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PII: S0223-5234(18)30735-9

DOI: 10.1016/j.ejmech.2018.08.063

Reference: EJMECH 10674

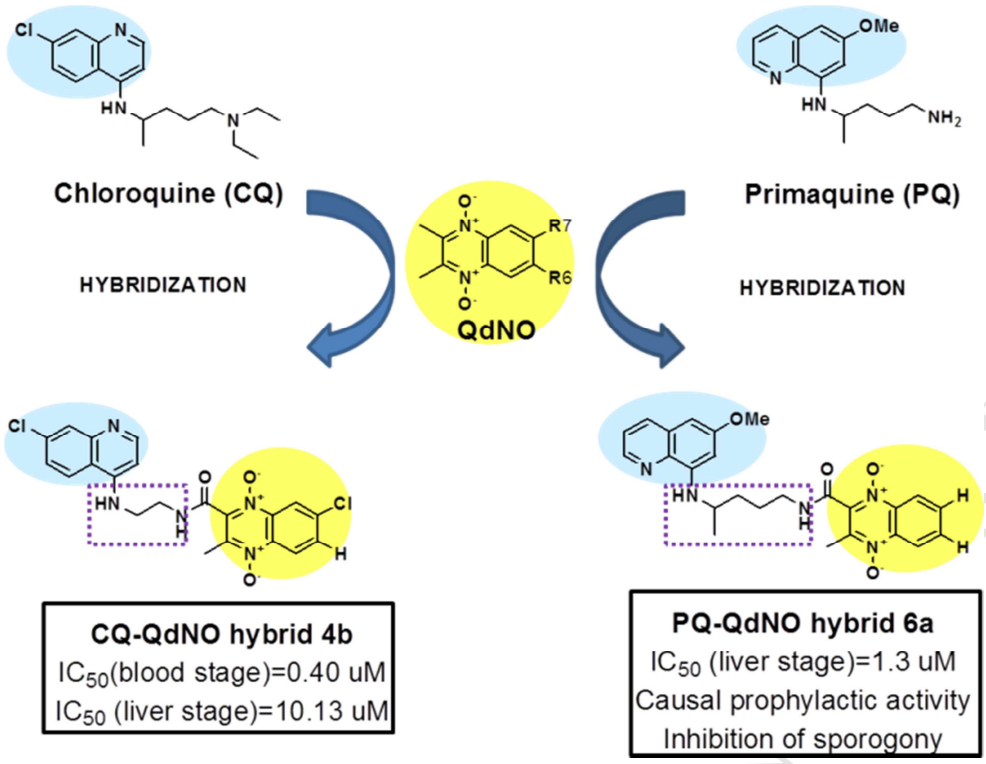
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 28 June 2018

Revised Date: 21 August 2018

Accepted Date: 23 August 2018

Please cite this article as: L. Bonilla-Ramírez, A. Rios, M. Quiliano, G. Ramírez-Calderon, Ivá. Beltrán-Hortelano, Jean.Franç. Franetich, L. Corcuera, M. Bordessoulles, A. Vettorazzi, A. López de Cerain, I. Aldana, D. Mazier, A. Pabón, S. Galiano, Novel antimalarial chloroquine- and primaquine-quinoxaline 1,4-di-*N*-oxide hybrids: Design, synthesis, *Plasmodium* life cycle stage profile, and preliminary toxicity studies, *European Journal of Medicinal Chemistry* (2018), doi: 10.1016/j.ejmech.2018.08.063.



Novel Antimalarial Chloroquine- and Primaquine-Quinoxaline 1,4-di-*N*-Oxide Hybrids: Design, Synthesis, *Plasmodium* Life Cycle Stage Profile, and Preliminary Toxicity Studies

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ABSTRACT:

Emergence of drug resistance and targeting all stages of the parasite life cycle are currently the major challenges in antimalarial chemotherapy. Molecular hybridization combining two scaffolds in a single molecule is an innovative strategy for achieving these goals. In this work, a series of novel quinoxaline 1,4-di-*N*-oxide hybrids containing either chloroquine or primaquine pharmacophores was designed, synthesized and tested against both chloroquine sensitive and multidrug resistant strains of *Plasmodium falciparum*. Only chloroquine-based compounds exhibited potent blood stage activity with compounds **4b** and **4e** being the most active and selective hybrids at this parasite stage. Based on their intraerythrocytic activity and selectivity or their chemical nature, seven hybrids were then evaluated against the liver stage of *Plasmodium yoelii*, *Plasmodium berghei* and *Plasmodium falciparum* infections. Compound **4b** was the only chloroquine-quinoxaline 1,4-di-*N*-oxide hybrid with a moderate liver activity, whereas compound **6a** and **6b** were identified as the most active primaquine-based hybrids against exoerythrocytic stages, displaying enhanced liver activity against *P. yoelii* and P. *berghei*, respectively, and better SI values than primaquine. Although both primaquine-quinoxaline 1,4-di-*N*-oxide hybrids slightly reduced the infection of mosquitoes, they inhibited sporogony of *P. berghei* and compound **6a** showed 92% blocking of transmission. *In vivo* liver efficacy assays revealed that compound **6a** showed causal prophylactic activity affording parasitaemia reduction of up to 95%

on day 4. Absence of genotoxicity and *in vivo* acute toxicity were also determined. These results suggest the approach of primaquine-quinoxaline 1,4-di-*N*-oxide hybrids as new potential dual-acting antimalarials for further investigation.

Keywords:

Chloroquine; Primaquine; Quinoxaline 1,4-di-*N*-oxide; Hybrid drugs; Blood stage; Liver stage

1. Introduction

Malaria is one of the most important and devastating parasitic diseases worldwide, affecting 91 countries. Despite the decreasing incidence of malaria globally over the last 15 years due to the use of strategies for vector control and artemisinin-based combination therapies (ACTs), an estimated 216 million cases and 445,000 deaths were caused by malaria worldwide in 2016 (WHO, 2017). The widespread drug resistance to most current antimalarial drugs and the complex life cycle of *Plasmodium spp*. remain the major impediments for the elimination of the disease (Wellems et Plowe, 2001; Ashley et al., 2014; Mbengue et al., 2015). In the context of the malaria eradication strategy, novel drugs effective against multidrug resistant strains and targeting all stages of the parasite life cycle for the prevention, treatment and transmission of disease are urgently required (Alonso et al., 2011).

The development of antimalarial chemotherapy has been traditionally focused on the symptomatic intraerythrocytic stage. Chloroquine (**CQ**, Fig. 1), has been the classical blood schizonticidal drug widely used as a standard therapy for decades based on its high clinical efficacy, limited toxicity, low cost, simple usage, and simple effective synthesis. Even though its clinical use has been restricted due to the emergence of resistance, the 7-chloro-4-aminoquinoline scaffold is critical for conferring antimalarial potency by inhibiting haemozoin formation, and consequently, accumulating the drug in the digestive vacuole of the parasites (Thomé et al., 2013). Additionally, a great number of chloroquine analogues have been reported with high antimalarial activity against chloroquine resistant strains of *P.*

falciparum (Guantai et al., 2011; de Souza et al., 2014), suggesting that the resistance mechanism seems to be compound specific and does not depend on the changes in the structure of the drug target. Therefore, 7-chloro-4-aminoquinoline continues to attract special attention as a privileged scaffold in current antimalarial drug discovery.

On the other hand, liver and vector stages have been underexplored. Currently, primaquine (**PQ**, Fig. 1) is the only licensed drug targeting these stages. The use of PQ does not only eliminate the liver forms of *Plasmodium*, including hypnozoites in *P. ovale* and *P. vivax* infections leading to a causal prophylaxis and avoiding relapse (Li et al., 2014), but PQ is also used as gametocytocidal blocking the transmission of the disease inside the mosquito vector (Kamtekar et al., 2004; Abay, 2013). Unfortunately, potential side effects such as methemoglobinemia and haemolytic anaemia in glucose-6-phosphate dehydrogenase-deficient patients (Devine et al., 2017) and the signs of emerging resistance to PQ (Ariey et al., 2014; Lu et al., 2017; Sutherland et al., 2017) have limited its clinical use. Moreover, recent studies show that primaquine is ineffective in people with low metabolizing cytochrome P450 2D6 genotypes (Potter et al., 2015).

The hybridization concept, which is the use of a combination of two (or more) active chemical scaffolds into a single molecule is an attractive approach for overcoming the challenges of multidrug resistance in *P. falciparum*, for further improving antimalarial activity and for reducing undesired side effects (Viegas-Junior et al., 2007; Agarwal et al., 2017). Additionally, the linking of dual-acting chemical moieties could be used to characterize new potential dual-stage antimalarial drugs (Meunier, 2008; Muregi and Ishih, 2010)

Various chloroquine- and primaquine-based hybrids have been reported to target multiple stage of *Plasmodium* life cycle in the past few years, namely *N*-cinnamoylated chloroquine analogues (Pérez et al., 2013), primaquine-chloroquine hybrid (Lödige et al., 2013), primaquine-pyrimidine hybrids (Kaur et al., 2015) and primaquine-artemisinin hybrids (Capela et al., 2011; Miranda et al., 2014).

As a part of our ongoing research on antimalarial compounds, we have recently reported that the fusion of quinoxaline 1,4-di-*N*-oxide (QdNO) core with a 7-chloro-4-hydrazinequinoline (CQ moiety) showed a significant increase in antiplasmodial activity in comparison to other previously synthesized analogues without a CQ core (**1,** Fig. 1) (Quiliano et al., 2017).

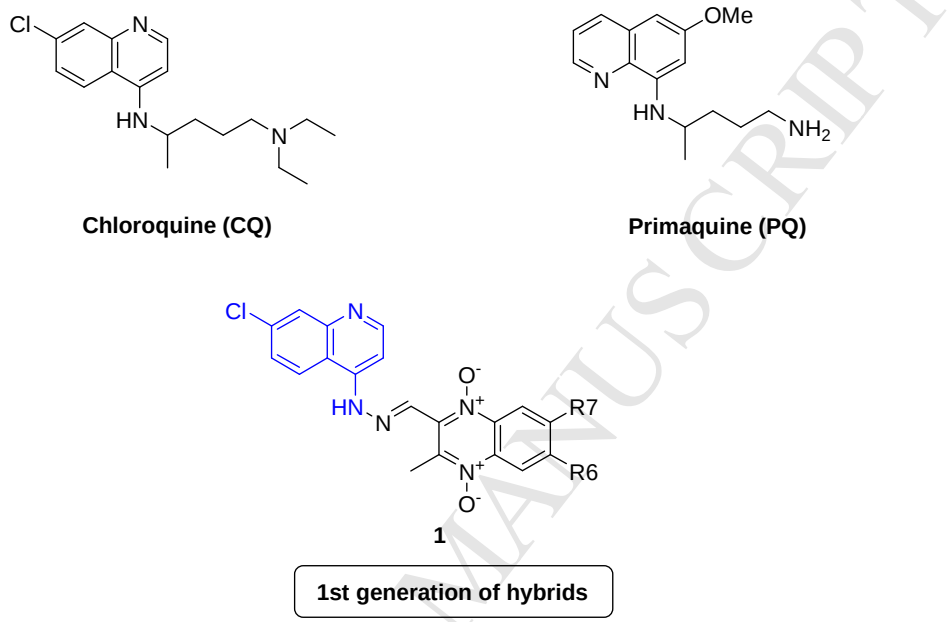


Figure 1. Chemical structures of chloroquine (CQ), primaquine (PQ) and the first generation of chloroquine-QdNO hybrids

Quinoxaline derivatives are well-known for their broad therapeutic potential (Hui et al., 2006; El Aissi et al., 2014; Cheng et al., 2016). In the last two decades, our group has synthesized a large number of quinoxaline 1,4-di-*N*-oxide derivatives (QdNO) with different substitutions at positions 2, 3, 6, and 7, displaying good activities for multiple infectious diseases (Vicente et al., 2011; Torres et al., 2013; Barea et al., 2013), and especially against the erythrocytic stage of malaria (Gil et al., 2014; Quiliano et al., 2017).

Taking all these requirements into account and based on this strategy, we designed and synthesized two new series of hybrids, covalently linking the scaffolds of two known antimalarial drugs, CQ and PQ with the QdNO core via an appropriate linker (Fig. 2). The *in vitro* antiplasmodial activities of all

synthesized hybrid compounds against 3D7 chloroquine sensitive and FCR-3 multidrug resistant strains of *P. falciparum* and cytotoxicity against HepG2 cell line are reported. Additionally, we present *in vitro* and *in vivo* liver stage efficacy and the transmission blocking potential results for the active compounds in the mosquito stage. Finally, an acute oral toxicity study in mice and the genotoxicity screening test SOS/*umu* test were also conducted for the best compound.

