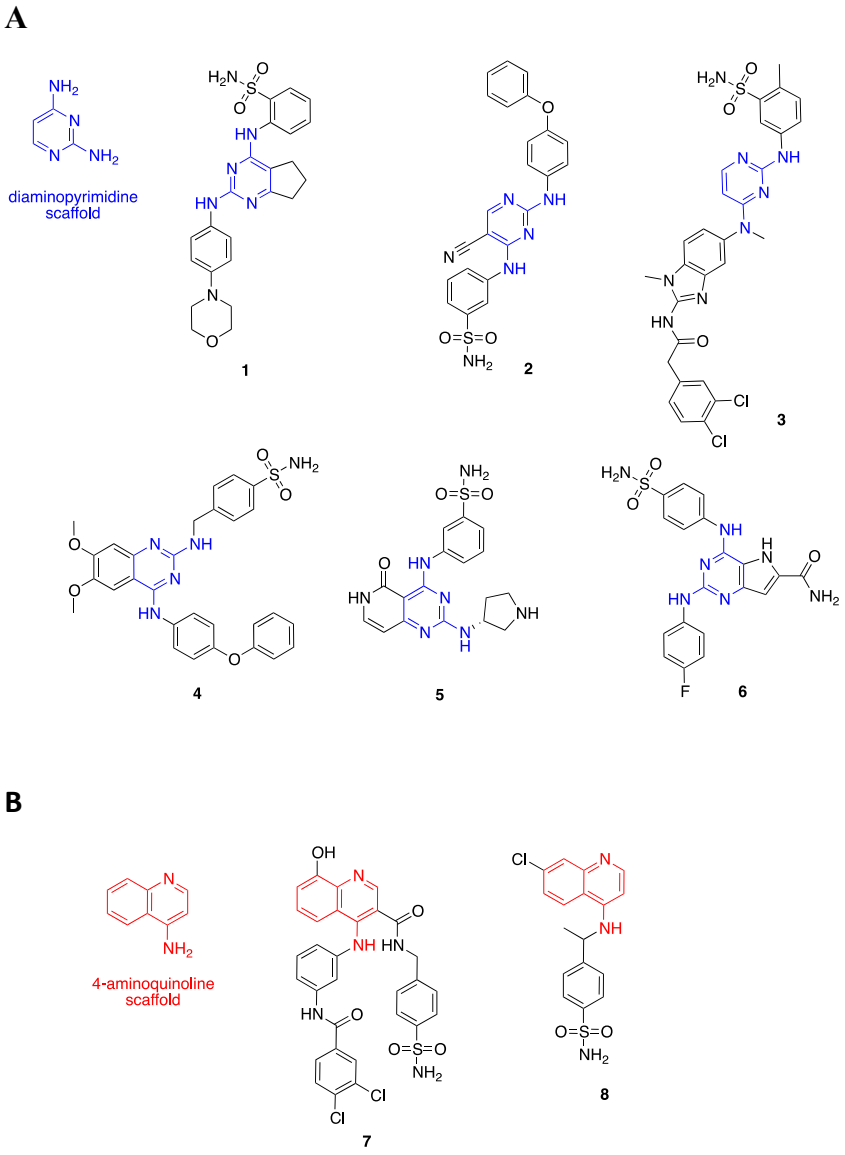

Fig. 1. Structures of TCAMS PS compounds with an antimalarial pharmacophore. Diaminopyrimidine based compounds highlighted in blue; 4-aminoquinoline based compounds highlighted in red. Compound **4** and **6** were tested as the formate salt, compounds **3**, **5**, **7** were tested as the trifluoracetate salt. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used for malaria prevention or treatment, but has a proven track record for treatment of other diseases, including glaucoma, renal disorders and epilepsy (Poulsen, 2010; Supuran, 2010; Pastorek and Pastorekova, 2015; Supuran and Winum, 2015).

In previous work we identified several PS glycosides with moderate *in vitro* antiplasmodial activity (50% growth inhibitory concentration (IC_{50}) ~1 μM) and selectivity for the parasite versus human cells (Selectivity Index (SI) > 40) (Andrews et al., 2013). Published work from another group identified a thioureido benzenesulfonamide with similar *in vitro* activity against *P. falciparum* (IC_{50} ~1 μM) and *in vivo* activity against *P. berghei* in a mouse malaria model (ID_{50} 10 mg/kg) (Krungkrai et al., 2008). Additional evidence that PS compounds have antimalarial potential comes from high throughput screening of a GlaxoSmithKline (GSK) library of ~2,000,000 compounds. The results of the GSK screen led to compilation of the Tres Cantos antimalarial set (TCAMS), with data made publicly available as a resource for antimalarial lead identification and basic research into the "druggable" genome of *P. falciparum* through deposition in the open access European Bioinformatics Institute ChEMBL Neglected Tropical Disease archive. The TCAMS dataset contains ~13,500 compounds that inhibit the *in vitro* growth of drug-sensitive (3D7) and multi-drug resistant (Dd2) *P. falciparum* parasites (≥80% and ≥50% at 2 μM, respectively) (Gamo et al., 2010). Following a substructure search of this open source malaria data, we identified 31 PS compounds (Figs. 1 and 2) that were subsequently provided by GSK and investigated in this study.

