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# 1 Antiplasmodial 2-Thiophenoxy-3-trichloromethyl 2 quinoxalines target the apicoplast of *Plasmodium* 3 *falciparum*.

4  
5 Dyhia Amrane<sup>1</sup>, Nicolas Primas<sup>1,2\*</sup>, Christophe-Sébastien Arnold<sup>3</sup>, Sébastien Hutter<sup>4</sup>, Béatrice  
6 Louis<sup>1</sup>, Julen Sanz-Serrano<sup>5</sup>, Amaya Azqueta<sup>5,6</sup>, Nadia Amanzougaghene<sup>7</sup>, Shahin Tajeri<sup>7</sup>, Dominique  
7 Mazier<sup>7</sup>, Pierre Verhaeghe<sup>8,9</sup>, Nadine Azas<sup>4</sup>, Cyrille Botté<sup>3,\*</sup> and Patrice Vanelle<sup>1,2\*</sup>

8 Dedicated to the memory of Professor José Maldonado, PharmD-PhD (1940 – 2020)

9 <sup>1</sup> Aix Marseille Univ, CNRS, ICR UMR 7273, Equipe Pharmaco-Chimie Radicalaire, Faculté de  
10 Pharmacie, 13385 Marseille cedex 05, France

11 <sup>2</sup> APHM, Hôpital Conception, Service Central de la Qualité et de l'Information Pharmaceutiques,  
12 13005 Marseille, France

13 <sup>3</sup> ApicoLipid Team, Institute for Advanced Biosciences, Université Grenoble Alpes, La Tronche, France.

14 <sup>4</sup> Aix Marseille Univ, IHU Méditerranée Infection, UMR VITROME, IRD, SSA, Mycology & Tropical  
15 Eucaryotic pathogens, 13005 Marseille cedex 05, France

16 <sup>5</sup> Department of Pharmacology and Toxicology, Faculty of Pharmacy and Nutrition, University of  
17 Navarra, C/ Irunlarrea 1, CP 31008, Pamplona, Navarra, Spain

18 <sup>6</sup> Navarra Institute for Health Research, IdiSNA, Irunlarrea 3, 31008 Pamplona, Spain

19 <sup>7</sup> Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses, CIMI,  
20 75013 Paris, France

21 <sup>8</sup> LCC-CNRS Université de Toulouse, CNRS, UPS, 31400 Toulouse, France

22 <sup>9</sup> CHU de Toulouse, Service Pharmacie, 330 avenue de Grande-Bretagne, 31059 Toulouse cedex 9,  
23 France

24 \* Correspondance: nicolas.primas@univ-amu.fr (N.P.); cyrille.botte@univ-grenoble-alpes.fr (C.B.);  
25 Patrice.vanelle@univ-amu.fr (P.V)

## 26 27 28 **Abstract**

29 The identification of a plant-like Achilles' Heel relict, i.e. the apicoplast, that is essential for  
30 *Plasmodium* spp., the causative agent of malaria lead to an attractive drug target for new  
31 antimalarials with original mechanism of action. Although it is not photosynthetic, the apicoplast  
32 retains several anabolic pathways that are indispensable for the parasite. Based on previously  
33 identified antiplasmodial hit-molecules belonging to the 2-trichloromethylquinazoline and 3-  
34 trichloromethylquinoxaline series, we report herein an antiplasmodial Structure-Activity  
35 Relationships (SAR) study at position two of the quinoxaline ring of 16 newly synthesized  
36 compounds. Evaluation of their activity toward the multi-resistant K1 *Plasmodium falciparum* strain  
37 and cytotoxicity on the human hepatocyte HepG2 cell line revealed a hit compound (**3k**) with a Pfk1  
38 EC<sub>50</sub> value of 0.3 μM and a HepG2 CC<sub>50</sub> value of 56.0 μM (selectivity index = 175). Moreover, hit-  
39 compound **3k** was not cytotoxic on VERO or CHO cell lines and was not genotoxic in the *in vitro*  
40 comet assay. Activity cliffs were observed when the trichloromethyl group was replaced by CH<sub>3</sub>, CF<sub>3</sub>  
41 or H, showing that this group played a key role in the antiplasmodial activity. Biological investigations  
42 performed to determine the target and mechanism of action of the compound **3k** strongly suggest  
43 that the apicoplast is the putative target as showed by severe alteration of apicoplast biogenesis  
44 and delayed death response. Considering that there are very few molecules that affect the

45 *Plasmodium* apicoplast, our work provides, for the first time, evidence of the biological target of  
46 trichloromethylated derivatives.