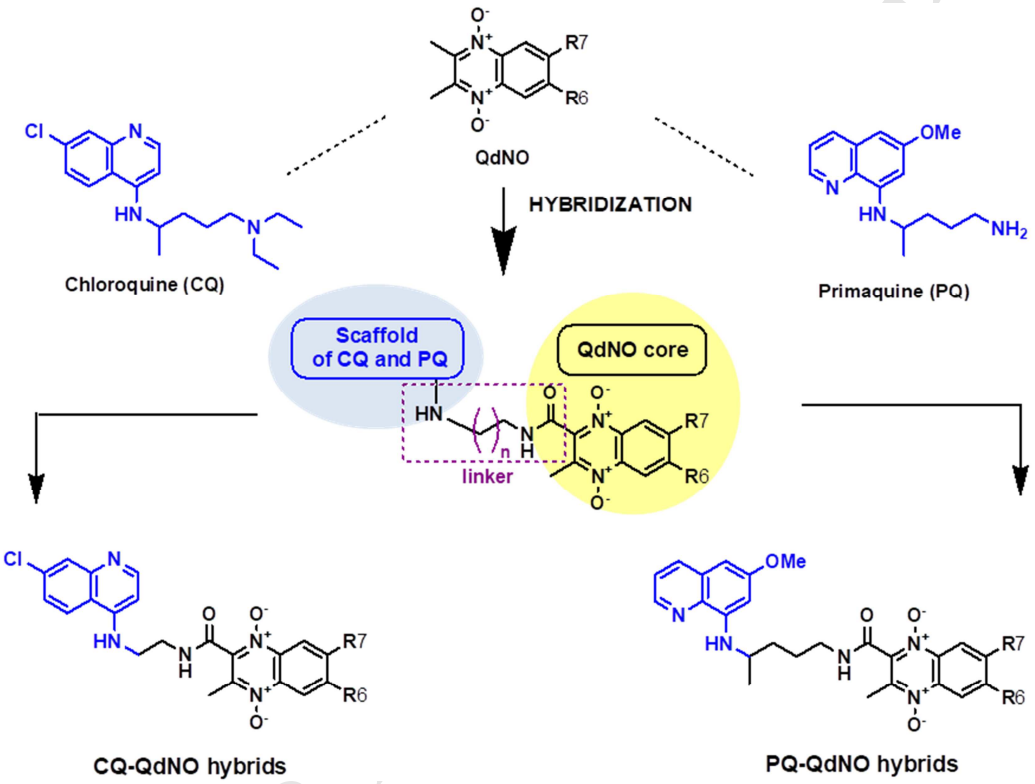


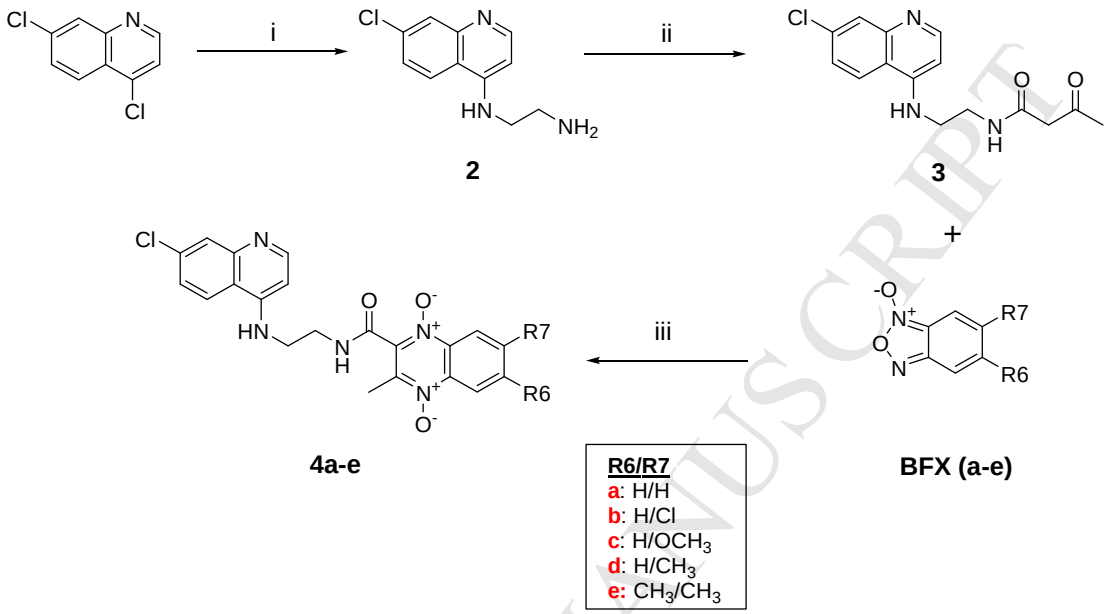
Figure 2. Design of chloroquine- and primaquine-quinoxaline 1,4-di-*N*-oxide (QdNO) hybrids

2. Results and discussion

2.1. Chemistry

The chloroquine-based hybrids **4a-e** were synthesized using a three-step procedure as outlined in Scheme 1. The commercially available 4,7-dichloroquinoline was reacted with an excess of ethylenediamine via a S_NAr to afford compound **2**. Subsequent acetoacetylation of **2** using diketene in the presence of methanol under a nitrogen atmosphere provided the desired derivative **3** (Clemens, 1986). Condensation of **3** with the corresponding benzofuroxans **BFX(a-e)** by a variation of the Beirut

reaction using calcium chloride and ethanolamine as catalysts give the final chloroquine-quinoxaline-1,4-di-*N*-oxide hybrids **4a-e** (Stumm and Niclas, 1989; Li, 2006)

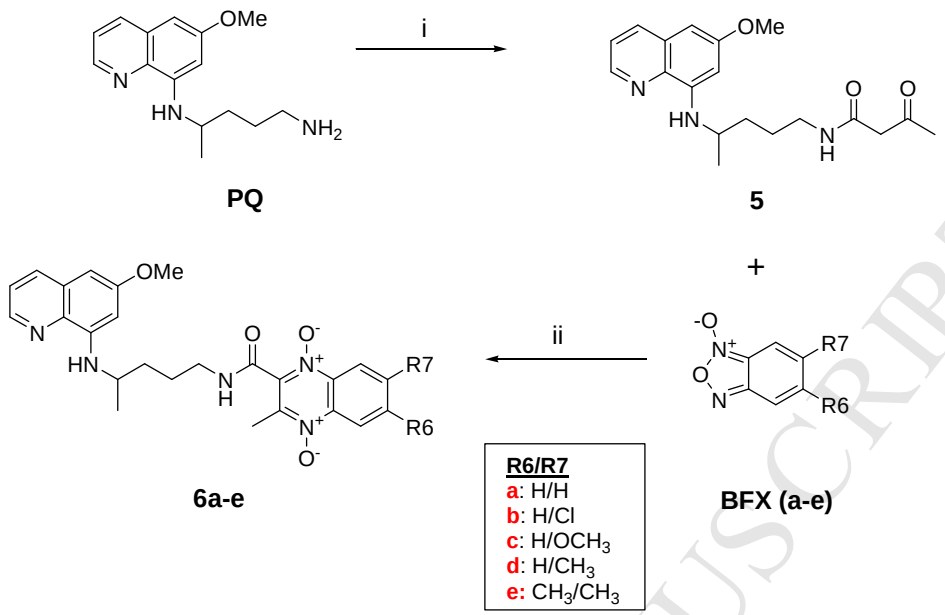


Reagents and conditions: (i) ethylendiamine, reflux, 1h, 92%; (ii) diketene, MeOH, N_2, 0ºC, 1h, 75%; (iii) MeOH, $CaCl_2$, ethanolamine, rt, 1-24 h

Scheme 1. Synthesis of Chloroquine-Quinoxaline 1,4-di-*N*-oxide hybrids

The general synthetic approach to primaquine-based hybrids **6a-e** is presented in Scheme 2. Primaquine **PQ** was obtained from the commercially available primaquine bisphosphate through an extraction using an aqueous solution of sodium bicarbonate to afford the free base of the compound. Treatment of **PQ** with diketene in the presence of methanol under a nitrogen atmosphere at 0°C provided the β-acetoacetamide derivative **5**. Finally, the primaquine-quinoxaline 1,4-di-*N*-oxide hybrids **6a-e** were obtained by a variation of the Beirut reaction of **5** with the appropriate **BFX(a-e)** in the presence of calcium chloride and ethanolamine as catalysts.

5,6-dimethylbenzofuroxan **BFXe** was obtained by previously described methods (Ortega et al., 2002; González and Cerecetto, 2007) whereas the rest of benzofuroxans **BFX(a-d)** were commercially available.



Reagents and conditions: (i) diketene, MeOH, N_2, 0ºC, 1h, 70%; (ii) MeOH, $CaCl_2$, ethanolamine, rt, 1-24 h

Scheme 2. Synthesis of Primaquine-Quinoxaline 1,4-di-*N*-oxide hybrids

2.2. In silico physicochemical properties (ADME profile)

The prediction of the ADME profile and the drug-likeness of all hybrid compounds were performed computationally and are outlined in Table 1. Topological polar surface area (TPSA) is a useful descriptor for human intestinal absorption, Caco-2 monolayers permeability, and blood-brain barrier penetration (Ertl et al., 2000). Furthermore, Lipinski's rule of five (Lipinski et al., 1997) and the number of rotatable bonds (n-ROTB) were also calculated since they are found to be important predictors for good oral bioavailability. Veber et al (2002) stated that compounds with ≤10 rotatable bonds and TPSA < 140 Å2 are more likely to show good bioavailability. In this study, all compounds exhibited high predicted gastrointestinal absorption. In addition, none of the hybrids violated either Lipinski's rule of five or Veber's criteria (i.e., all except **6d** with 10 rotatable bonds). Therefore, the oral bioavailability of these hybrid compounds could be considered promising.

Table 1. *In silico* physicochemical properties of hybrid compounds (ADME profile)

Comp.	ALOGPs 2.1	MW	n-ON acceptors	n-OHNH donors	LV	n-ROTB	GI	TPSA (Å^2)
Rule	**< 5**	**< 500**	**< 10**	**< 5**	**≤ 1**	**≤ 10**		**≤ 140**
4a	1.55	423.85	4	2	0	6	High	104.94
4b	2.20	458.30	4	2	0	6	High	104.94
4c	1.69	453.88	5	2	0	7	High	114.17
4d	1.70	437.88	4	2	0	6	High	104.94
4e	1.80	451.91	4	2	0	6	High	104.94
6a	2.01	461.51	5	2	0	9	High	114.17
6b	2.61	495.96	5	2	0	9	High	114.17
6c	2.12	475.54	5	2	0	9	High	114.17
6d	2.11	491.54	6	2	0	10	High	123.40
6e	2.22	489.57	5	2	0	9	High	114.17
CQ	5.28	319.87	2	1	1	8	High	28.16
PQ	2.76	259.35	3	2	0	6	High	60.17

ALOGPs (LogP): logarithm of compound partition coefficient between n-octanol and water; MW: molecular weight expressed in Daltons; n-ON: number of hydrogen bond acceptors; n-OHNH: number of hydrogen bond donors; LV: Lipinski's violations; n-ROTB: number of rotatable bonds; GI: human gastrointestinal absorption; TPSA: topological polar surface area; CQ: chloroquine; PQ: primaquine

2.3. Blood Stage Activity

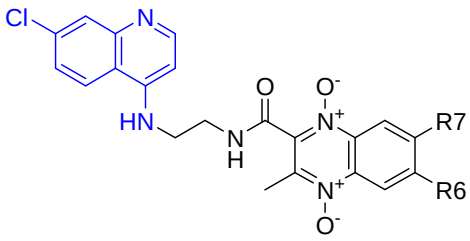
2.3.1. In vitro antiplasmodial activity (3D7 and FCR-3 strains of P. falciparum)

The antiplasmodial activity of all synthesized hybrid compounds was determined against the 3D7 chloroquine sensitive (CQS) strain and the FCR-3 multidrug resistant (MDR) strain of *P. falciparum* using the hypoxanthine incorporation assay (Tables 2 and 3). Chloroquine (CQ) and primaquine (PQ) were used as reference drugs.