PS compounds are known to inhibit carbonic anhydrase (CA) enzyme activity in many organisms (Supuran, 2008). CA enzymes maintain an important physiological equilibrium: the hydration of carbon dioxide to bicarbonate anion and a proton: $H_2O + CO_2 \leftrightarrows HCO_3^- + H^+$ and are responsible for HCO_3^- and pH homeostasis, including within erythrocytes. Malaria parasite CA inhibitors were first suggested as a potential new class of antimalarials in 1998 (Sein and Aikawa, 1998) and later the esterase activity of *P. falciparum* CA (*Pf*CA; PlasmoDB (Aurrecoechea et al., 2009) gene

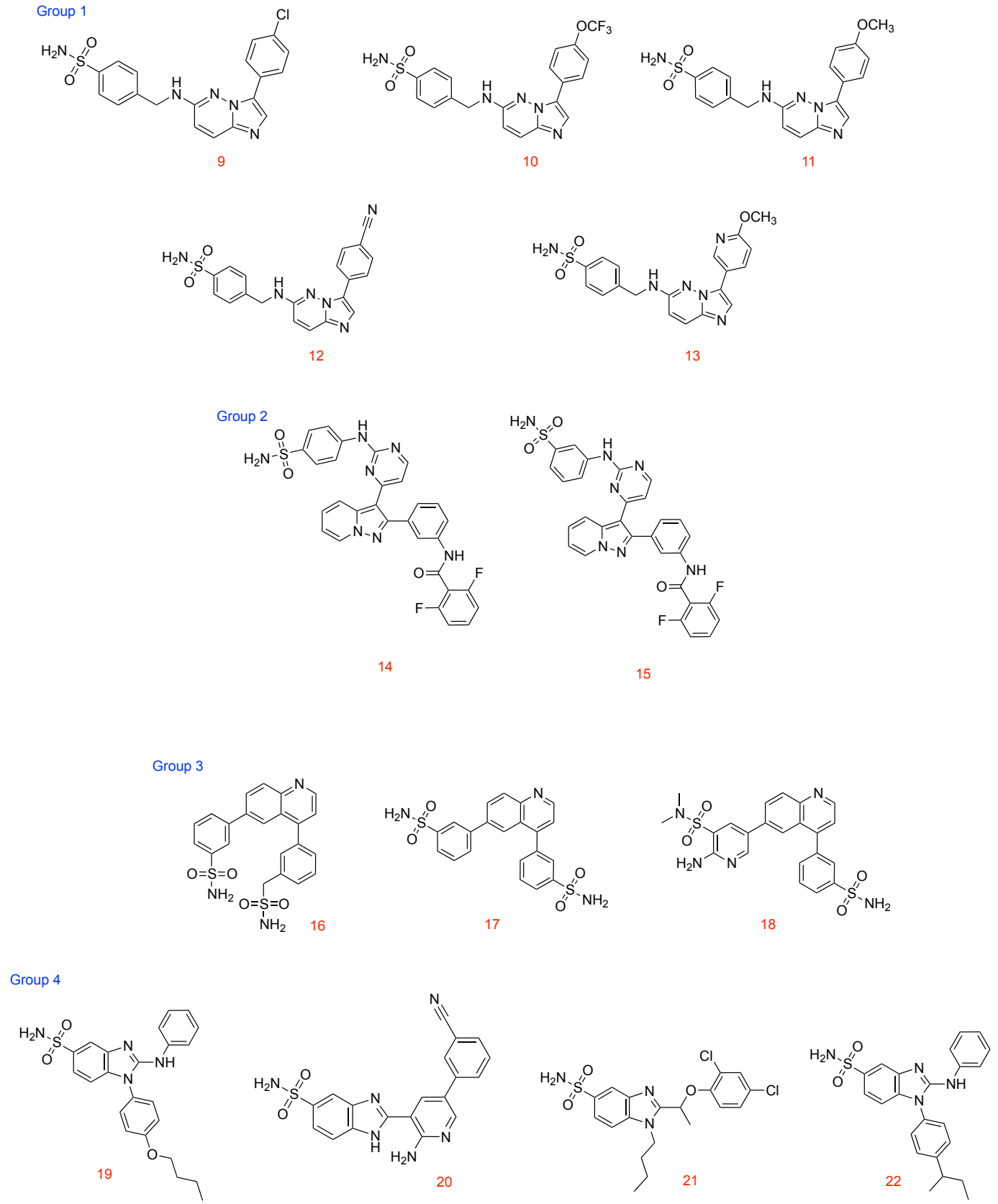

Fig. 2. Structures of TCAMS PS compounds with no antimalarial pharmacophore. Group 1 - Imidazo[1,2-*b*]pyridazines; Group 2 - 2-Pyrimidinyl pyrazolopyridines; Group 3 – Quinolines; Group 4 – Benzimidazoles; Group 5 – Quinazolinamines and Group 6. Trifluoracetate salt - **18**, **24** Hydrochloride salt -**23**.