47

#### 48 Key words

49 Quinoxaline

50 Trichloromethyl group

51 *Plasmodium falciparum*

52 In vitro antiplasmodial activity

53 Structure-activity relationships

54 Apicoplast

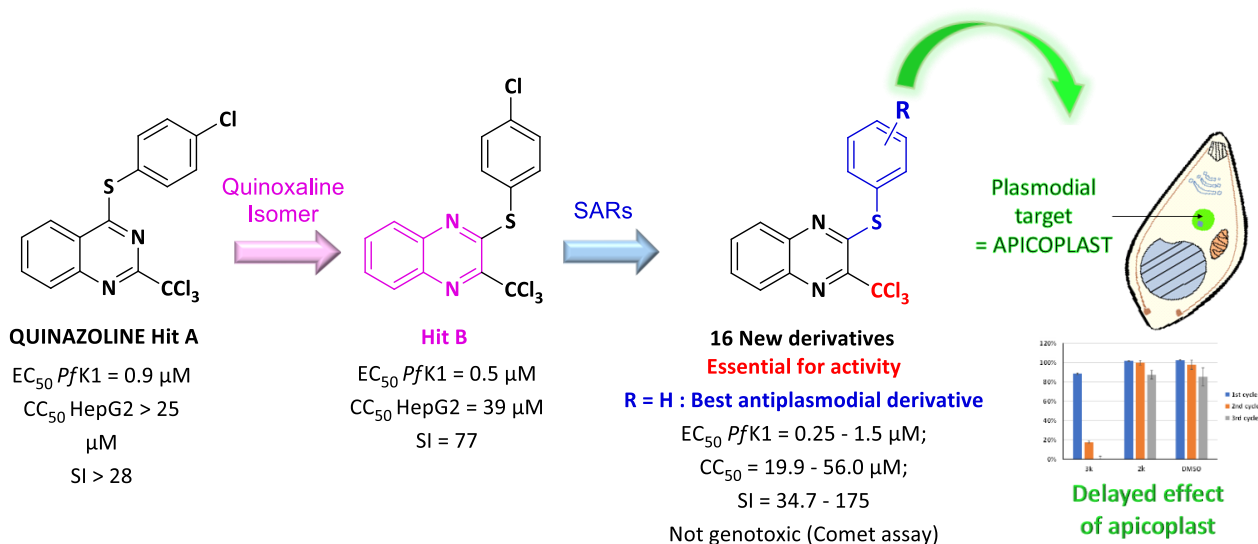
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#### 56 Highlights

- 57 • 16 new compounds were obtained in the 2-thiophenoxy-3-trichloromethylquinoxaline series
- 58 • **3k** showed good and selective antiplasmodial activity and was not genotoxic
- 59 • The trichloromethyl group is essential for providing the antiplasmodial activity
- 60 • The hit molecule **3k** showed typical anti-apicoplast activity
- 61 • Size of schizonts were affected at high concentration of **3k** without hepatotoxicity

62

63 *Graphical abstract*



64

65

66

#### 67 Introduction

68 Malaria remains the leading cause of death among parasitic infections worldwide, mainly  
69 affecting children under the age of 8, the elderly and pregnant women. According to the World  
70 Malaria Report 2020 [1], malaria caused an estimated 229 million clinical cases leading to 409 000  
71 deaths. Eighty nine countries from the sub-tropical zone are endemic, where the mosquito vectors  
72 are present. Most cases and deaths occur in sub-Saharan Africa and South-East Asia. Malaria is  
73 caused by a unicellular eukaryote, i.e. protist, of the Apicomplexa phylum and the *Plasmodium*  
74 genus, among which the species *falciparum* is responsible for most fatal malaria cases, including  
75 cerebral malaria. The parasite is transmitted to humans by a bite of an infected female *Anopheles*  
76 mosquito during a blood meal. Despite numerous and ambitious malaria control efforts, *Plasmodium*