All CQ-QdNO hybrids except **4c** showed submicromolar activity in the CQS strain (3D7 IC_{50} < 1 μM), whereas only two showed IC_{50} in the same range against the MDR strain. Compounds **4b** and **4e** were found to be the most active CQ-based hybrids with IC_{50} values ranging from 0.40 to 0.90 μM in both strains. Although none of these hybrids exhibited better erythrocytic activity than CQ, most of them resulted in a moderate to strong increase of antiplasmodial activity in the MDR strain in comparison with previous CQ-QdNO hybrids (*) synthesized by our group (Table 4: **4b** vs ***1b** and **4c** vs ***1c**: FCR-3 IC_{50}= 0.40-2.07 μM vs 2.56-2.80 μM). In contrast, they resulted slightly less active in

the CQS strain. These results revealed that the length and chemical nature of the linker may contribute to the biological activity of this kind of hybrid.

Table 2. *In vitro* antiplasmodial activity against blood stage of *P.falciparum* 3D7 and FCR-3 strains and cytotoxicity on HepG2 cell line of CQ-QdNO hybrids



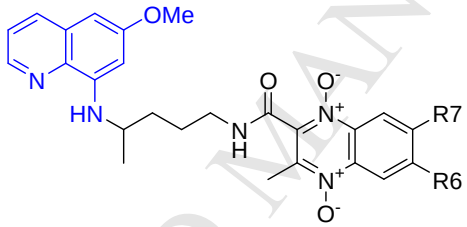
Comp.	Substituents		*P.falciparum* IC_{50} (µM)[a]		Cytoxicity CC_{50} (µM)[b]	SI[d]
	R6	R7	3D7	FCR-3	HepG2[c]	
4a	H	H	0.78 ± 0.20	1.90 ± 0.72	58.22 ± 0.11	30.64
4b	H	Cl	0.52 ± 0.11	0.40 ± 0.23	21.83 ± 0.28	54.58
4c	H	OCH_3	2.11 ± 0.99	2.07 ± 0.12	92.69 ± 2.55	44.77
4d	H	CH_3	0.57 ± 0.08	2.24 ± 0.67	31.41 ± 4.43	14.02
4e	CH_3	CH_3	0.68 ± 0.25	0.90 ± 0.29	64.54 ± 5.07	71.71
CQ			0.026 ± 0.003	0.207 ± 0.015	137.42 ± 0.02	663.86

Mean values of three independent experiments performed in triplicate ± standard deviation (SD)
[a]IC_{50}: Concentration inhibiting 50% of the parasite growth;
[b]CC_{50}: Concentration producing 50% of cytotoxicity;
[c]HepG2: Hepatocellular carcinoma cells;
[d]SI: Selectivity index= CC_{50} (cytotoxicity on HepG2)/IC_{50} (FCR-3)

As can be seen from Table 2, this set of CQ-QdNO hybrids is much less active than CQ. This fact is significantly correlated with the lower selectivity indices (although still reasonable) which fall into a somewhat less comfortable range. The research group of Egan and co-workers provided evidence that drug accumulation in the acidic food vacuole of the parasite though pH trapping is essential for strong antiplasmodial activity and the need of a basic nitrogen atom attached to the aminoalkyl side chain of the quinoline to assist this accumulation at the site of action (Egan et al., 2000; Kaschula et al., 2002). These findings appear to be the explanation for the reduced activity of these hybrids in comparison with those obtained by CQ and this represents a feature that must be explored in depth in further studies (i.e. changing ethylendiamine with diethylentriamine as a linker).

Regarding PQ-QdNO hybrids (Table 3), all compounds showed weak activity in the erythrocytic stage, not exhibiting better activity than PQ (only 2-6-fold less than PQ in CQS) and CQ. This fact is not particularly surprising because PQ is itself a modest blood schizonticidal drug (Vale et al., 2009) and the lack of a basic amino group in these hybrids linked to PQ was established for obtaining blood stage activity with PQ derivatives (Philip et al., 1988). In contrast to the CQ-based hybrids, the antiplasmodial activities of PQ-based hybrids were comparable in both strains, with the exception of **6b**.

Table 3. *In vitro* antiplasmodial activity against blood stage of *P.falciparum* 3D7 and FCR-3 strains and cytotoxicity on HepG2 cell line of PQ-QdNO hybrids



Comp.	Substituents		*P.falciparum* IC_{50} (μM)		Cytoxicity CC_{50} (μM)	SI
	n	R7	3D7	FCR-3	HepG2	
6a	H	H	67.18 ± 3.12	68.20 ± 3.26	39.48 ± 1.07	0.58
6b	H	Cl	62.62 ± 7.85	36.05 ± 3.56	154.69 ± 12.08	3.34
6c	H	OCH_3	26.98 ± 4.27	30.71 ± 3.97	>203.66	6.63
6d	H	CH_3	44.85 ± 5.82	50.64 ± 5.36	109.75 ± 13.12	2.17
6e	CH_3	CH_3	30.83 ± 2.47	52.87 ± 3.29	>202.02	3.82
CQ			0.026 ± 0.003	0.207 ± 0.015	137.42 ± 0.02	663.86
PQ			9.86 ± 1.85	1.10 ± 0.22	120.03 ± 11.89	109.11

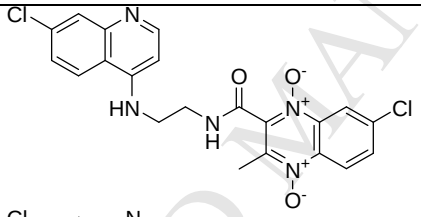
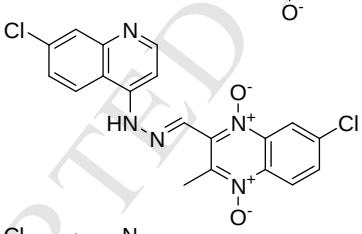
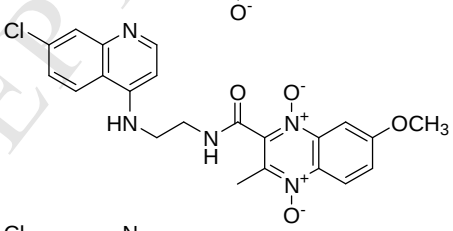
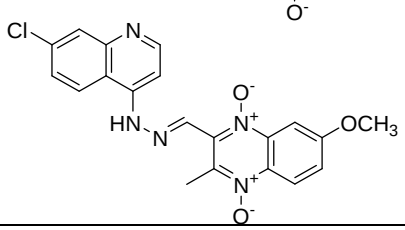
Mean values of three independent experiments performed in triplicate ± standard deviation (SD)
[a]IC_{50}: Concentration inhibiting 50% of the parasite growth;
[b]CC_{50}: Concentration producing 50% of cytotoxicity;
[c]HepG2: Hepatocellular carcinoma cells;
[d]SI: Selectivity index= CC_{50} (cytotoxicity on HepG2)/IC_{50} (FCR-3)

Thus, both CQ- and PQ- based hybrids were found to be a less effective blood schizonticidal drug against 3D7 and FCR-3 strains than the reference drugs, revealing no additional or synergistic effects.

Additionally, the poor results obtained for PQ hybrids could be an indication of the resistance-reversing effect of primaquine (Bray et al., 2005).

Furthermore, no correlation between the electronic pattern of substituents R6 and R7 of the quinoxaline ring and the antiplasmodial activity was found. In the CQ hybrids, substitution at position 7 with a chlorine group led to an increase of blood stage activity, whereas PQ hybrids bearing an electron-releasing group such as a methoxy were the most active compounds in this series.

Table 4. Comparison of new CQ-QdNO hybrids with previous hybrids

Comp.	Structure	P. falciparum IC_{50} (µM)	
		3D7	FCR-3
4b		0.52	0.40
***1b**		0.24	2.80
4c		2.11	2.07
***1c**		1.40	2.56

[a]IC_{50}: Concentration inhibiting 50% of the parasite growth;
*Compounds from Quiliano et al (2017)

2.3.2. Cytotoxicity studies

The cytotoxicity of the ten newly synthesized hybrids (**4a-e** and **6a-e**) was evaluated by measuring the metabolic activity of HepG2 cell line using the MTT assay. The cytotoxicity and selectivity index (SI) results are outlined in Tables 2 and 3.

CQ-QdNO hybrid compounds exhibited low toxicity against this hepatocellular carcinoma cell line, with a CC_{50} ranging between 21 and 92 μM and only 1-6-times less than CQ. The most active CQ-based hybrids (**4b** and **4e**) showed high SI values (SI> 55 μM).

Most PQ-QdNO hybrids showed comparable or lower cytotoxicity than the parent compound PQ (i.e., all except **6a**). However, PQ-based hybrids displayed a low selectivity (SI < 6.63).

2.4. Liver Stage Activity

2.4.1. In vitro inhibitory activity in Plasmodium liver stage

To evaluate the ability of these compounds to inhibit the liver stage of *Plasmodium spp in vitro.*, cell lines (HepG2-CD81 and HepG2) and primary culture of human's hepatocytes were infected with *P. yoelii*, *P. berghei* and *P. falciparum* sporozoites, respectively and assayed by immunofluorescence microscopy against *Plasmodium* Hsp70 after 2-7 days of treatment with seven selected compounds (**4b**, **4e**, **6a**, **6b**, **6c**, **6d** and **6e**). The compounds were selected on the basis of: (i) better antiplasmodial activity and selectivity index in the blood stage for CQ-QdNO hybrids; and (ii) chemical nature of the hybrid with the presence of PQ in their structure due to its ability to eliminate liver forms of *Plasmodium* for PQ-QdNO hybrids. Thus, two CQ-QdNO (**4b** and **4e**) and five PQ-QdNO (**6a**, **6b**, **6c**, **6d** and **6e**) hybrids were chosen.

The data for *in vitro* liver stage activity are presented in Table 5. Compounds **6a** and **6b** were identified as the most active hybrid compounds against the exoerythrocytic forms (EEFs), compared to PQ control. Compound **6a** was 1.5-fold more active than PQ against *P.yoelii* (IC_{50}= 1.39 vs 2.13 μM),

248 while **6b** showed 3.2-fold higher activity against *P. berghei* than the reference drug (IC_{50}= 1.14 vs 3.65
249 μM). In contrast, hybrids **6a** and **6b** exhibited only moderate activity against the liver stage of *P.*
250 *falciparum.* We found that other hybrids with primaquine have been synthesized and evaluated as
251 antiplasmodial agents. For example, primaquine-artemisinin hybrids showed an increase in their
252 activity compared to the parent drugs against exoerythrocytic stages (Capela et al., 2011). Similarly,
253 two series of primaquine-derived hybrids synthesized based on endoperoxides (trioxane-primaquine
254 and tetraoxane-primaquine) showed a potent activity in the asexual phases of parasitic development
255 (Miranda et al., 2014). Likewise, promising new primaquine-imidazolidinone hybrids showed
256 antimalarial activity in the exoerythrocytic phase (Aguiar et al., 2017).

257 Compound **4b** was the only CQ-QdNO hybrid with a moderate activity against all liver stage
258 parasites. This result was surprising because compounds structurally related to chloroquine (7-chloro-4-
259 aminoquinolines scaffold) are remarkably active against blood stages but not against liver stage malaria
260 (Delves et al., 2012; Derbyshire et al., 2011).