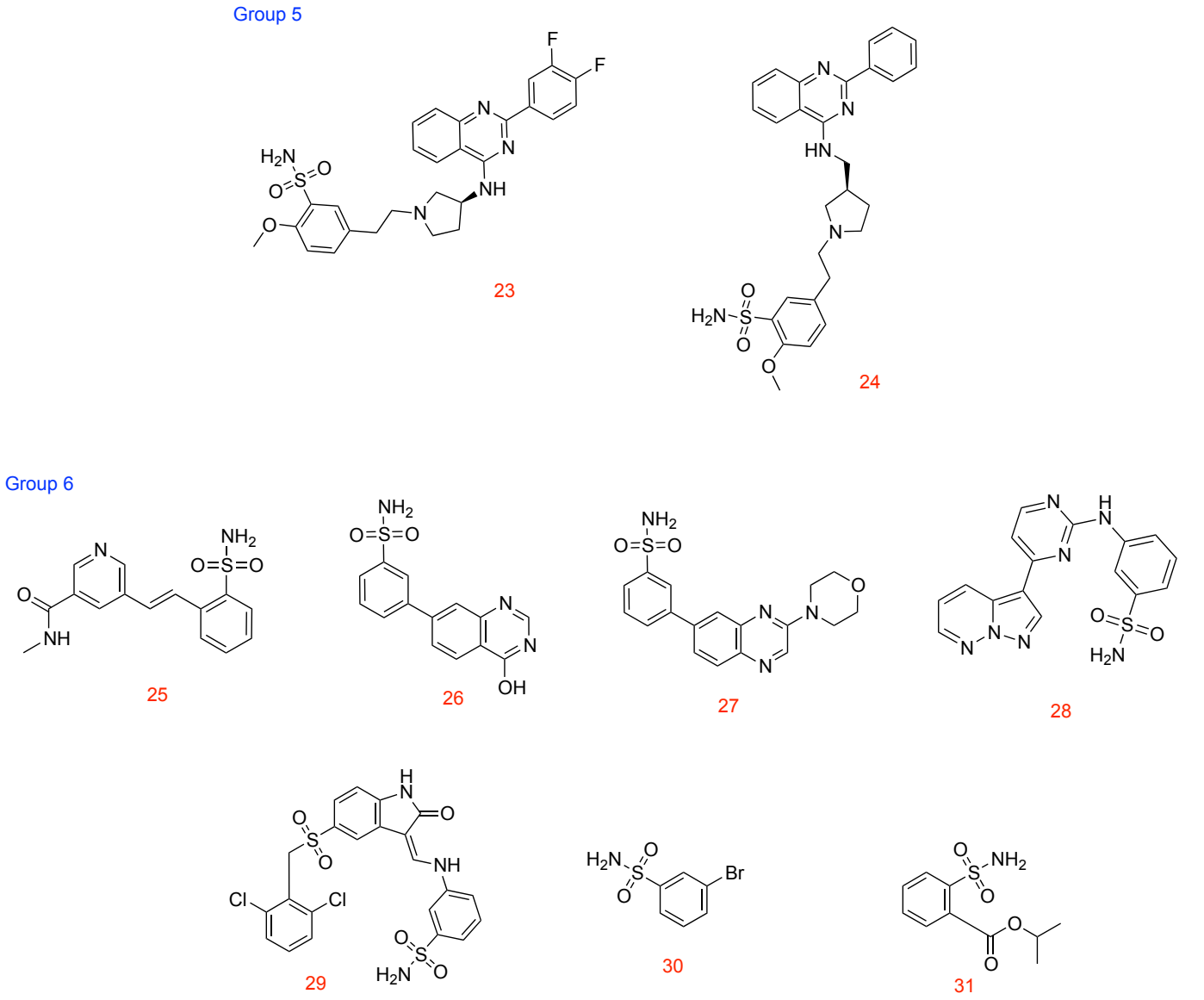

Fig. 2. (*continued*).

ID number PF3D7_1140000) was shown to be inhibited by a series of PS compounds (Krungkrai et al., 2008). We further characterized *Pf*CA from *P. falciparum* 3D7, identifying this protein as the first member of a new family of CAs (the η-CA family) (Del Prete et al., 2014). Using purified recombinant *Pf*CA expressed in *E. coli*, hydration of CO_2 was shown to be inhibited by clinically used PS compounds (Vullo et al., 2015). However, a limitation of previous studies was that *in vitro* antiplasmodial activity had not been correlated with inhibition of *Pf*CA-mediated CO_2 hydration. In this study the antiplasmodial potency (*in vitro* IC_{50}) of PS compounds from the TCAMS set were determined and compared with enzymatic activity against human CA I (hCA I), human CA II (hCA II) and *Pf*CA. Based on these data, structure activity relationship (SAR) analysis was carried out in order to assess potential correlation of *in vitro* IC_{50} values with recombinant *Pf*CA inhibition data. Finally, to assess the ability of PS compounds to target different *Plasmodium* species, three of the most potent antiplasmodial PS compounds were also assessed for activity against laboratory adapted *P. knowlesi* A1H.1 (Moon et al., 2013), a species that normally causes malaria in macaque monkeys but that can also cause severe disease and death in humans (Jongwutiwes et al., 2004, 2011; Luchavez et al., 2008; Kantele and Jokiranta, 2011; Khim et al., 2011; Barber et al., 2013; Lee et al., 2013).

2. Materials and methods

2.1. Compounds

2.1.1. GSK PS compounds

31 PS compounds identified in the European Bioinformatics Institute ChEMBL Neglected Tropical Disease TCAMS database (www.ebi.ac.uk/chemblntd/download/#tcams) were supplied by GSK (Gamo et al., 2010) under a Material Transfer Agreement (MTA). All compounds were provided as 10 mM stocks diluted in 100% DMSO and diluted as required. Chloroquine and pyrimethamine were purchased from Sigma-Aldrich®, USA and stock solutions of 10 mM were prepared in 100% DMSO (Sigma-Aldrich®, USA) and diluted as required for assays.

2.2. In vitro P. falciparum growth inhibition assays

The antiplasmodial activity of compounds was tested against *P. falciparum* using a 72 h isotopic microtest, as previously described (Fisher et al., 2014). Briefly, synchronous ring-stage *P. falciparum* infected RBCs (0.25% parasitemia and 2.5% final hematocrit) were seeded into 96-well tissue culture plates (3596 Costar®, Corning, USA) containing serial dilutions of control or test compounds. After incubating for 48 h under standard *P. falciparum* culture conditions in hypoxanthine-free parasite culture media, 0.5 μCi [^{3}H]-hypoxanthine (Perkin Elmer®, USA) was added to each well followed by culturing for a further 24 h. Cells were harvested onto 1450 MicroBeta filter mats (Wallac, USA) and [^{3}H]-incorporation was determined using a 1450 MicroBeta liquid scintillation counter (Perkin Elmer®, USA). The percentage inhibition of growth compared to that of matched DMSO controls (0.5%; Sigma-Aldrich, USA) was determined for at least three independent experiments, each carried out in triplicate wells. IC_{50} values were calculated using linear interpolation of inhibition curves (Huber and Koella, 1993). Chloroquine was used as a positive control in all assays.

2.3. In vitro P. knowlesi growth inhibition assays

The *in vitro* antimalarial growth inhibition activity of compounds was tested against *P. knowlesi* infected erythrocytes using a modified [^{3}H]-hypoxanthine incorporation assay (Arnold et al., 2016). Briefly, asynchronous *P. knowlesi* A1H.1 parasites (0.25% parasitemia and 2% final hematocrit) were seeded into 96-well tissue culture plates (3596 Corning®, USA) containing serial dilutions of control or test compounds in parasite culture media (Moon et al., 2013). Assay plates were incubated for 48 h with 0.5 μCi [^{3}H]-hypoxanthine (PerkinElmer®, USA) added at the 24 h time point. All other assay procedures, including data analysis, controls and independent replicates, were as described for *P. falciparum* above.