77 strains that resist commercial anti-malarial drugs such as chloroquine [2], quinine [3], atovaquone  
78 [4], mefloquine [5], piperazine [6] and associations of pyrimethamine-cycloguanil [7] or  
79 sulfadoxine-pyrimethamine [8] are spreading worldwide. In addition, the emergence of resistances  
80 toward the first line treatments against severe *P. falciparum* infections, Artemisinin-based  
81 Combination Therapies (ACTs), is now threatening the efficacy of malaria treatment particularly in  
82 the Greater Mekong Subregion [9], Africa [10,11] and India [12]. Therefore, new chemotypes with  
83 original modes of action, for use in combination therapies, are urgently needed to overcome these  
84 resistances and move towards malaria eradication.

85

86 Working on the synthesis and reactivity of new heterocycles bearing a trichloromethyl group  
87 as potential antiparasitic agents [13-15], our research group has previously reported an  
88 antiplasmodial 2-trichloromethylquinazoline derivative (Hit A, Table 1) bearing a thiophenoxy group  
89 at position 4 [16]. From this initial compound, a quinoxaline position isomer (Hit B, **Table 1**) was  
90 prepared and evaluated, showing an improved antiplasmodial profile [17]. Based on this encouraging  
91 result, we decided to synthesize a series of original quinoxaline derivatives bearing a thiophenoxy  
92 moiety at the 2-position of the quinoxaline ring, with the aim of identifying a new optimized hit-  
93 compound and investigating the antiplasmodial structure-activity relationships.

94

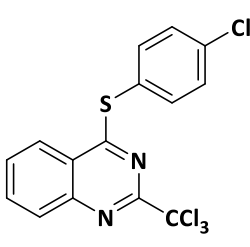
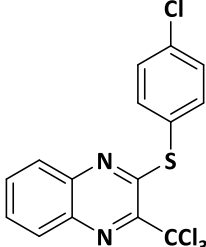
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**Table 1.**

Comparison between antiplasmodial Hit A and position isomer Hit B

96

97

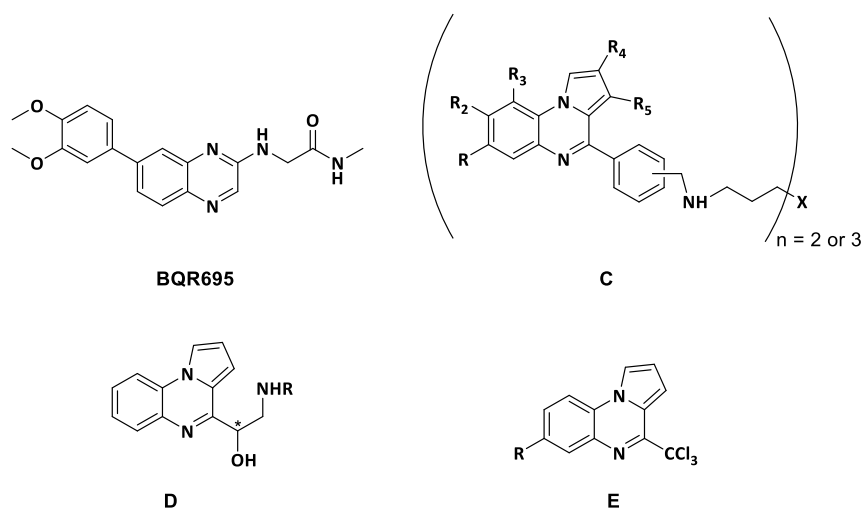
		
	<b>Hit A</b>	<b>Hit B</b>
EC <sub>50</sub> <i>PfK1</i> (μM)	0.9	0.5
CC <sub>50</sub> HepG2 (μM)	>25	38.6
Selectivity index (SI)	>28	77.2