Table 5. *In vitro* antiplasmodial activity against liver stage of *P.yoelii*, *P. berghei and P. falciparum* and cytotoxicity on HepG2-CD81, HepG2 cell lines and human's hepatocytes of hybrids

Comp.	IC_{50} (µM)[a]			CC_{50} (µM)[b]			SI[c]		
	P. yoelii	***P. berghei***	***P. falciparum***	**HepG2-CD81**	**HepG2**	**Human's hepatocytes**	***P. yoelii***	***P. berghei***	***P.falciparum***
4b	14.59	10.13 ± 1.61	6.39	69.86 ± 3.25	21.83 ± 0.28	61.13	4.78	2.15	9.56
4e	NA[d]	NA	NA	ND[e]	64.54 ± 5.07	ND	-	-	-
6a	1.39	5.19 ± 0.13	5.38 ± 0.03	>212.76	39.48 ± 1.07	>212.76	153.02	7.59	39.53
6b	5.71	1.14 ± 0.26	3.64 ± 2.46	>198.21	154.69 ± 12.08	>198.21	34.71	136.32	54.45
6c	NA	14.05 ± 5.63	NA	>203.66	>203.66	>203.66	-	14.49	-
6d	14.02	6.34 ± 0.46	7.21	>206.61	109.75 ± 13.12	>206.61	14.74	17.32	28.65
6e	ND	11.33 ± 1.16	8.11	>202.02	>202.02	>202.02	-	17.83	20.22
PQ	2.13	3.65 ± 0.73	0.40 ± 0.19	69.80 ± 4.87	120.03 ± 11.89	54.21 ± 1.23	32.76	32.95	135.53

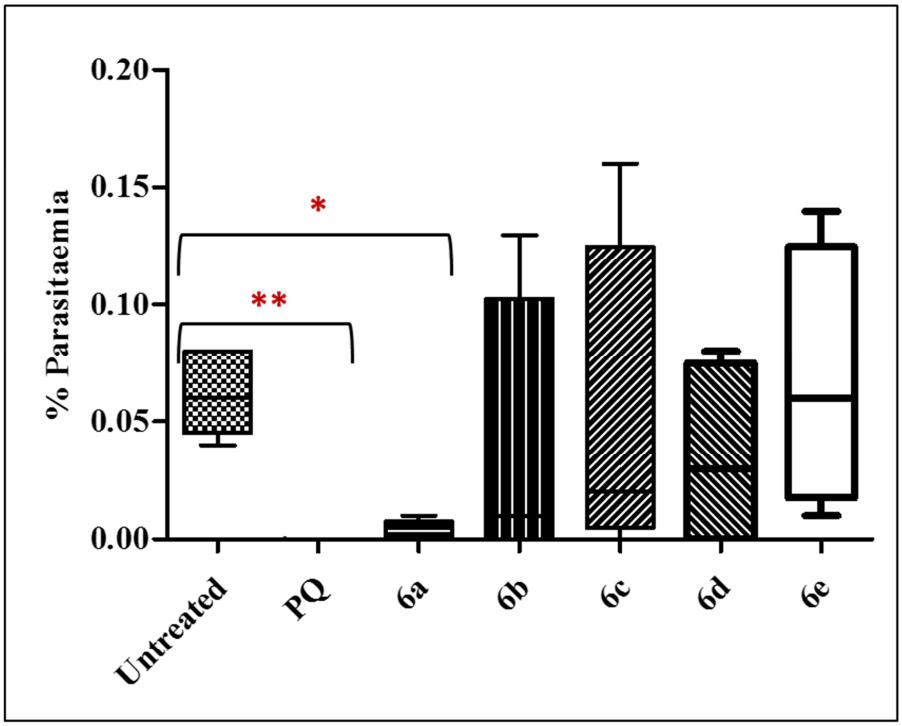
Mean values of two independent experiments performed in triplicate ± standard deviation (SD).
[a]IC_{50}: Concentration inhibiting 50% of the parasite growth
[b]CC_{50}: Concentration producing 50% of cytotoxicity
[c]Selectivity Index (SI)= CC_{50} (corresponding cell line)/IC_{50} (corresponding parasite); HepG2-CD81 cell line for *P.yoelii*, HepG2 cell line for *P.berghei*, and human's hepatocytes for *P.falciparum*
[d]NA: Not active at the highest concentration used (10 µg/mL).
[e]ND: Not determined.

2.4.2. Cytotoxicity assays

All of these hybrid compounds were evaluated for mammalian cytotoxicity against different cell lines (HepG2-CD81, HepG2 and human's hepatocytes). As can be observed in Table 5, most compounds showed very low toxicity against all cell lines that were much lower than those for the reference drug PQ. Remarkably, no cytotoxic activity was exhibited by the most active hybrids in the liver stage, **6a** and **6b** (CC_{50} > 154 μM). More importantly, high SI values were displayed by **6a** and **6b** in *P.yoelii* and *P. berghei*, respectively, resulting in a 4-fold higher SI value than that of PQ alone (SI= 153.02 and 136.32 vs 32.76 and 32.95).

2.4.3. In vivo causal prophylaxis efficacy

To assess the causal chemoprophylactic potential *in vivo*, mice were treated with the selected hybrids (**6a**, **6b**, **6c**, **6d** and **6e**) prior to being infected with *P.yoelii* 17XNL sporozoites. The prophylactic activity was measured as the percentage of inhibition of parasitaemia, detected microscopically between 3 and 4 day post infection compared to the negative control group. Compound **6a** showed a median parasitaemia of 0% on day 4 of follow-up, whereas the values of median parasitaemia for **6b**, **6c**, **6d** and **6e** were 0.01, 0.02, 0.03 and 0.06%, respectively. The negative control exhibited a median parasitaemia of 0.06%. No significant difference was found ($p < 0.05$) (Fig. 3). For the positive control group (PQ), no parasites were observed on day 1 of the follow-up and a significant difference was observed compared to the negative control ($p=0.0236$). In addition, 1 out 4 (25%) mice treated with compound **6a** showed peripheral blood parasites (0.007 parasites/μL) on day 1 with significant difference relative to the negative control ($p<0.05$). Although the percentage of blood parasites in mice treated with **6a** remained lower than that of the negative control on day 5, the significant difference was lost ($p>0.05$).



294

295 **Figure 3**. **Comparison of parasitaemias in BALB/c mice on day 4 post infection with *P.yoelii***
296 **17XNL and treated with the hybrids and with primaquine.** Negative control group (vehicle) n=5,
297 positive control group (primaquine) n=5, treatment hybrid group n=4. Significance was calculated
298 using the Kruskal-Wallis test (p=0.0284)

299 Interestingly, on day 4, compound **6a** displayed excellent reduction parasitaemia of 95.97%, very
300 similar to that of PQ (100%) (Fig. 4) and the pre-patent period (the time from inoculation until parasites
301 become microscopically detectable) was extended by approximately 24 h over that of the negative
302 control. This fact underlines the prophylactic activity of hybrid **6a.**

303 Others hybrid molecules with covalently linked primaquine related to the results of this work have
304 been reported. Lodige et al. showed that the hybrid with primaquine administered at 20 mg/kg in mice
305 infected with 10.000 sporozoites of *P. berghei* was able to extend the prepatent period of parasitaemia,
306 similar to our assays, but only for 7 days (Lödige et al., 2013). Hybrids composed of primaquine and
307 derivatives of artemisinins have shown antiplasmodial activity. However, the combination with

inhibitors of erythrocytes and hepatics forms mask the chemoprophylactic causal activity, because these compounds inhibit 60% of parasitaemia in mice infected with 10,000 sporozoites of *P. berghei* at day 4 post-infection and after this day (Capela et al., 2011).

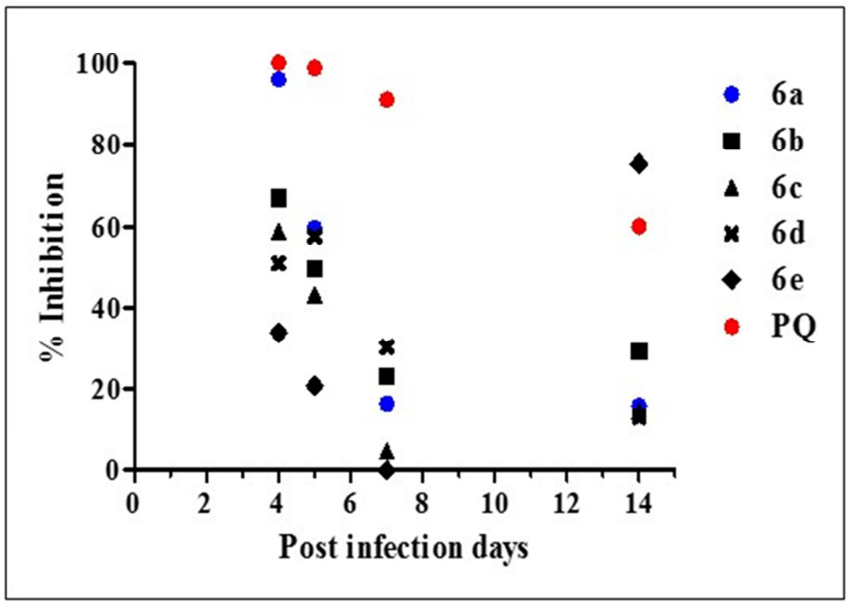


Figure 4. **Percentage inhibition of parasitaemia of the treatment groups in different post infection days**. Each point represents the mean parasitaemia value of mice treated with four doses of hybrid compounds (n=4) and primaquine (n=5)

2.5. Effects on Transmission Stages

2.5.1. Inhibition of sporogonic development of Plasmodium in mosquitoes.

We evaluated the effect of **6a** and **6b** on *in vivo P. berghei* oocyst formation in *Anopheles stephensi* mosquitoes. These compounds were selected based on their better selectivity index against EEFs of *P. falciparum*. *A. stephensi* mosquitoes were allowed to feed on *Pb*GFP-infected mice 1.5 h after the treatment. Mosquito midguts were evaluated for two parameters: (i) the presence of oocysts, and (ii) the number of oocysts per mosquito, compared to the mosquito control group. Compound **6a** displayed a 20% reduction in infected mosquitoes and demonstrated potent activity with a 92% reduction in the

oocyst numbers per midgut ($p<0.001$) compared to mosquitoes fed on untreated mice (Figs. 5A and 5B). On the other hand, compound **6b** reduced the infection of *A.stephensi* and number of oocysts in midguts by 23% and 77%, respectively. DMSO group (vehicle) showed no alteration in the formation of oocysts.

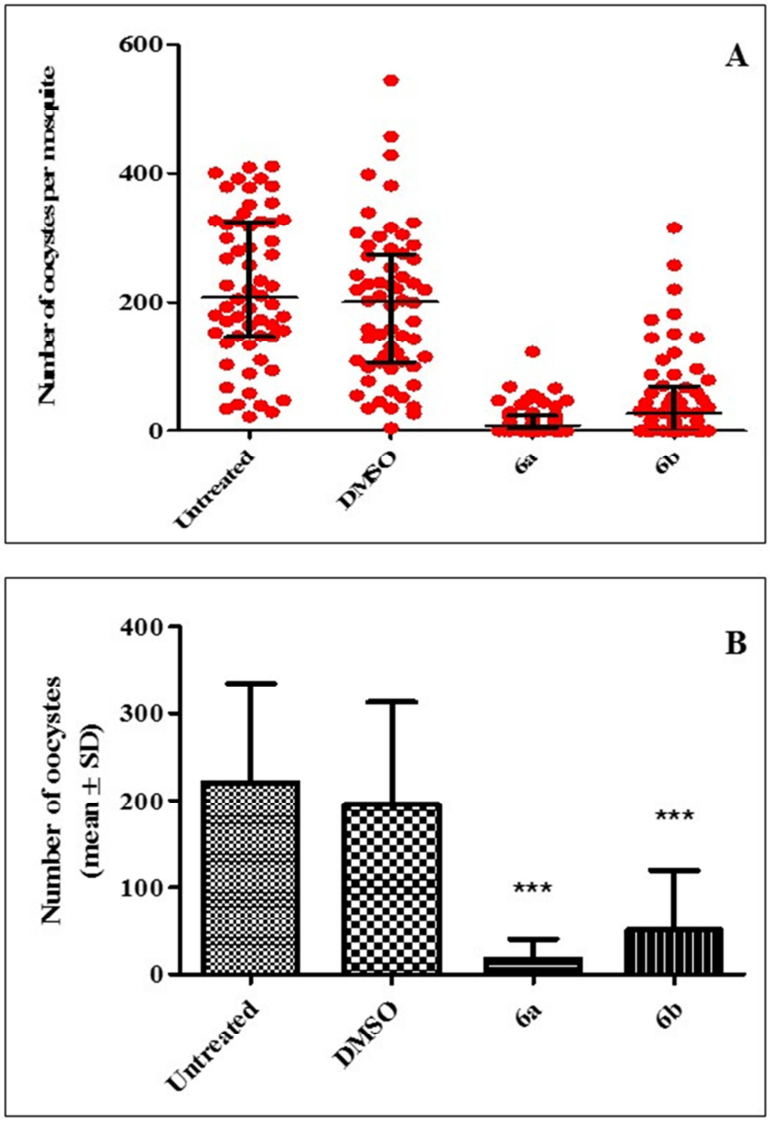


Figure 5. Hybrids 6a and 6b reduce the number of oocysts in midgut of *Anopheles stephensi*. Number of oocysts of *Pb*GFP in midgut of *A. stephensi* (n=60 per group) was quantificated using fluorescence microscopy. (A) Scatter dot plot shows the number of oocyst per mosquito; black line

represents median with interquartile range; (B) Bars plot shows the mean of number of oocysts per group of treatment. Data are expressed as the mean ± SD *** $p<0.001$

*2.6. Preliminary Toxicity evaluation of compound **6a***

2.6.1. In vivo acute oral toxicity

On the basis of its high antimalarial activity *in vitro* and *in vivo* against liver stage, significant *in vitro* selectivity index and promising transmission blocking activity, additional safety profiling of **6a** was performed. First, we evaluated the *in vivo* acute oral toxicity of **6a**. All mice showed normal behaviour and feeding habits without any signs of toxicity specific to its species when a dose of 300 mg/kg of **6a** was administered. In clinical examination, the mice exhibited good general condition, clear and bright eyes, normal coloured mucosa and bright hair without erection. Same behaviour was observed in two of the three mice administered with 500 mg/kg of this hybrid compound, while one of them was euthanized to be prostrated after 20 hours of hybrid administration. At the necropsy, the organs were observed to be normal in appearance and size. In addition, the microscopic findings were acute necrosis of liver and kidney. In contrast, one mouse was sacrificed to be unconscious and convulsing 20 hours after the administration of 500 mg/kg of PQ. At the necropsy, a degree of severe and ulcerated congestion in the stomach was observed, whereas the rest of the organs were normal.