2.4. CA enzyme assays

An SX.18MV-R Applied Photophysics (Oxford, UK) stopped-flow instrument was used to assay inhibition of CA isozymes for each test compound (Khalifah, 1971). A saturated CO_2 solution in water at 25 °C was used as substrate. Phenol Red (0.2 mM) was used as

Table 1
In vitro antiplasmodial activity and selectivity of PS compounds 1–31.

CPD #	CHEMBL ID	MW	cLogP	HepG2[a] %Inhib @ 10μM	Pf3D7[b] IC_{50} μM	PfDd2[c] IC_{50} μM	SI[e]
DAP/PS							
1	546456	467	3.2	21	0.62 ± 0.14	0.42 ± 0.01	>16
2	587256	459	3.7	74	1.31 ± 0.18	<2[d]	<8
3	524967	626	4.8	71	>5	<2[d]	<2
4	547936	558	4.5	14	0.28 ± 0.04	0.37 ± 0.09	>36
5[f]	548709	401	−0.5	7	0.26 ± 0.06	0.22 ± 0.09	>38
6	588030	441	1.9	2	0.84 ± 0.02	0.48 ± 0.08	>12
AQ/PS							
7	548576	637	4.9	56	0.57 ± 0.12	>2[d]	<18
8	589061	362	3.2	5	1.57 ± 0.02	>2[d]	>6
Group 1							
9	530148	414	2.9	26	0.84 ± 0.01	<2[d]	>12
10	537064	463	4.3	10	0.47 ± 0.01	0.41 ± 0.04	>21
11	533004	410	2.2	19	1.89 ± 0.13	<2[d]	>5
12	530711	404	2.1	7	0.46 ± 0.01	0.43 ± 0.02	>22
13	536144	410	1.6	16	0.46 ± 0.01	0.86 ± 0.08	>22
Group 2							
14[f]	587021	598	4.5	83	1.09 ± 0.13	<2[d]	<9
15[f]	582203	598	4.5	81	3.04 ± 0.01	<2[d]	<3
Group 3							
16	530087	454	2.5	39	3.21 ± 0.03	>2[d]	>3
17	533876	440	2.5	40	0.79 ± 0.03	<2[d]	>13
18	529796	484	1.5	35	>5	<2[d]	<2
Group 4							
19	586746	437	4.6	0	1.59 ± 0.15	<2[d]	>6
20	529534	390	2.0	16	0.85 ± 0.05	<2[d]	>12
21	528642	442	5.2	24	2.00 ± 0.22	>2[d]	>5
22	529358	420	4.9	0	3.57 ± 0.11	>2[d]	>3
Group 5							
23	587392	540	4.4	18	>5	<2[d]	<2
24	587392	518	4.2	54	1.92 ± 0.39	ND	<5
Group 6							
25	533539	317	0.6	17	0.69 ± 0.15	<2[d]	>14
26	586070	301	1.9	6	0.81 ± 0.02	<2[d]	>12
27	580443	370	1.3	17	1.55 ± 0.12	<2[d]	>6
28	533079	367	0.9	39	0.23 ± 0.01	0.16 ± 0.04	>43
29	548077	538	3.2	1	3.02 ± 0.23	<2[d]	>3.3
30	531249	236	1.3	0	>5	>2[d]	<2
31	530277	442	5.2	24	>5	>2[d]	<2
CQ	76	320	4.3	0	0.01 ± 0.003	0.05 ± 0.01	>1000
PYR	36	249	2.8	0	0.03 ± 0.01	17.28 ± 6.90	>333

[a]HepG2% inhibition at 10 μM data derived from ChEMBL GSK TCAMS data (Gamo et al., 2010); Mean 50% inhibitory concentration (IC_{50}) ± standard deviation against *P. falciparum* line 3D7[b] and Dd2[c] for three independent experiments, each performed in triplicate (this study); [d]*P. falciparum* Dd2 IC_{50} data derived from ChEMBL GSK TCAMS data (Gamo et al., 2010) [e]SI selectivity index (HepG2 IC_{50}/Pf IC_{50}); [f]Compounds have potential serine/threonine protein kinase activity. All other data derived from ChEMBL GSK TCAMS data (Gamo et al., 2010). **DAP/PS** PS compounds with a diaminopyrimidine group; **AQ/PS** PS compounds with a 4-aminoquinoline group; **CQ** chloroquine; **PYR** pyrimethamine.

indicator, working at the absorbance maximum of 557 nm, 10 mM Hepes (pH 7.4) as buffer, Na_2SO_4 or $NaClO_4$ (0.1 M, anions are not inhibitory at this concentration) to maintain constant ionic strength was used to follow the CA-catalyzed CO_2 hydration reaction for 5–10 s. A stock solution of each test compound was prepared in DMSO-water 1:1, v/v (10 mM). At least 7 different inhibitor concentrations (prepared by serial dilution of stock solutions with the assay buffer) in triplicate were used to measure the CA inhibition constant. Inhibitor (I) and enzyme (E) solutions were preincubated for 10 min at room temperature prior to assay to allow for the formation of the E-I complex. The IC_{50} values were obtained by non-linear least-squares methods as reported earlier (Del Prete et al., 2014; Vullo et al., 2015), and represent the mean from at least three different determinations. All CA isozymes were recombinant proteins obtained as reported earlier by our group (Del Prete et al., 2014; Vullo et al., 2015). The concentration of enzyme in the assay system was: 12.3 nM for hCA I, 8.7 nM for hCA II and 14.5 nM for *Pf*CA.