98

99

100

101 Indeed, several quinoxalines have already been identified as promising antiplasmodial  
102 molecules, including BQR695 (**Figure 1**), identified from a phenotypic screening of the Novartis  
103 chemical library, exerting antiplasmodial activity through inhibition of the plasmodial protein kinase  
104 PfPI(4)K [18]. Guillon et al. [19] previously described a series of bis- and tris-pyrrolo[1,2-*a*]quinoxaline  
105 derivatives (**C**, Figure 1) showing good antiplasmodial activity during the intraerythrocytic stage of *P.*  
106 *falciparum* W2 and 3D7 with EC<sub>50</sub> in the micromolar range. In addition, (*R*)-enantiomers of  
107 pyrrolo[1,2-*a*]quinoxalines (**D**) showed good antiplasmodial activities [20]. In addition, our lab has  
108 previously described 2-trichloromethylpyrroloquinoxaline derivatives (**E**) which also show selective  
109 antiplasmodial activity [21], illustrating the potential of quinoxaline ring in antiplasmodial drug-  
110 design.

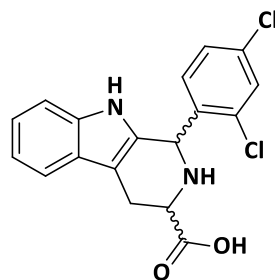


**Figure 1.** Structures of some previously described antiplasmodial derivatives containing a quinoxaline moiety.

With the aim of discovering and developing new antimalarial compounds, Medicines for Malaria Ventures (MMV) defined target candidates (TCPs) and target product profiles (TPPs) for the treatment and chemoprevention of malaria [22]. MMV currently lists four molecules in pre-clinical trials and 14 compounds in clinical trials [23]. Therefore, new compounds with novel mechanisms of action are urgently required to overcome the resistance of *Plasmodium* against commercial drugs. In this context, apicoplast, a plant plastid-like organelle that is essential for parasite survival provide potentially an interesting antimalarial target.

The discovery in Australian corals of a photosynthetic apicomplexan *Chromera velia* confirmed the common plant and photosynthetic evolutionary origin of *P. falciparum* and its peculiar organelle, the apicoplast [24]. Indeed, the apicoplast is a non-photosynthetic plastid acquired by secondary endosymbiosis from a plastid-containing red algal ancestor [25]. As a consequence of this secondary endosymbiotic origin, the apicoplast is delimited by four membranes [26]. This *P. falciparum* organelle possesses a housekeeping machinery maintained in its small 35 kb circular genome, which encodes for tRNAs, ribosomal proteins and a handful of proteins that are targeted to the apicoplast [27]. Despite being non photosynthetic, the apicoplast retains several anabolic pathways that are essential for parasite survival. These pathways include fatty acids biosynthesis, isoprenoid biosynthesis precursors, iron-sulfur cluster assembly and heme biosynthesis [27]. These metabolic pathways have no equivalent in the human host. Isoprenoid precursors such as isopentenyl pyrophosphate (IPP) have previously been described as the only required function of the apicoplast in the asexual blood stage of malaria parasite *in vitro* under rich culture conditions [28]. Thus, it was confirmed that IPP inhibitors, such as fosmidomycin, mainly target the apicoplast. On the other hand, the apicoplast fatty acid biosynthesis plays a vital role during the *in vitro* liver stage [29], and can be reactivated during the blood stages and become critical in a low host nutritional/lipid environment [30, 31]. Furthermore, even if disrupted and absent in blood stage parasites by anti-plastidial treatment (azithromycin, doxycycline, fosmidomycin), the apicoplast remains metabolically active in the form of vesicles, showing once again that the IPP pathway is not the only essential pathway of the apicoplast [32]. Moreover, Prigge et al. [33] have recently demonstrated the importance of carbon metabolism in the apicoplast of the malaria parasite. Indeed, a loss of pyruvate