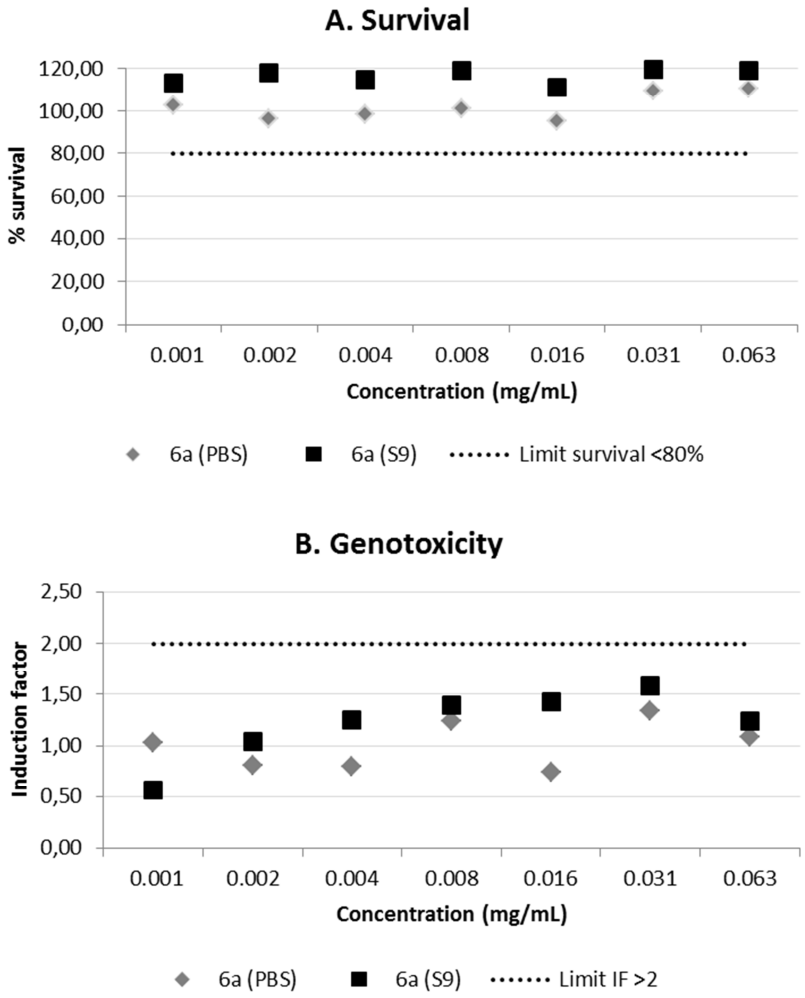
On the other hand, all surviving mice treated with **6a** at both 300 (3/3 mice) and 500 mg/kg (2/3 mice) showed the same weight gain as the untreated animals. Additionally, haematological parameters were not significantly different from those of the untreated mice (Kruskal-Wallis test, $p=0.098$) and blood urea nitrogen, creatinine, and total bilirubin were similar to those of the untreated mice. The alanine transferase enzyme in a mouse treated with 500 mg/kg was the only enzyme displaying a slight increase (82 U/L) compared to the range of enzyme values in untreated animals (34-78 U/L).

355 *2.6.2. Genotoxicity assay*

356 We also established the DNA-damaging effect of compound **6a** using the SOS/*umu* test as a
357 preliminary genotoxicity assay due to the good agreement between the SOS/*umu* test and the
358 standardized Ames test (OECD guideline 471) (OECD, 1997; Reifferscheid and Heil, 1996). All
359 controls used for the SOS/*umu* test were correct (IF< 2 for negative and IF>2 for positive controls).
360 From the eleven concentrations tested, the compound **6a** precipitated in the wells containing the highest
361 concentrations tested (1, 0.5; 0.25 and 0.125 mg/mL); thus, these wells were not considered in the
362 analysis.

363 The rest of the tested concentrations (from 0.001 to 0.063 mg/mL) were not toxic for the bacteria
364 because the survival percentages were always higher than 80% with or without S9 (Fig. 6).

365 Compound **6a** was considered non-genotoxic as its induction factor was always lower than 2 at non-
366 cytotoxic concentrations with or without the S9 fraction (Fig. 6). Even if a high degree of agreement
367 between the SOS/*umu* test and the standardized Ames test (OECD, 1997) was found (Reifferscheid et
368 al., 1996), it should be noted that the SOS/*umu* test is used for screening purposes. For regulatory
369 purposes, further evaluation with the standardized Ames test should be performed.



370

371 **Figure 6. Results from SOS/*umu* test with (black) o without S9 (grey) activation.** A) Bacterial
372 survival is shown as percentage. Concentrations are considered non-cytotoxic if survival is > than 80%.
373 B) Genotoxicity. A compound is considered genotoxic if the induction factor is ≥ 2 at non-cytotoxic
374 concentrations for the bacteria in any of the conditions tested

375

376 **3. Conclusions**

377 In this paper, two new classes of QdNO hybrids have been described. Chloroquine-based hybrids
378 displayed potent *in vitro* blood stage antiplasmodial activity in CQS and MDR strains. Remarkably,
379 compounds **4b** and **4e** were the most active and selective hybrids against erythrocytic stage of malaria

parasite with a moderate liver stage activity for the compound **4b** that may represent an opportunity for the further development of CQ-related molecules targeting both liver and blood stage parasites.

Novel primaquine-QdNO hybrids have been found to exhibit preferential inhibition of parasite growth in the liver versus blood stage parasite. Compounds **6a** and **6b** were identified as the most active hybrids against exoerythrocytic stages, enhancing the parent drug's liver activity against *P.yoelii* and P. *berghei*, respectively, as well as demonstrating better SI values than the reference primaquine drug. Furthermore, both compounds reduced the number of oocysts in the mosquito midgut. Significantly, compound **6a** resulted in high inhibition of *P.berghei* oocyst formation, inhibiting sporogony and demonstrating 92% blocking of transmission. Interestingly, compound **6a** was also the only PQ-based hybrid to show causal prophylactic activity displaying excellent parasitaemia inhibition (95.97%), slightly less than primaquine and extending the prepatent period of parasitaemia by approximately 24 hours.

A preliminary toxicity evaluation of **6a** was performed. No signs of genotoxicity and *in vivo* acute toxicity at doses of 300 mg/kg were found. At doses of 500 mg/kg, compound **6a** exhibited fewer histopathological alterations than primaquine. Further evaluations at higher doses must be performed.

In addition, because PQ-QdNO hybrids are racemic mixtures as the classical arylamino alcohol primaquine, it is necessary to confirm the absence of significant potency differences between both enantiomers. Further chiral separation and enantiomeric testing of PQ-QdNO hybrids must be done in future studies.

Overall, this study led to the identification of a PQ-QdNO hybrid as a dual stage antimalarial agent. Its effects on the liver stage and mosquito stages suggest not only a possible role in chemoprophylaxis but also a potential to reduce the transmission of malaria, two fundamental properties in the current agenda for the treatment and eradication of this disease. These findings establish the hybridization of primaquine and QdNO scaffolds as a valuable approach for the development of dual-action antimalarial drugs and provide a good basis for further drug development.

4. Experimental section

4.1. Chemicals and instrumentation

All solvents and chemicals were obtained from commercial sources and used as supplied. Diketene (acetyl ketene) was purchased from BOC Sciences (Shirley, NY, USA).

Melting points were determined using a Mettler FP82+FP80 apparatus and are uncorrected. Infrared (IR) spectra were obtained with a Nicolet Nexus FTIR using KBr pellets. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 Ultrashield spectrometer at 400 and 100 MHz, respectively, with tetramethylsilane (TMS) as the internal standard and DMSO-d_6 as the solvent. The chemical shifts, δ, are expressed in ppm and the coupling constants, J, are given in Hz. Signal multiplicities are represented as s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (doublet of doublets). Elemental microanalyses were performed using a Leco CHN-900 Elemental Analyzer and were within ±0.4 of the theoretical values. All final compounds were confirmed to have >96% purity. The reactions were monitored by thin-layer chromatography (TLC) on Alugram SIL G/UV 254 sheets (Layer: 0.2 mm) and visualized under UV light. All final compounds were purified by automated flash chromatography with a binary gradient of dichloromethane and methanol and UV variable dual-wavelength detection. Flash chromatography was run on a Teledyne Isco Combiflash® Rf using DCM/MeOH as solvents in the gradient mode and the normal phase of 12 g Silica RediSep® Rf columns.

4.2. General synthetic methods for Chloroquine-Quinoxaline-1,4-di-N-oxide hybrids

*4.2.1. General method for the synthesis of N-(7-chloroquinolin-4-yl)ethane-1,2-diamine (**2**).* 4,7-dichloroquinoline (1.0 equiv, 3.00 g, 15 mmol) was stirred with ethylenediamine (6.0 equiv, 6.01 mL, 90 mmol) under reflux for 1 h. The mixture was allowed to cool to room temperature and then was poured into a water-ice mixture. The precipitate was filtered off. The resulting residue was washed with diethyl ether and used in the next step without further purification. Yield: 92%. IR (KBr) ν cm^{-1}: 3315

(m, N-H); 2950 (w, C-Halip); 1587 (s, C=C); 1020 (m, Ar-Cl). ^{1}H-RMN (400 MHz, DMSO-d_6) δ (ppm): 8.38 (d, J = 5.4 Hz, 1H), 8.31 (d, J = 9.0 Hz, 1H), 7.77 (d, J = 2.4 Hz, 1H), 7.43 (dd, J= 9.0, 2.2 Hz, 1H), 7.31 (bs, 1H), 6.49 (d, J= 5.5 Hz, 1H), 3.27 (t, J = 6.4 Hz, 2H), 2.83 (t, J= 6.5 Hz, 2H), 2.61 (bs, 2H).

*4.2.2. General method for the synthesis of N-[2-(7-chloroquinolin-4-ylamino)ethyl]-3-oxobutanamide (**3**).* Diketene was added (1.2 equiv, 1.56 mL, 20.29 mmol) dropwise to a solution of **2** (1.0 equiv, 3.60 g, 16.23 mmol) in methanol (30 mL) cooled in an ice bath under N_2 atmosphere. The reaction mixture was stirred for 1 h and the residue was suspended in cold diethyl ether. The white precipitate was filtered and used without purification for further reactions. Yield: 75%. IR (KBr) *v*: 3335 (m, N-H); 1718 (s, C=O ketone); 1672 (s, C=O amide); 1579 (s, C=C); 1035 (m, Ar-Cl) cm^{-1}. ^{1}H-RMN (400 MHz, DMSO-d_6) δ (ppm): 8.41 (d, J = 5.3 Hz, 1H), 8.31 (t, J= 5.5 Hz, 1H), 8.18 (d, J= 9.0 Hz, 1H), 7.79 (d, J= 1.8 Hz, 1H), 7.46 (dd, J= 8.9, 1.9 Hz, 1H), 7.38 (t, J= 5.2 Hz, 1H), 6.56 (d, J= 5.4 Hz, 1H), 3.36 (bs, 6H), 2.13 (s, 3H).

*4.2.3. General method for the synthesis of Chloroquine-Quinoxaline-1,4-di-N-oxide hybrids (**4a-e**).* A mixture of **3** (1.2 equiv) and the appropriate benzofuroxan **BFX(a-e)** (1.0 equiv) was dissolved in methanol (30 mL). Calcium chloride (0.1 equiv) and ethanolamine (0.1 equiv) were then added to the solution and the reaction mixture was stirred at room temperature for 2-3 h. The obtained solid was then filtered and washed with cold diethyl ether. The crude product was purified by flash chromatography eluting with DCM/MeOH (9:1) to afford the desired hybrid.