3. Results and discussion

3.1. Identification of PS compounds in the Tres Cantos antimalarial set (TCAMS)

Data mining of the GSK TCAMS open source antimalarial data using the PS chemotype ($-SO_2NH_2$) as the search fragment resulted in identification of 31 PS-containing compounds (Figs. 1 and 2). Of these, seven compounds contain a known antimalarial pharmacophore, either the diaminopyrimidine scaffold (diaminopyrimidine group; compounds **1-6**) or a 4-aminoquinoline scaffold (4-aminoquinoline group; compounds **7** and **8**) (Fig. 1A and B, respectively). Compounds lacking a known antimalarial pharmacophore were grouped where possible according to structural similarity (Groups 1-5; Fig. 2). Seven TCAMS PS compounds did not share structural similarity with other compounds and were separately grouped (Group 6, Fig. 2). As published data for GSK TCAMS compounds was limited to percentage inhibition at 2 μM, the *in vitro* IC_{50} of compounds was determined against *P. falciparum* drug sensitive (3D7) and multi-drug resistant (Dd2) parasite lines (Table 1).

3.2. Antiplasmodial structure activity relationship (SAR) analysis of compounds containing an antimalarial pharmacophore

3.2.1. Diaminopyrimidine group

Diaminopyrimidines are a class of compound that comprise two amino substituents directly attached to a pyrimidine ring (Fig. 1A; highlighted in blue), and include the antimalarial drug pyrimethamine which is a dihydrofolate reductase inhibitor (Falco et al., 1951; Gregson and Plowe, 2005). Six of the PS compounds (**1-6**) contained a diaminopyrimidine scaffold as part of their structure. Of these, compounds **4** and **5** displayed the most potent IC_{50}s against *P. falciparum* 3D7 parasites (IC_{50} 0.28 and 0.26 μM, respectively; Table 1) and the highest selectivity for *P. falciparum* parasites versus the mammalian cell line HepG2 (SI > 35; Table 1). It should be noted that compounds **4** and **5** have opposing cLog P values (+4.5 and −0.5, respectively) indicating that there may be potential to improve the potency and selectivity of compound **5** via rational medicinal chemistry approaches to improve membrane permeability. Compound **1** (Pf3D7 IC_{50} 0.62 μM) and **6** (Pf3D7 IC_{50} 0.84 μM) showed lower antiplasmodial activity and selectivity (SI < 20; HepG2/Pf3D7; Table 1) compared to **4** and **5**. Compound **2** had the poorest antiplasmodial activity and selectivity of the diaminopyrimidines (Pf3D7 IC_{50} 1.3 μM; HepG2, 74% inhibition at 10 μM; Table 1). Compounds **1**, **4**, **5**, and **6** also displayed submicromolar antiplasmodial activity against the *P. falciparum* Dd2 parasite line (IC_{50}s 0.22–0.48 μM), which is resistant to drugs including chloroquine, pyrimethamine and sulfadoxine (Noedl et al., 2003). The resistance index (Ri), which is the ratio of the IC_{50}s of the resistant line Dd2 to the sensitive line 3D7, for these compounds ranged from 0.6 to 1.3, indicating a lack of cross resistance with Dd2.

3.2.2. 4-Aminoquinoline group

The 4-aminoquinoline scaffold is another known antimalarial pharmacophore (Fig. 1B; highlighted in red), with the antimalarial drug chloroquine being a 4-aminoquinoline derivative (Greenwood, 1992). Compounds **7** and **8** contain a 4-aminoquinoline and PS group (IC_{50} 0.57 and 1.57 μM, respectively; Table 1). Although compound **7** has a submicromolar IC_{50} against *P. falciparum* 3D7, it has only moderate selectivity for the parasite versus the HepG2 mammalian cell line (56% inhibition at 10 μM; SI ~18; Table 1) and the TCAMS database data indicates that **7** is less active against chloroquine resistant *P. falciparum* Dd2 parasites (IC_{50} > 2 μM; Ri > 3.5). Given that *P. falciparum* Dd2 is resistant to chloroquine, (Noedl et al., 2003), it is possible that the 4-aminoquinoline contributes to its activity. As the *P. falciparum* Dd2 IC_{50} was not calculated for this group, the activity data for compound **8** are not sufficient to be able to make any conclusions about cross resistance.

3.3. SAR analysis of GSK PS compounds lacking an antimalarial drug pharmacophore

GSK PS compounds lacking a known antimalarial drug pharmacophore (Fig. 2) were categorised into groups according to structure similarity.

3.3.1. Group 1 - Imidazo[1,2-b]pyridazines

Group 1 compounds **9-13** are all differently substituted phenyl/pyridinyl imidazo[1,2-*b*]pyridazines with the PS attached to a benzyl amine substituent. The common imidazopyridazine core has previously been associated with inhibition of *P. falciparum* calcium dependent protein kinase 1 (*Pf*CDPK1) inhibition, however compounds **9-13** were found not to inhibit several *Plasmodium* kinases including *Pf*CDPK1 and *Pf*CDPK4 (Crowther et al., 2016). Of the five compounds in this group, compounds **10** (trifluoromethoxyphenyl substituent), **12** (nitrilephenyl substituent), and **13** (methoxypyridinyl substituent) displayed the greatest level of antiplasmodial activity (Pf3D7 IC_{50} 0.46–0.47 μM; PfDd2 IC_{50} 0.41–0.86 μM; Table 1) and moderate parasite-specific selectivity (SI > 20 HepG2/Pf3D7; Table 1) while, the chlorophenyl and methoxyphenyl variants (**9** and **11**) had reduced antiplasmodial activity (Pf3D7 IC_{50} 0.84 μM and 1.89 μM respectively; Table 1) indicating that the different substituents of these compounds had a small impact on antiplasmodial activity.