145 kinase II activity which is an essential *Plasmodium* enzyme providing the pyruvate necessary for the  
146 generation of essential isoprenoid precursors, leads to a reduction of apicoplast RNA, following by  
147 apicoplast disruption and parasite death. Seven enzymes of the isoprenoid precursors pathway are  
148 drug-target in the apicoplast, which are DOXP synthase, IspC, IspD, IspE, IspF, IspG and IspH. Knowing  
149 that the isoprenoid precursors biosynthesis is localized only to apicoplast, chemical rescue by adding  
150 IPP to the growth medium identified **MMV-08138** as a potent IspD inhibitor during the blood stage of  
151 *P. falciparum* with an EC<sub>50</sub> of 110 nM (Figure 2) [34].



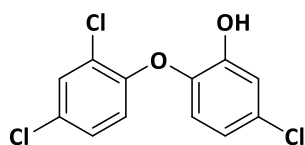
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**Figure 2.** MMV-08138 acting at the level of the *Plasmodium* apicoplast.

154 Currently very few molecules targeting the apicoplast have been reported, but some known  
155 antibiotics have been studied, such as ciprofloxacin and doxycycline belonging to the fluoroquinolone  
156 and tetracycline classes respectively, which target DNA replication and rRNA translation respectively  
157 in *P. falciparum* apicoplast, leading to a delayed death drug-response [26].

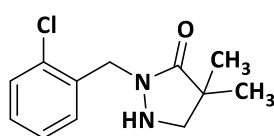
158 The delayed death phenomenon in the apicomplexan parasite is well studied and its  
159 molecular mechanism appears to be related to the inhibition of functions in the apicoplast that  
160 induce death of daughter cell after one life cycle. It is related to apicoplast housekeeping processes  
161 such as DNA replication, transcription and translation mechanisms [27].

162 In addition to these antibiotics, several biocides such as triclosan (Figure 3) are reported to  
163 be active against *Plasmodium* through the metabolic pathway of fatty acid synthesis located in the  
164 apicoplast [26] although it was further proved that the apicoplast is not the main target [35]. As *P.*  
165 *falciparum* apicoplast is an essential chloroplast-like organelle, studies have demonstrated that  
166 herbicides active against plants are also active against *P. falciparum* and therefore could act as  
167 antimalarials [36,37]. Indeed, clomazone [38] and fluridone [39] affect DOXP synthase and block  
168 plastidial abscisic acid synthesized by the plastid DOXP pathway respectively, localized in the  
169 apicoplast (**Figure 3**).

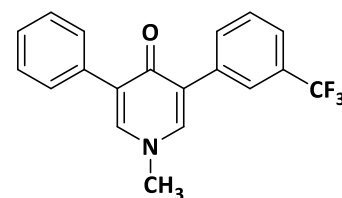


**Triclosan**

EC<sub>50</sub> Pf3D7 = 1.55 μM



**Clomazone**



**Fluridone**

170

171

**Figure 3.** Chemical structures of antiapicomplex biocids.

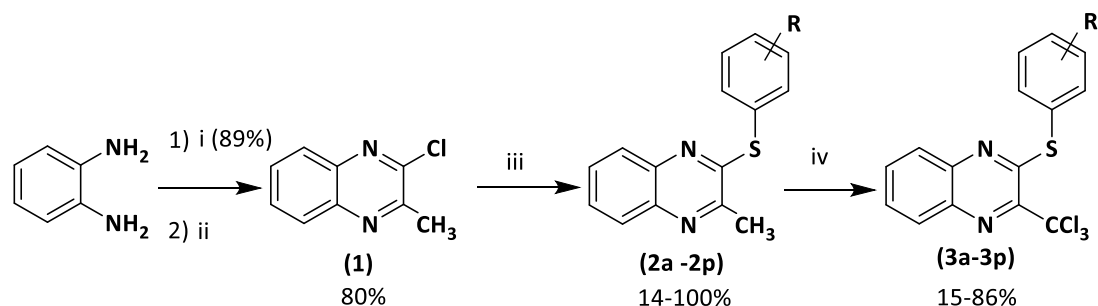
172 After working for 15 years on the development of trichloromethylated heterocycles without  
173 identifying their mechanism of action, we decided herein to evaluate the effect of our molecules

174 toward this vital organelle in *P. falciparum* in order to look for the mechanism of action of such  
175 trichloromethyl derivatives.