*4.2.3.1. N-[2-(7-chloroquinolin-4-ylamino)ethyl]-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**4a**).* The title compound was synthesized from **3** (1.50 g, 4.90 mmol) and **BFX(a)** (0.70 g, 4.09 mmol) according to the general procedure described above. Yield: 73%, mp: 199-200°C. IR (KBr) *v*: 3421 (m, N-H); 1676 (m, C=O); 1578 (s, C=C); 1333 (s, N-O); 1082 (s, Ar-Cl) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.10 (t, J= 5.7 Hz, 1H), 8.52-8.45 (m, 2H), 8.44 (d, J= 5.4 Hz, 1H), 8.23 (d, J= 9.1 Hz, 1H), 8.03-7.92 (m, 2H), 7.80 (d, J= 2.1 Hz, 1H), 7.48 (dd, J= 9.0, 2.1 Hz, 1H), 7.40 (t, J=

5.2 Hz, 1H), 6.64 (d, *J*= 5.5 Hz, 1H), 3.66 (q, *J*= 6.1 Hz, 2H), 3.53 (q, *J*= 5.8 Hz, 2H), 2.38 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 159.56; 152.00; 150.05; 149.02; 139.07; 137.87; 137.07; 136.36; 133.56; 132.56; 131.73; 127.54; 124.32; 124.02; 119.86; 119.60; 117.51; 98.96; 41.66; 37.60; 14.36. Anal. calcd for $C_{21}H_{18}ClN_5O_3 \cdot$ ½ H_2O: C, 58.27%; H, 4.42%; N, 16.18%; Found: C, 58.38%; H, 4.30%; N, 15.95%.

*4.2.3.2. N-[2-(7-chloroquinolin-4-ylamino)ethyl]-7-chloro-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**4b**).* The title compound was synthesized from **3** (0.96 g, 3.13 mmol) and **BFX(b)** (0.44 g, 2.61 mmol) according to the general procedure described above. Yield: 69%, mp: 146-148°C. IR (KBr) *v*: 3325 (m, N-H); 3096 (m, C-Harom); 1671 (m, C=O); 1578 (s, C=C); 1327 (s, N-O); 1085 (m, Ar-Cl) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.11 (t, *J*= 5.6 Hz, 1H), 8.45 (dd, *J*= 9.2, 2.7 Hz, 2H), 8.42 (d, *J*= 2.4 Hz, 1H), 8.22 (d, *J*= 9.1 Hz, 1H), 8.00 (dd, *J*= 9.2, 2.3 Hz, 1H), 7.80 (d, *J*= 2.2 Hz, 1H**),** 7.48 (dd, *J*= 9.0, 2.1 Hz, 1H), 7.45 (bs, 1H), 6.64 (d, *J*= 5.5 Hz, 1H), 3.67 (q, *J*= 5.7 Hz, 2H), 3.53 (q, *J*= 6.0 Hz, 2H), 2.37 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 159.24; 151.57; 150.32; 148.48; 139.57; 138.59; 136.88; 136.69; 136.02; 133.78; 132.88; 127.11; 124.43; 124.08; 122.02; 118.96; 117.39; 98.94; 41.73; 37.62; 14.33. Anal. calcd for $C_{21}H_{17}Cl_2N_5O_3$: C, 55.02%; H, 3.71%; N, 15.28%; Found: C, 54.62%; H, 4.10%; N, 14.92%.

*4.2.3.3. N-[2-(7-chloroquinolin-4-ylamino)ethyl]-7-methoxy-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**4c**).* The title compound was synthesized from **3** (0.92 g, 3.00 mmol) and **BFX(c)** (0.42 g, 2.50 mmol) according to the general procedure described above. Yield: 68%, mp: 153-155°C. IR (KBr) *v*: 3305 (s, N-H); 3093 (m, C-Harom); 1655 (s, C=O); 1559 (s, C=C); 1328 (s, N-O); 1085 (m, Ar-Cl) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.13 (t, *J*= 5.6 Hz, 1H), 8.44 (d, *J*= 5.4 Hz, 1H), 8.37 (d, *J*= 9.5 Hz, 1H), 8.25 (d, *J*= 9.0 Hz, 1H), 7.80 (d, *J*= 1.9 Hz, 1H), 7.74 (d, *J*= 2.5 Hz, 1H), 7.57 (dd, *J*= 9.5, 2.6 Hz, 1H), 7.48 (dd, *J*= 9.1, 2.0 Hz, 1H), 7.43 (bs, 1H), 6.64 (d, *J*= 5.5 Hz, 1H), 3.97 (s, 3H), 3.67 (q, *J*= 5.8 Hz, 2H), 3.54 (q, *J*= 5.7 Hz, 2H), 2.35 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ

(ppm): 161.54; 159.60; 151.65; 150.19; 148.65; 140.55; 138.16; 137.64; 136.91; 133.64; 132.31; 127.21; 124.30; 124.05; 123.68; 121.31; 117.41; 98.87; 56.42; 41.70; 37.57; 13.93. Anal. calcd for $C_{22}H_{20}ClN_5O_4 \cdot 1/3\ H_2O$: C, 57.45%; H, 4.42%; N, 15.25%; Found: C, 57.30%; H, 4.70%; N, 15.05%.

*4.2.3.4. N-[2-(7-chloroquinolin-4-ylamino)ethyl]-3,7-dimethylquinoxaline-2-carboxamide-1,4-di-N-oxide (**4d**).* The title compound was synthesized from **3** (0.92 g, 3.00 mmol) and **BFX(d)** (0.38 g, 2.50 mmol) according to the general procedure described above. Yield: 71%, mp: 146-148°C. IR (KBr) *v*: 3318 (m, N-H); 3059 (m, C-Harom); 1670 (m, C=O); 1579 (s, C=C); 1329 (s, N-O); 1079 (m, Ar-Cl) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.13 (t, *J*= 5.7 Hz, 1H), 8.44 (d, *J*= 5.4 Hz, 1H), 8.32 (dd, *J*= 8.8, 2.2 Hz, 1H), 8.24 (bs, 1H), 8.22 (s, 1H), 7.79 (d, *J*= 2.2 Hz, 1H), 7.77 (dd, *J*= 8.7, 1.7 Hz, 1H), 7.47 (dd, *J*= 9.0, 2.0 Hz, 1H), 7.43 (t, *J*= 4.9 Hz, 1H), 6.64 (d, *J*= 5.5 Hz, 1H), 3.67 (q, *J*= 6.0 Hz, 2H), 3.54 (q, *J*= 5.8 Hz, 2H), 2.55 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 159.63; 151.70; 150.17; 148.70; 142.50; 138.20; 137.84; 136.44; 135.38; 134.14; 133.63; 127.27; 124.31; 124.01; 119.30; 118.54; 117.42; 98.89; 41.70; 37.60; 21.17; 14.16. Anal. calcd for $C_{22}H_{20}ClN_5O_3 \cdot 1/3\ H_2O$: C, 59.53%; H, 4.66%; N, 15.78%; Found: C, 59.21%; H, 4.81%; N, 15.43%.

*4.2.3.5. N-[2-(7-chloroquinolin-4-ylamino)ethyl]-3,6,7-trimethylquinoxaline-2-carboxamide-1,4-di-N-oxide (**4e**).* The title compound was synthesized from **3** (1.21 g, 3.52 mmol) and **BFX(e)** (0.44 g, 2.94 mmol) according to the general procedure described above. Yield: 73%, mp: 177-179°C. IR (KBr) *v*: 3348 (m, N-H); 3066 (m, C-Harom); 1670 (m, C=O); 1579 (s, C=C); 1330 (s, N-O); 1080 (m, Ar-Cl) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 9.12 (t, *J*= 5.8 Hz, 1H), 8.43 (d, *J*= 5.4 Hz, 1H), 8.25-8.20 (m, 2H), 8.19 (bs, 1H), 7.79 (d, *J*= 2.2 Hz, 1H), 7.47 (dd, *J*= 9.0, 2.2 Hz, 1H), 7.38 (t, *J*= 5.3 Hz, 1H), 6.63 (d, *J*= 5.5 Hz, 1H), 3.67 (q, *J*= 6.2 Hz, 2H), 3.53 (q, *J*= 5.9 Hz, 2H), 2.47 (s, 6H), 2.35 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 159.70; 151.92; 150.00; 148.97; 143.32; 142.38; 138.17; 137.18; 135.27; 134.51; 133.49; 127.49; 124.22; 123.94; 118.67; 118.54; 117.45; 98.87; 41.68;

37.62; 19.87; 19.76; 14.18. Anal. calcd for $C_{23}H_{22}ClN_5O_3$: C, 61.13%; H, 4.87%; N, 15.50%; Found: C, 61.02%; H, 4.95%; N, 15.27%.

4.3. General synthetic methods for Primaquine-Quinoxaline-1,4-di-N-oxide hybrids

*4.3.1. General method for the extraction of primaquine (**PQ**).* Primaquine bisphosphate (1.0 equiv, 1.50 g, 3.30 mmol) was dissolved in an aqueous solution of sodium bicarbonate $NaHCO_3$ (50 mL) and extracted with dichloromethane (2 x 100 mL). The combined organic phases were dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated in vacuo. The free primaquine **PQ** was obtained as a beige oil and used in the subsequent reaction without further purification.

*4.3.2. General method for the synthesis of N-[4-(6-methoxyquinolin-7-ylamine)-4-pentyl]-3-oxobutanamide (**5**).* In a round-bottom flask, 1.0 equiv of **PQ** (0.93 g, 3.59 mmol) was dissolved in methanol (30 mL) cooled in an ice bath under N_2 atmosphere and stirred at 0°C. After 30-45 minutes, diketene (1.2 equiv, 0.35 mL, 4.49 mmol) was added to the solution and the reaction was carried out under stirring for 1-2 h. The residue was concentrated under reduced pressure and the crude product was used without purification for further reactions. Yield: 87%. IR (KBr) *v*: 3387 (m, N-H); 3081 (w, C-H aromatic); 2935 (w, C-H aliphatic); 1718 (s, C=O ketone); 1650 (s, C=O amide); 1520 (vs, C=C); cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.54 (dd, *J*= 4.2, 1.5 Hz, 1H), 8.08 (dd, *J*= 8.3, 1.6 Hz, 1H), 8.04 (t, *J*= 5.3 Hz, 1H), 7.43 (dd, *J*= 8.2, 4.2 Hz, 1H), 6.48 (d, *J*= 2.5 Hz, 1H), 6.27 (d, *J*= 2.5 Hz, 1H), 6.12 (d, *J*= 8.8 Hz, 1H), 3.82 (s, 3H), 3.12-3.08 (m, 1H), 3.33 (bs, 2H), 3.29 (s, 2H), 2.11 (s, 3H), 1.71-1.44 (m, 4H), 1.21 (d, *J*= 6.3 Hz, 3H).

*4.3.3. General method for the synthesis of Primaquine-Quinoxaline-1,4-di-N-oxide hybrids (**6a-e**).* A mixture of **5** (1.2 equiv) and the appropriate benzofuroxan **BFX(a-e)** (1.0 equiv) was dissolved in methanol (30 mL). Calcium chloride (0.1 equiv) and ethanolamine (0.1 equiv) were then added to the solution and the reaction mixture was stirred at room temperature for 1-2 h. The reaction mixture was

concentrated under reduced pressure and the crude product was purified by flash chromatography eluting with DCM/MeOH to afford the desired compound.

*4.3.3.1. Synthesis of N-[4-(6-methoxyquinolin-7-ylamino)-4-pentyl]-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**6a**).* The title compound was synthesized from **5** (0.93 g, 2.71 mmol) and **BFX(a)** (0.31 g, 2.26 mmol) according to the general procedure described above. Purified by flash chromatography eluting with DCM/MeOH (99:1). Yield: 16%, mp: 96-98°C. IR (KBr) *v*: 3384 (m, N-H); 3076 (w, C-H aromatic); 2936 (w, C-H aliphatic); 1678 (s, C=O); 1518 (s, C=C); 1333 (vs, N-O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.91 (t, *J*= 5.6 Hz, 1H), 8.52 (dd, *J*= 4.1, 1.5 Hz, 1H), 8.46 (dd, *J*= 8.1, 1.3 Hz, 1H), 8.12 (dd, *J*= 8.1, 1.3 Hz, 1H), 8.06 (dd, *J*= 8.2, 1.3 Hz, 1H), 7.95-7.83 (m, 2H), 7.41 (ddd, *J*= 8.2, 4.2, 1.1 Hz, 1H), 6.44 (d, *J*= 2.0 Hz, 1H), 6.27 (d, *J*= 2.0 Hz, 1H), 6.16 (d, *J*= 8.7 Hz, 1H), 3.77 (s, 3H), 3.72-3.67 (m, 1H), 3.38 (bs, 2H), 2.59 (s, 3H), 1.84-1.51 (m, 4H), 1.24 (d, *J*= 6.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 164.58; 158.98; 150.46; 144.63; 144.25; 141.62; 138.95; 136.07; 134.82; 134.56; 131.38; 131.20; 129.90; 129.59; 122.12; 118.24; 96.21; 91.57; 54.96; 46.98; 33.37; 25.69; 20.25; 15.22; 13.77. Anal. calcd for $C_{25}H_{27}N_5O_4 \cdot$ ½ H_2O: C, 63.83%; H, 5.96%; N, 14.89%; Found: C, 63.49%; H, 5.62%; N, 15.24%.