3.3.2. Group 2 - 2-pyrimidinyl pyrazolopyridines

Group 2 comprises compounds **14** and **15** (Pf3D7 IC_{50} 1.09 and 3.04 μM, respectively; PfDd2 IC_{50} < 2 μM; Table 1), which are 2-pyrimidinyl pyrazolopyridines. The compounds differ in the position of the PS group substituent of the benzene ring. Position 4 (**14**) on the benzene ring may be responsible for the somewhat better Pf3D7 activity with respect to position 5 (**15**) for the placement of the PS group, however additional analogues would be required for SAR to be confirmed. However, these compounds have poor selectivity for Pf3D7 versus human cells (SI < 10 HepG2/Pf3D7; Table 1) and therefore are of limited interest as lead compounds. No SAR conclusions can be made for compounds **14** and **15** with respect to PfDd2 activity as accurate IC_{50}s were not determined.

3.3.3. Group 3 - quinolines

Group 3 consists of substituted quinoline compounds **16**, **17** and **18**. Of this group compound **17**, a 3-[4-(3-sulfamoylphenyl)quinolin-6-yl]benzenesulfonamide, displayed the best antiplasmodial activity (Pf3D7 IC_{50} 0.79 μM; Table 1) and moderate selectivity (SI > 10 HepG2/Pf3D7; Table 1) and minimal cross-resistance (IC_{50} < 2 μM; Ri < 2.5). In contrast, compound **16** which is a 3-[4-[3-(sulfamoylmethyl)phenyl]quinolin-6-yl]benzenesulfonamide displayed approximately 4-fold decrease in antiplasmodial activity (Pf3D7 IC_{50} 3.2 μM; PfDd2 IC_{50} > 2 μM; Table 1) and poor selectivity (SI ~3: HepG2/Pf3D7; Table 1), indicating that the additional methylene group of **16** caused reduced antiplasmodial activity and lower parasite selectivity. Compound **18** comprises a dimethylaminosulfonamide pyridine moiety and has the lowest antiplasmodial activity of the group (Pf3D7 IC_{50} > 5 μM; PfDd2 IC_{50} < 2 μM; Table 1).

3.3.4. Group 4 - benzimidazoles

Group 4 consists of compounds **19**-**22** all of which contain a benzimidazole-5-sulfonamide group with different substitutions. Of this group compound **20**, 2-[2-amino-5-(3-cyanophenyl)pyridin-3-yl]-3*H*-benzimidazole-5-sulfonamide, displayed the best antiplasmodial activity (Pf3D7 IC_{50} 0.85 μM; Table 1) and moderate parasite selectivity (SI > 12 HepG2/Pf3D7; Table 1) with no cross resistance to *P. falciparum* Dd2 (Table 1; IC_{50} < 2 μM; Table 1). Compound **20** is a novel benzimidazolyl-pyridine and it should be noted that this class of compound has also been implicated in the inhibition of mammalian serum and glucocorticoid-regulated kinase 1 (SGK-1) activity (Gaulton et al., 2012). However, this target has yet to be validated in *Plasmodium*. The remaining compounds in Group 4 (**19**, **21** and **22**) showed poor antiplasmodial activity and parasite selectivity (Pf3D7 IC_{50} 1.59–3.57 μM; SI > 3 - >6 HepG2/Pf3D7; Table 1).

3.3.5. Group 5 - Quinazolinamines

Group 5 comprised two compounds, **23** and **24**, that contain a 4-quinazolinamine pharmacophore. The compounds differ by fluorination on the 2-phenyl substituent, with two hydrogens of **24** replaced with fluorine in **23**. Additionally the pyrrolidine fragment of compound **24** has an additional methylene spacer to the 4-quinazolinamine pharmacophore.

Compound **24** displayed better antiplasmodial activity (Pf3D7 IC_{50} 1.92 μM; Table 1) than compound **23** (Pf3D7 IC_{50} > 5 μM; Table 1), suggesting that the fluorine groups of compound **23** may contribute to reduced antiplasmodial activity.

3.3.6. Group 6

The remaining seven compounds from the GSK PS compounds (Group 6; **25-31**; Fig. 2) do not share structural similarity with a known antimalarial pharmacophore or with other PS compounds to allow informative SAR analysis. Of this group, compound **28**, a 3-[(4-pyrazolo[1,5-*b*]pyridazin-3-ylpyrimidin-2-yl)amino]benzenesulfonamide had the best antiplasmodial activity (Pf3D7 IC_{50} 0.23 μM), highest selectivity (SI > 44: HepG2/Pf3D7) and no

Table 2
Carbonic anhydrase enzyme inhibition of PS compounds.

Compd	IC_{50} (μM)			Selectivity	
	hCA I[a]	hCA II[b]	*Pf*CA[c]	hCA I/*Pf*CA	hCA II/*Pf*CA
DAP/PS					
1	4.40	2.47	8.00	0.55	0.31
2	2.29	6.39	8.63	0.27	0.74
4	8.59	2.54	7.48	1.15	0.34
5	9.52	7.30	6.81	1.40	1.07
AQ/PS					
7	1.69	3.98	8.52	0.20	0.47
8	0.21	0.35	0.53	0.39	0.67
Group 1					
10	0.89	3.59	6.16	0.14	0.58
11	3.11	0.69	6.37	0.49	0.11
13	0.76	0.64	1.90	0.40	0.34
Group 2					
14	3.96	0.70	8.69	0.46	0.08
15	3.14	1.17	8.13	0.39	0.14
Group 3					
16	9.40	1.27	0.91	10.29	1.39
17	0.95	0.17	5.19	0.18	0.03
18	0.75	0.31	6.10	0.12	0.05
Group 4					
20	8.401	0.44	0.70	11.99	0.62
21	6.23	6.01	5.30	1.18	1.14
22	3.39	2.60	2.63	1.29	0.99
Group 5					
23	0.69	7.29	0.90	0.76	8.06
24	6.13	7.48	6.91	0.89	1.08
Group 6					
25	4.31	4.83	4.73	0.91	1.02
26	0.38	0.65	4.21	0.09	0.16
27	0.71	2.58	5.63	0.13	0.46
28	0.91	2.21	6.78	0.13	0.33
29	8.03	5.08	7.75	1.04	0.66
30	0.40	0.29	0.78	0.52	0.38
31	9.19	9.66	0.90	10.27	10.80
AZA	0.28	0.02	0.37	0.76	0.05