## 176 1. Results

### 177 1.1. Synthesis

178  
179 As shown in **Scheme 1**, the key substrate (**1**) was easily obtained by reacting *o*-  
180 phenylenediamine with ethyl pyruvate to provide the corresponding lactam which was then  
181 chlorinated using POCl<sub>3</sub> (Scheme 1) [15]. Next, chlorimine (**1**) was reacted through a nucleophilic  
182 aromatic substitution reaction (S<sub>N</sub>Ar) with various thiophenol derivatives in the presence of cesium  
183 carbonate in DMF at 70 °C. The desired intermediates (**2a-2p**) were obtained in low to quantitative  
184 yields (14 to 100%). Yield variation does not seem to be correlated with the electron-donating or -  
185 withdrawing behavior of the different thiophenol substituents. Finally, from the methylated  
186 precursors (**2a-2p**), a chlorination reaction using PCl<sub>5</sub> and POCl<sub>3</sub> was performed under microwave  
187 heating at 100 °C for 30 min, according to a previously reported method [40], leading to the target  
188 compounds (**3a-3p**) in low to very good yields (15-86%). A series of 16 original derivatives (**3a-3p**)  
189 was then prepared, bearing electron-donating or electron-withdrawing substituents in *ortho*, *meta*  
190 and *para* positions of the thiophenol moiety. It is to note that attempt to chlorinate the intermediate  
191 (**2i**), bearing a 3'-OMe substituent on the thiophenoxy group, led to the only the unexpected 2'-  
192 chlorinated product.



**Scheme 1.** Preparation of 2-thiophenoxy-3-trichloromethylquinoxaline derivatives (**3a-3p**). *Reagents and conditions:* (i) ethyl pyruvate 1 equiv, H<sub>2</sub>O, 50 °C, 15 min; (ii) POCl<sub>3</sub>, reflux, 2 h; (iii) appropriate thiophenol derivative 1 equiv, Cs<sub>2</sub>CO<sub>3</sub> 1 equiv, anh. DMF, 70 °C, 12 h, sealed vial, N<sub>2</sub>; (iv) PCl<sub>5</sub> 6 equiv, POCl<sub>3</sub> as solvent, 100 °C, MW, 20 min, 800 W.

### 200 1.2. Biological results

#### 201 Structure-Activity Relationship (SAR) study

202 These new derivatives were evaluated *in vitro* against the K1 multidrug-resistant *P.*  
203 *falciparum* strain, by determining their 50% efficacy concentration (EC<sub>50</sub>), and compared with three  
204 reference antimalarial drugs: chloroquine, atovaquone and doxycycline. The *in vitro* 50% cytotoxic  
205 concentrations (CC<sub>50</sub>) were assessed on the HepG2 human hepatocyte cell line and compared with  
206 cytotoxic reference drug: doxorubicin. Selectivity indexes were calculated: SI = CC<sub>50</sub>/EC<sub>50</sub>. The results  
207 are presented in **Table 2**.

208 All the 16 newly synthesized compounds showed good aqueous solubility except for  
209 compound (**3j**), which precipitated above 1.9 μM in the cell viability assay. The derivatives showed  
210 cytotoxicity values ranging from 19.9 to 69.7 μM, comparable or better than chloroquine (30 μM) or  
211 doxycyclin (20 μM). It appears that strong electron-withdrawing groups (EWG) (**3m-3o**) at 4-position

212 of 2-thiophenoxy group led to slightly more cytotoxic compounds (19.9-29.9  $\mu\text{M}$ ), contrary to  
 213 electron-donating groups (EDG) (**3p**) or the unsubstituted thiophenoxy moiety (**3k**) (69.7-56.0  $\mu\text{M}$ ,  
 214 respectively).