*4.3.3.2. Synthesis of N-[4-(6-methoxyquinolin-7-ylamino)-4-pentyl]-7-chloro-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**6b**).* The title compound was synthesized from **5** (1.10 g, 3.20 mmol) and **BFX(b)** (0.46 g, 2.67 mmol) according to the general procedure described above. Purified by flash chromatography eluting with DCM/MeOH (98:2). Yield: 43%, mp: 102-104°C. IR (KBr) *v*: 3379 (m, N-H); 3075 (w, C-H aromatic); 2960 (w, C-H aliphatic); 1679 (s, C=O); 1518 (vs, C=C); 1327 (vs, N-O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.86 (t, *J*= 5.6 Hz, 1H), 8.53 (dd, *J*= 4.2, 1.6 Hz, 1H), 8.46 (d, *J*= 9.1 Hz, 1H), 8.42 (d, *J*= 2.2 Hz, 1H), 8.06 (dd, *J*= 8.3, 1.6 Hz, 1H), 7.98 (dd, *J*= 9.2, 2.3 Hz, 1H), 7.41 (dd, *J*= 8.2, 4.2 Hz, 1H), 6.47 (d, *J*= 2.5 Hz, 1H), 6.30 (d, *J*= 2.4 Hz, 1H), 6.16 (d, *J*= 8.8 Hz, 1H), 3.82 (s, 3H), 3.76-3.62 (m, 1H), 3.39 (m, 2H), 2.37 (s, 3H), 1.78-1.58 (m, 4H), 1.24 (d, *J*= 6.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 159.02; 158.56; 144.62; 144.24; 139.47;

138.76; 136.87; 136.57; 135.88; 134.79; 134.55; 132.73; 129.58; 122.09; 121.92; 118.96; 96.29; 91.65; 54.98 (2C); 46.97; 33.38; 25.55; 20.24; 15.16; 14.19. Anal. calcd for $C_{25}H_{26}N_5O_4Cl$: C, 60.54%; H, 5.25%; N, 14.13%; Found: C, 60.70%; H, 5.61%; N, 13.70%.

*4.3.3.3. Synthesis of N-[4-(6-methoxyquinolin-7-ylamino)-4-pentyl]-7-methoxy-3-methylquinoxaline-2-carboxamide-1,4-di-N-oxide (**6c**).* The title compound was synthesized from **5** (0.86 g, 2.50 mmol) and **BFX(c)** (0.35 g, 2.09 mmol) according to the general procedure described above. Purified by flash chromatography eluting with DCM/MeOH (98:2). Yield: 47% mp: 95-97°C. IR (KBr) *v*: 3221 (m, N-H); 3049 (w, C-H aromatic); 2936 (w, C-H aliphatic); 1677 (s, C=O); 1520 (vs, C=C); 1328 (vs, N-O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.88 (t, *J* = 5.6 Hz, 1H), 8.53 (dd, *J*= 4.2, 1.1 Hz, 1H), 8.38 (d, *J*= 9.5 Hz, 1H), 8.08 (dd, *J*= 8.2, 1.3 Hz, 1H), 7.75 (d, *J*= 2.7 Hz, 1H), 7.58 (dd, *J*= 9.5, 2.7 Hz, 1H), 7.42 (dd, *J*= 8.2, 4.2 Hz, 1H), 6.48 (d, *J*= 2.3 Hz, 1H), 6.30 (d, *J*= 2.3 Hz, 1H), 6.16 (d, *J*= 8.8 Hz, 1H), 3.97 (s, 3H); 3.82 (s, 3H), 3.76-3.61 (m, 1H), 3.30 (bs, 2H), 2.35 (s, 3H), 1.80-1.52 (m, 4H), 1.24 (d, *J*= 6.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 161.55; 159.04; 158.98; 144.66; 144.30; 138.41; 137.73; 136.89; 134.86; 134.57; 132.26; 129.63; 123.72; 122.17; 121.32; 98.93; 96.27; 91.68; 56.47; 55.02; 46.96; 33.38; 25.62; 20.27; 15.54; 13.92. Anal. calcd for $C_{26}H_{29}N_5O_5$: C, 63.54%; H, 5.91%; N, 14.25%; Found: C, 63.27%; H, 6.31%; N, 13.86%.

*4.3.3.4. Synthesis of N-[4-(6-methoxyquinolin-7-ylamino)-4-pentyl]- 3,7-dimethylquinoxaline-2-carboxamide-1,4-di-N-oxide (**6d**).* The title compound was synthesized from **5** (1.21 g, 3.52 mmol) and **BFX(d)** (0.44 g, 2.94 mmol) according to the general procedure described above. Purified by flash chromatography eluting with DCM/MeOH (99:1). Yield: 18%, mp: 100-101°C. IR (KBr) *v*: 3221 (m, N-H); 3077 (w, C-H aromatic); 2963 (w, C-H aliphatic); 1678 (s, C=O); 1519 (vs, C=C); 1329 (vs, N-O) cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.87 (t, *J*= 5.6 Hz, 1H), 8.53 (dd, *J*= 4.2, 1.6 Hz, 1H), 8.33 (t, *J*= 9.1 Hz, 1H), 8.24 (bs, 1H), 8.07 (dd, *J*= 8.3, 1.6 Hz, 1H), 7.77 (dd, *J*= 9.0, 1.6 Hz, 1H), 7.42 (dd, *J*= 8.2, 4.2 Hz, 1H), 6.47 (d, *J*= 2.5 Hz, 1H), 6.31 (d, *J*= 2.5 Hz, 1H), 6.16 (d, *J*= 8.8 Hz, 1H), 3.82 (s, 3H), 3.77-3.62 (m, 1H), 3.42-3.36 (m, 2H), 2.56 (d, *J*= 4.4 Hz, 1H), 2.38 (s, 3H), 1.90-1.51 (m,

4H), 1.24 (d, *J*= 6.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 159.03; 158.96; 144.64; 144.25; 142.41; 138.14; 136.18; 135.29; 134.80; 134.56; 134.06; 133.23; 129.60; 122.10; 119.27; 118.60; 96.27; 91.66; 54.98; 46.97; 33.39; 25.57; 21.14; 20.24; 15.16; 14.08. Anal. calcd for $C_{26}H_{29}N_5O_4$: C, 65.68%; H, 6.10%; N, 14.74%; Found: C, 65.98%; H, 5.92%; N, 14.56%

*4.3.3.5. Synthesis of N-[4-(6-methoxyquinolin-7-ylamino)-4-pentyl]- 3,6,7-trimethylquinoxaline-2-carboxamide-1,4-di-N-oxide (**6e**).* The title compound was synthesized from **5** (1.00 g, 2.91 mmol) and **BFX(e)** (0.40 g, 2.43 mmol) according to the general procedure described above. Purified by flash chromatography eluting with DCM/MeOH (99:1).Yield: 11%, mp: 94-95.8°C. IR (KBr) *v*: 3369 (m, N-H); 3064 (w, C-H aromatic); 2955 (w, C-H aliphatic); 1679 (s, C=O); 1518 (vs, C=C); 1330 (vs, N-O) cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.88 (t, *J*= 5.7 Hz, 1H), 8.53 (dd, *J*= 4.2, 1.6 Hz, 1H), 8.18 (s, 1H), 8.15 (s, 1H), 8.06 (dd, *J*= 8.3, 1.6 Hz, 1H), 7.41 (dd, *J*= 8.2, 4.2 Hz, 1H), 6.47 (d, *J*= 2.5 Hz, 1H), 6.30 (d, *J*= 2.5 Hz, 1H), 6.16 (d, *J*= 8.8 Hz, 1H), 3.82 (s, 3H), 3.76-3.61 (m, 1H), 3.43-3.35 (m, 2H), 2.45 (d, *J*= 3.9 Hz, 6H), 2.37 (s, 3H), 1.84-1.57 (m, 4H), 1.24 (d, *J*= 6.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 159.02; 144.62; 144.24; 143.23; 142.31; 138.13; 137.40; 135.18; 134.80; 134.56; 129.59; 122.10; 118.73; 118.52; 96.26; 91.66; 54.98; 46.97; 33.40; 25.58; 20.24; 19.86; 19.72; 15.16; 14.11. Anal. calcd for $C_{27}H_{31}N_5O_4 \cdot 1/3\,H_2O$: C, 65.45%; H, 6.40%; N, 14.14%; Found: C, 65.51%; H, 6.76%; N, 13.86%.

4.4. Parasites, cell lines and primary cultures

GFP-expressing *P. berghei* (*Pb*GFP) and *P. yoelii* (*Py*GFP) (Manzoni et al., 2014) were used. *Pb*GFP and *Py*GFP blood stage parasites were propagated in female Swiss mice (6–8 weeks old). *Anopheles stephensi* mosquitoes were fed on *Pb*GFP or *P. yoelii*-infected mice and kept at 21°C and 24°C, respectively. *Pb*GFP and *Py*GFP sporozoites were freshly isolated from the salivary glands of infected mosquitoes 21 or 15 days post-feeding, respectively. *A. stephensi* mosquitoes infected with *P. falciparum* sporozoites (NF54 strain) were obtained from the Department of Medical Microbiology,

University Medical Centre, St Radboud, Nijmegen, Netherlands. *P. yoelii* 17XNL cryopreserved sporozoites were obtained in the Sanaria® Company. BALB/c mice were obtained from Animal Facilities University of Antioquia. These experiments were conducted according to Colombian legislation on laboratory animal use and care (No. 008430) and the institutional ethical committee for experimentation in animals approved all assays performed in the animal model (Act, June 25, 2015). HepG2 (ATCC HB-8065) and HepG2-CD81 cells (Yalaoui et al., 2008) were cultured in 96 well culture plates coated with rat tail collagen I (Becton-Dickinson, Le Pont de Claix, France) at 37°C under 5% CO_2 in DMEM supplemented with 10% fetal calf serum and antibiotics (Life Technologies). Primary human hepatocytes were isolated and cultured as described previously (Silvie et al., 2003). Human liver fragments used to prepare primary hepatocyte cultures were collected after written informed consent from patients undergoing a partial hepatectomy. The collection and use of these tissues were undertaken in accordance with French national ethical regulations and have been approved by the Ethic Committee of the Centre Hospitalo-Universitaire Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, France.

4.5. In silico physicochemical properties calculation

Topological Polar Surface Area (TPSA) (Ertl et al., 2000), ALOGPs2.1, number of rotatable bonds and violations of Lipinski´s rule of five (Lipinski et al., 1997) were calculated using Virtual Computational Chemistry Laboratory (Tetko et al., 2005) (http://www.vcclab.org/) and SwissADME web tool (http://www.swissadme.ch/) (Daina et al., 2017).

4.6. Blood Stage Activity Assays

4.6.1. In vitro antiplasmodial activity (3D7 and FCR-3 strains of P.falciparum)

The chloroquine sensitive 3D7 strain and the multidrug resistant FCR-3 of *P. falciparum* were cultured at 37°C in 5% CO_2, 5% O_2 in a balanced N_2 atmosphere environment on RPMI 1640 medium

supplemented with 25 mM HEPES, 5% (w/v) $NaHCO_3$ and 0.1 mg/mL gentamicin 0.1 mg/mL and 10% heat-inactivated A^+ human serum, as previously described (Desjardins et al, 1979). Chloroquine-QdNO and primaquine-QdNO hybrids were dissolved in dimethyl sulfoxide and then added at final concentrations ranging from 1.56 to 100 μg/mL (range of 3.5-198 μM). The final DMSO concentration was never greater than 1%. *In vitro* antimalarial activity was measured using Hoechst® 33342 (Thermo Fisher Scientific) nuclei acid staining according to Malleret et al. (2011). Briefly, 250 μL of the total culture medium with the diluted drug and the suspension of human red blood cells in medium (A^+ group, 2% haematocrit) with 1% parasitaemia and predominance of rings (>80%) were placed into the wells of 96-well microtiter plates. On the second day of the test (48 h), analysis of $1x10^4$ cells by flow cytometry was performed using a FACS CantoTMII FlowCytometer, Becton Dickinson (San Jose´ CA). All experiments were performed in triplicate. The results were expressed as the concentration resulting in 50% inhibition (IC_{50}), which was calculated by a nonlinear regression logistic dose response-sloped variable model. The mean IC_{50} values and standard deviation for each compound were calculated.