Mean % inhibition against human [a]human CA I, [b]human CA II and [c]*P. falciparum* CA (±standard deviation) for three independent experiments, each performed in triplicate. **DAP/PS** PS compounds with a diaminopyrimidine group; **AQ/PS** PS compounds with a 4-aminoquinoline group; **AZA** acetazolamide.

evidence of cross resistance to *P. falciparum* Dd2 parasites (IC_{50} 0.16 μM) (Table 1). Compound **25**, a *N*-methyl-5-[(*E*)-2-(2-sulfamoylphenyl)ethenyl]pyridine-3-carboxamide and compound **26** a 4-hydroxyquinazoline displayed 3- to 4-fold lower antiplasmodial activity (Pf3D7 IC_{50} 0.69–0.81 μM) and selectivity (SI > 12: HepG2/Pf3D7) than **28** (Table 1). The remaining compounds **27**, **29**, **30** and **31** showed poor antiplasmodial activity (Pf3D7 IC_{50} 1.55 - >5 μM) and low selectivity (SI > 3 to >7 HepG2/Pf3D7). Compounds **25**-**29** also demonstrated some antiplasmodial activity against the drug resistant *P. falciparum* parasites (PfDd2 IC_{50} < 2 μM; Table 1).

3.4. Relationship between PS compound inhibition of P. falciparum growth in vitro and biochemical potency against PfCA

The PS moiety is in the structure of several drugs where the mechanism of action is attributed to CA inhibition (Supuran, 2008). CAs from several pathogens including *Plasmodium* are now recognised as potential anti-infective drug targets (Capasso and Supuran, 2013). This, together with the current dogma that *Pf*CA is likely to be essential for parasite survival as it is believed to be involved in the first step of the crucial pyrimidine synthesis pathway in *P. falciparum* (Krungkrai et al., 2003), led us to investigate *Pf*CA as a possible target of 26 (compounds **3**, **6**, **9**, **12** and **19** were not assayed due to insufficient stocks) of the 31 GSK PS compounds in this study. In addition, a potential limitation of PS-based drugs for malaria use is that they may also inhibit hCA I and hCA II found in erythrocytes (and/or other human CAs), hence CA inhibition data was also investigated against hCA I and hCA II (Table 2). Acetazolamide (**AZA**), the *par excellence* therapeutically established CA inhibitor, was included as a reference compound (Supuran, 2008).

*Pf*CA inhibition for PS compounds ranged from IC_{50} 0.53–8.69 μM, with six compounds (**8**, **16**, **20**, **23**, **30** and **31**) having submicromolar activity against the enzyme (Table 2). Only one of these contained an antimalarial pharmacophore (4-aminoquinoline, compound **8**) and this compound displayed 16-fold greater inhibition for *Pf*CA (IC_{50} 0.53 μM) than the other 4-aminoquinoline, compound **7** (IC_{50} 8.52 μM). However compound **8** showed no selectivity at the enzyme level for *Pf*CA compared to hCA I or hCA II (IC_{50} 0.21 μM and 0.35 μM, respectively; Table 2). Group 3 quinoline compound **16** has submicromolar inhibitory activity against *Pf*CA, ~5- to 6-fold better than for quinolines **17** and **18**. Of note, compound **16** is 10-fold more selective for *Pf*CA over hCA I and non-selective for hCA II, while the two analogues **17** and **18** have 5- to 31-fold and 8- to 20-fold, respectively, better inhibition for the human CAs. At least for compounds **16** and **17**, this may suggest that the different orientation of one of the benzenesulfonamide groups leads to improved *Pf*CA inhibitory activity and selectivity over human CA I for **16** (*Pf*CA IC_{50} 0.91 μM SI; hCA I IC_{50}/*Pf*CA IC_{50} ~10) compared to **17** (*Pf*CA IC_{50} 5.19 μM SI; hCA I IC_{50}/*Pf*CA IC_{50} < 1). Group 4 benzimidazole **20** has submicromolar inhibitory activity against *Pf*CA (IC_{50} 0.71 μM; Table 2) and like **16** from Group 3, is more selective for *Pf*CA over hCA I but not hCA II (SI; hCA I IC_{50}/*Pf*CA IC_{50} ~12; SI; hCA II IC_{50}/*Pf*CA IC_{50} < 1). In Group 5, the submicromolar inhibitory activity of quinazolinamine **23** compared with ~8-fold less activity for analogue **24** (IC_{50} 0.90 and 6.90 μM, respectively) indicates that replacement of hydrogen with fluorine may improve *Pf*CA inhibition. In contrast to the *Pf*CA selectivity of **16** and **20** for hCA I over hCA II, **23** shows the opposite profile having 8-fold better selectivity for *Pf*CA over hCA II (IC_{50} 0.90 and 7.29 μM, respectively) and no selectivity for *Pf*CA over hCA I (IC_{50} 0.90 and 0.69 μM, respectively). Two Group 6 compounds, structurally unrelated compounds **30** and **31**, exhibited submicromolar *Pf*CA inhibition activity with **31** being ~10-fold more selective for *Pf*CA over both human CAs. Additionally, it should be noted that

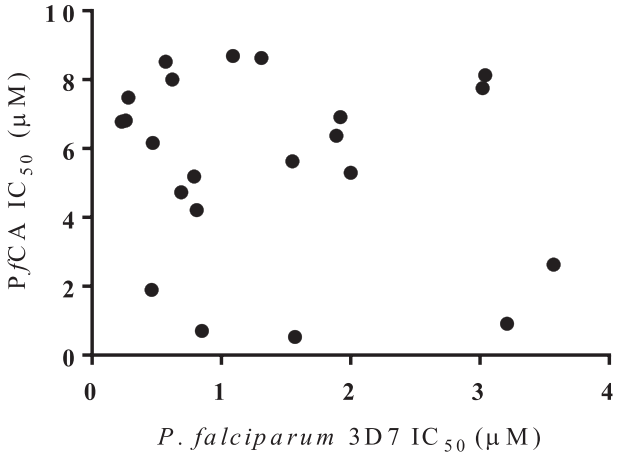

Fig. 3. Relationship between inhibitor potency against *P. falciparum* infected erythrocytes and *Pf*CA enzyme activity. Spearman correlation (two-tailed) analysis of the mean IC_{50} against *P. falciparum* infected erythrocytes and *Pf*CA enzyme for 22 PS compounds (r = −0.1316; P = 0.556).