4.7. In vitro cytotoxicity assays (HepG2, HepG2-CD81 and human hepatocytes)

Hybrids cytotoxicity for the HepG2, HepG2-CD81 cell lines or primary human hepatocytes was evaluated by a colorimetric test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described by Mosmann (1983). In brief, the cells were cultured in a 96-well flat-bottomed plate (2 × 105 cells/well in 100 μL complete medium) and incubated for 24 h at 37°C in a 5% CO_2 humidified atmosphere to allow monolayer formation. An aliquot of each compound dilution was then added to the wells in triplicate. The plates were incubated for another 48 h and then 30 μL MTT (2 mg/mL) was added and the plates were incubated again for 4 h; DMSO (96%, 130 μL) was added and plates incubated for a further 20 min at room temperature. The DMSO diluent agent and untreated cells in complete medium as negative controls were included. Absorbance was measured at 550 nm and

Statistical Prism version 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used to calculate the toxic concentration (CC_{50}).

4.8. Liver Stage Activity Assays

4.8.1. In vitro exoerythrocytic activity (P. yoelii, P. berghei and P. falciparum)

To assess liver stage development, HepG2 or HepG2-CD81 cells (3×10^4 per well in collagen-coated 96-well plates) were infected with GFP-expressing sporozoites (5×10^3 to 1×10^4 per well) and cultured for 40 h prior to analysis by fluorescence microscopy, after fixation with cold methanol and immunolabelling of exoerythrocytic forms (EEFs) with antibodies specific for *Plasmodium* HSP70. Primary human hepatocytes (8×10^4 per well in collagen-coated 96-well plates) were infected with *P. falciparum* sporozoites (2.5×10^4 per well) and cultured for 8 days prior to fixation with cold methanol and immunolabelling of EEFs with antibodies specific for *Plasmodium* HSP70. Primaquine was used as the reference drug in all experiments. Hybrids were diluted in DMEM at 10 µg/mL as the maximum concentration and seven serial dilutions were performed. The treatments of the cells were simultaneously with infection. The culture medium was changed after 3 h and every 24 h post infection and fresh compounds were added at the same concentration to maintain exposure. The cultures were allowed to grow at 37°C in 5% CO_2. After the time necessary for the development of each parasite, the cells were fixed with cold methanol, and then incubated for 1 h with antibodies specific to *Plasmodium* HSP70 at 37°C. The plates were washed three times with PBS and incubated with secondary antibodies coupled to Alexa 594 (Life Technologies) for 1 h at 37°C, using DAPI (Life Technologies) as nuclei staining. Analysis of liver stage parasites was performed using a CellInsight NXT HCS Platform (Thermo Scientific). All experiments were performed in triplicate. The results were expressed as the concentration resulting in 50% inhibition (IC_{50}), which was calculated by a nonlinear regression logistic dose response-sloped variable model. The mean IC_{50} values and standard deviation for each compound were calculated.

4.8.2. In vivo mouse causal chemoprophylaxis efficacy

The causal prophylaxis efficacy was determined according to the method modified by Peters (Peters, 1970). BALB/c mice (n=5 per negative and positive control group; n=4 per treatment group; 6-8 weeks old; 20-23 g) were randomly allotted to five treatment groups and two control groups and orally administered either 100 µL of the tested hybrid compounds (**6a**, **6b**, **6c**, **6d** and **6e**) at the dose of 100 mg/kg dose on days -1, 0, +1, and +2 or DMSO 100% (negative control group). Primaquine diphosphate 98% (Sigma Aldrich) was used as a positive control at the dose of 30 mg/kg daily. On day 0, the mice were infected by intravenous inoculation of 5 x 10^3 *P.yoelii* 17XNL cryopreserved sporozoites obtained from Sanaria Company. Blood samples were obtained from each mouse on days 4, 5, 7 and 14 and the infection was monitored by a blood smear stained with Giemsa and flow cytometry using Sybr Green as a DNA marker. The mice were observed daily for clinical signs and mortality. The prophylactic activity was expressed in terms of the absence or decrease of parasitaemia compared to the negative control group. Statistical analysis was undertaken via one-way analysis of variance (ANOVA) tests coupled to Tukey HSD tests, using GraphPad Prism™ program version 5.01. The exact p-value is given only if it exceeded 0.01.

4.9. Effects on Transmission Stages

4.9.1. P. berghei sporogonic assays

Two Swiss mice from the experimental group (female; 6 weeks old, 25 g) were inoculated intraperitoneally (i.p.) with 200 µL of *Pb*GFP infected red blood cells (3000 parasites/µL). For 4 days after the infection, parasitaemia was monitored using Giemsa-stained blood smear and examined for the presence of gametocytes and testing for exflagellating gametocytes. *A. stephensi* mosquitoes were blood-fed from the mice whose blood showed 1% of gametocytes during 1 h. Hybrids **6a** and **6b** were administered (i.p) at 100 mg/kg in DMSO 1.5 h before infection of mosquitoes. Mice untreated and treated with DMSO (96%, 50 µL) were used as controls. After 8 days, 30 mosquitoes per group were

dissected. Midguts were removed and the number of oocysts was determined by fluorescence microscopy. Inhibition of sporogony was calculated based on the number of oocysts in the control mosquitoes considered as 100% infection. Statistical Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA, USA) was used for comparison between the groups.

4.10. Preliminary Toxicity Studies

4.10.1. In vivo mouse acute oral toxicity testing

The acute oral toxicity of hybrid **6a** was evaluated according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD, 2001). BALB/c (female mice; 6-8 weeks old; 20-23 g and variation in weight lower ± 20%) were divided into groups of three and each mouse was treated with a single oral dose of 300 mg/kg or 500 mg/kg body weight of this hybrid and the control group received the vehicle (DMSO 100%). Another control group was treated with 2000, 500 and 300 mg/kg body weight doses of primaquine. The animals were observed for signs of toxicity for 4 h after the administration of the dose and changes in physical appearance, injury, pain and signs of illness were recorded daily for the 14 days of the study. On day 14 or in the case of signs of suffering, the animals were euthanized in CO_2 chamber. After the death of the animals, the analysis of macroscopic pathological anatomy was carried out and tissue samples from the spleen, liver and kidney were fixed in formaldehyde 37% and taken for microscopic evaluation using haematoxylin and eosin staining performed in the Pathology Laboratory of the Faculty of Veterinary Sciences of the University of Antioquia. Blood samples were also taken by cardiac puncture to measure biochemical parameters of renal function (urea nitrogen and creatinine) and hepatic (alanine aminotransferase: ALT and total bilirubin) and Lab tests-complete blood count using a Mindray-2800vet.

4.10.2. Genotoxicity assay

The SOS/*umu* test was used to determine the DNA damage effect and was carried out according to the method of Oda et al. (1985) and Reifferscheid et al. (1991) with some modifications. The test strain *Salmonella typhimurium* TA1535/pSK1002 (German Collection for microorganisms and Cell cultures (DSMZ)) from stock (-80°C; in TGA medium containing 10% DMSO as cryoprotective agent) was thawed and 0.5 mL of bacteria were suspended in 100 mL TGA medium supplemented with ampicillin (50 µg/mL). The tester strain suspension was incubated overnight at 37°C with slight orbital shaking (155 rpm) until optical density was reached (OD_{600} between 0.5 and 1.5). Then, the overnight culture was diluted with fresh (not supplemented with ampicillin) TGA medium and incubated for 2 h at 37°C, 155 rpm in order to obtain log-phase bacteria exponential growth culture (OD_{600} between 0.15 and 0.4).

The test was performed in the absence and presence of an external metabolic activation system (10% of rat S9 mix, prepared from S9 SD rat liver Aroclor KCl frozen, Trinova, Germany) in order to also determine the possible genotoxic effects of any metabolite. Negative and positive controls were included in each test performed, with DMSO used as the solvent control (negative control), and 4-nitroquinoline-N-oxide (4-NQO) (Sigma-Aldrich, China) and 2-aminoanthracene (2-AA) (Sigma-Aldrich, Germany) used as positive controls in the absence and presence of the S9 mix, respectively.

The test procedure was as follows: first, each tested compound was dissolved in DMSO at 40 mg/mL (for the final concentration in the assay of 1 mg/mL) and 11 serial ½ dilutions were prepared in a 96-well plate (plate A; final volume in each well was 10 µL). The highest concentrations in DMSO used for the positive controls were 100 µg/mL for 4-NQO (final concentration: 2.5 µg/mL) and 0.5 mg/mL for 2-AA (final concentration: 0.0125 mg/mL). Then, 70 µL of water was added to each well. At this point, each well was checked in order to detect any precipitation of the compounds. In other two 96-well plates (plates B; one for the test with S9 and the other without S9), 10 µL S9 mix or 10 µL PBS, respectively, were added followed by the addition of 25 µL of each concentration of the compound previously prepared. Finally, 90 µL of exponentially growing bacteria was added to each

751 well and both plates were incubated for 4 h by shaking (500 rpm) at 37°C. After the incubation period,
752 absorbance at 600 nm was measured in order to evaluate the toxicity on *S. typhimurium*
753 TA1535/pSK1002.

754 Toxicity was calculated as follows:

$$\text{Survival percentage} = \left(\frac{\text{A}_{600\text{nm}} \text{ for each concentration tested}}{\text{Media A}_{600\text{nm}} \text{ for negative control}}\right) \times 100$$

755

756 Afterwards, for the determination of β-galactosidase activity, in two new 96-well plates (plates C)
757 150 µL ONPG solution (2-nitrophenyl-β-D-galactopyranoside, Sigma-Aldrich, Switzerland) (0.9
758 mg/mL in B-buffer prepared according to Reifferscheid et al. (1991) was added to each well and 30 µL
759 of the content of each well of the plates B was transferred to these plates C. Both plates were incubated
760 for 30 minutes by shaking (500 rpm) at 28°C avoiding direct light exposure. After the incubation
761 period, 120 µL of the stop reagent (Na_2CO_3, 1 M) was added to stop the reaction. Absorbance at 420
762 nm was then measured immediately, and β-galactosidase activity (relative units; RU) was calculated as
763 follows:

$$\beta \text{ galactosidase enzymatic units} = \frac{\text{A}_{420\text{ nm}} \text{ for each concentration tested}}{\text{A}_{600\text{ nm}} \text{ for each concentration tested}}$$

764

765 And finally, the induction factor (IF) was calculated as:

$$\text{IF} = \frac{\beta \text{ galactosidase RU for each concentration tested}}{\text{Average } \beta \text{ galactosidase RU for negative control}}$$

766

767 Where:

$$\text{Average } \beta \text{ galactosidase RU for negative control} = \frac{\text{Average A}_{420\text{ nm}} \text{ for negative control}}{\text{Average A}_{600\text{ nm}} \text{ for negative control}}$$

768

In the same way, β-galactosidase relative units were calculated for both positive controls and the test was only considered valid if the positive controls reached an induction factor ≥2 under the given test conditions.

Thus, a compound was considered genotoxic when in any of the conditions studied (with or without metabolic activation) the induction factor was ≥2 at non-toxic concentrations (bacteria survival percentage ≥ 80%). Any well where compound precipitation was observed was discarded from the analysis.

Author's Contributions

The manuscript was written through contributions of all authors. All authors approved the final version of the manuscript. The authors declared that there is no conflict of interest.

Acknowledgements

This work was supported by Colciencias grant #111526934273 (contract RC-597-2013), PIUNA Project-University of Navarra and Foundation CAN (grant number: 70391). The authors are grateful to Universidad de Antioquia for its Sustainability Strategic Plan and the Institute of Tropical Health (ISTUN) of University of Navarra for their financial support and help. Leonardo Bonilla-Ramírez is funded by Colciencias Grants (528-2011), and Miguel Quiliano is grateful to "Programa Nacional de Innovación para la competitividad y productividad" (Innóvate-Perú) for his PhD scholarship (grant 065-FINCYT-BDE-2014). The authors greatly acknowledge Centre d'Immunologie et des Maladies Infectieuses-UPMC for the intership of BR-L. This work benefited from equipment and services from the CELIS cell culture core facility (Institut du Cerveau et de la Moelle Epinière, Paris), a platform supported through the ANR grants, ANR-10-IAIHU-06 and ANR-11-INBS-0011-NeurATRIS. We are particularly grateful to David Akbar for his valuable assistance regarding automated fluorescence.

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Highlights

- New chloroquine/primaquine–quinoxaline 1,4-di-*N*-oxide (QdNO) hybrids were synthesized
- Chloroquine-based **4b** was the most active in blood stage with a moderate liver activity
- Primaquine-QdNO **6a**/**6b** displayed better liver *Py/Pb* activity and SI than primaquine
- Hybrid **6a** showed causal prophylactic activity and high inhibition of sporogony (92%)
- Absence of genotoxicity and *in vivo* acute toxicity for primaquine-QdNO **6a** was found