two of the most potent antiplasmodial compounds that did not contain an antimalarial pharmacophore (**10** and **28;** Pf3D7 IC_{50} 0.47 and 0.23 μM, respectively) had poor *Pf*CA inhibition (IC_{50} 6.16–6.78 μM) but displayed submicromolar inhibition for hCA I (IC_{50} 0.89–0.91 μM). This potential off target effect needs to be taken in to consideration if these compounds are to be further investigated as candidates for malaria therapy.

Despite individual inhibitors having inhibitory activity in *Pf*CA enzymatic assays, no correlation between *Pf*CA enzymatic IC_{50}s and those determined against *P. falciparum* infected erythrocytes was found for compounds for which IC_{50}s were available for both assays (i.e. for 22 of the PS compounds (**1**, **2**, **4**, **5**, **7**, **8**, **10**, **11**, **13**-**17**, **20**-**22**, **24**-**29**); Fig. 3). At the Group level, either no correlation was found or numbers were too small to analyse. Likewise, there was no correlation between either of the human CA IC_{50}s and the *P. falciparum* 3D7 IC_{50}s (not shown).

3.5. Antiplasmodial activity of selected GSK PS compounds against P. knowlesi

One of the desired attributes of new antimalarial leads is the ability to target multiple human infecting *Plasmodium* species. *P. knowlesi* is a zoonotic species that can cause death in humans (Barber et al., 2011; William et al., 2011) and is also a possible model for *P. vivax* which causes significant malaria morbidity (WHO, 2015). *P. knowlesi* is the only *Plasmodium* species other than *P. falciparum* that is amenable to long term continuous culture *in vitro* (Moon et al., 2013). In addition, of relevance to this study, whole genome sequencing has failed to identify a *Pf*CA homologue in *P. knowlesi* (Aurrecoechea et al., 2009). Three of the GSK PS compounds that lack an antimalarial drug pharmacophore and

Table 3
Comparison of the activity of selected PS compounds against *P. falciparum* and *P. knowlesi in vitro*.

Compound	*P. falciparum* IC_{50} (μM)[a]	*P. knowlesi* IC_{50} (μM)[b]
10	0.47 ± 0.01	0.46 ± 0.07
12	0.46 ± 0.01	0.62 ± 0.15
28	0.23 ± 0.01	0.19 ± 0.05
CQ	0.02 ± 0.001	0.01 ± 0.003

Mean % inhibition against [a]*P. falciparum* line 3D7 and [b]*P. knowlesi* (±standard deviation) for three independent experiments, each performed in triplicate.

with the highest potency against *P. falciparum* (**10**, **12** and **28**) were selected for *in vitro* testing for activity against *P. knowlesi* infected erythrocytes. No significant difference ($P > 0.05$) in IC_{50}s was seen for the three compounds for *P. knowlesi* (IC_{50} 0.46 ± 0.07, 0.62 ± 0.15 and 0.19 ± 0.05 μM, respectively) compared to those obtained for *P. falciparum* 3D7 (IC_{50} 0.47 ± 0.01, 0.46 ± 0.01 and 0.23 ± 0.01 μM, respectively) (Table 3). Together with the lack of correlation of biological activity with enzyme activity discussed above, and the absence of a *Pf*CA homologue in *P. knowlesi*, these data are consistent with the PS compounds in this study having an alternative target to *Pf*CA. It should be noted, however, that we cannot discount the presence of an orthologue of *Pf*CA in *P. knowlesi* given that a large proportion of the genome cannot yet be annotated based on amino acid sequence homology (Aurrecoechea et al., 2009). In addition, this study focuses on asexual intraerythrocytic stage *Plasmodium* parasites and it may be that the enzymatic activity observed here is of relevance to other *Plasmodium* lifecycle stages as *Pf*CA is also reported to be expressed in the ookinete life cycle stage in the mosquito (Aurrecoechea et al., 2009).

4. Conclusion

Information sharing of antimalarial high throughput screening data by pharmaceutical company GSK allowed us the opportunity to source and investigate the activity and potential mode of action of a panel of compounds containing a novel pharmacophore, the primary sulfonamide (PS) moiety. Whilst the PS moiety is known to target CA in other organisms (Supuran, 2008) most compounds did not selectively inhibit *Pf*CA over human red blood cell CAs, with compound **31** being an exception. However the poor antiplasmodial activity of **31** (Pf3D7 $IC_{50} > 5\mu M$) and the good cell permeability properties (LogP 5.2) suggests no correlation with *Pf*CA inhibition, as least in asexual blood stage parasites. Nonetheless, several PS compounds with submicromolar antiplasmodial activity (IC_{50} 0.16–0.47 μM) and some selectivity (SI > 21 - >43) were identified and these compounds could be investigated further for their potential to be developed as antimalarial lead candidates. This includes PS diaminopyrimidines **4** and **5** and compounds **10**, **12** and **28**. While compounds **4** and **5** are structurally similar to the antimalarial drug pyrimethamine they did not exhibit cross resistance with a pyrimethamine resistant parasite line. It should be also noted that compounds **10**, **12** and **28** displayed promising *in vitro* activity and selectivity across two *Plasmodium* species (*P. falciparum* and *P. knowlesi* $IC_{50} < 0.5$ μM; SI > 21). This, along with structural novelty, warrants investigating these three compounds further to determine if they possess the appropriate pharmacokinetic properties and *in vivo* efficacy to progress as antimalarial drug leads.

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