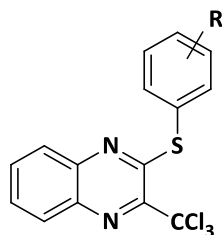
215 With regard to the antiplasmodial activity, all derivatives showed submicromolar activity  
 216 except for (**3p**) bearing a 4-Me group (1.5  $\mu\text{M}$ ). A strong EWG such as 4-NO<sub>2</sub> group (**3o**) gave the best  
 217 antiplasmodial activity with only 0.2  $\mu\text{M}$ . The 2, 3 or 4 fluoro-substituted thiophenoxy moiety (**3e-3g**)  
 218 led to quite similar activities (0.32, 0.31, 0.36  $\mu\text{M}$  respectively). Other 4-EWG such as 4-CF<sub>3</sub> (**3m**) and  
 219 4-OCF<sub>3</sub> (**3n**) gave quite similar activities to the fluoro-substituted thiophenoxy group (0.40 and 0.30  
 220  $\mu\text{M}$  respectively). Substitution by a methoxy group was slightly detrimental to the activity, especially  
 221 for the 2-OMe compound (**3h**) (0.74  $\mu\text{M}$ ). The disubstituted 2-chloro-3-methoxy-thiophenoxy moiety  
 222 (**3i**) led to a better antiplasmodial activity than only 2- or 4-methoxy substitution (**3h**, **3j**). For the  
 223 dichloro-containing molecules (**3c**, **3d**), 2-3 substitutions were better than the 2-4 substitutions (0.31  
 224 vs 0.90  $\mu\text{M}$ ).

225 Finally, the best compound in this series was the unsubstituted thiophenoxy group (**3k**) which  
 226 combined both good antiplasmodial activity and low cytotoxicity, leading to the highest selectivity  
 227 index in the series (175) (**Table 2**). It is to note that there is no correlation between the calculated  
 228 logP and activity.

229

230 **Table 2.** Antiplasmodial activity, cLogP and human cell toxicity on HepG2 cell line of the 2-phenoxy-3-  
 231 trichloro-methylquinoxaline compounds (**3a-3p**).

232



233

R	N°	Yields (%)	EC <sub>50</sub> PfK1 ( $\mu\text{M}$ )	CC <sub>50</sub> HepG2 ( $\mu\text{M}$ )	SI <sup>d</sup>	cLogP <sup>e</sup>
<b>2-Cl</b>	3a	62	0.50 $\pm$ 0.10	36.9 $\pm$ 8.1	73.8	5.15
<b>3-Cl</b>	3b	74	0.41 $\pm$ 0.08	38.3 $\pm$ 8.1	93.4	5.16
<b>2,3-Cl</b>	3c	61	0.31 $\pm$ 0.06	34.6 $\pm$ 7.1	111.6	5.68
<b>2,4-Cl</b>	3d	46	0.90 $\pm$ 0.10	>31.2 <sup>c</sup>	>34.7	5.69
<b>2-F</b>	3e	56	0.32 $\pm$ 0.07	40.5 $\pm$ 8.1	126.6	4.94
<b>3-F</b>	3f	63	0.31 $\pm$ 0.07	35.9 $\pm$ 7.9	115.8	4.94
<b>4-F</b>	3g	51	0.36 $\pm$ 0.08	30.8 $\pm$ 7.0	85.6	4.95
<b>2-OMe</b>	3h	64	0.74 $\pm$ 0.16	42.6 $\pm$ 5.2	56.8	4.63
<b>2-Cl-3-OMe</b>	3i	15	0.46 $\pm$ 0.15	58.4 $\pm$ 5.8	126.9	5.14
<b>4-OMe</b>	3j	59	0.65 $\pm$ 0.15	>1.9 <sup>c</sup>	>3.0	4.61
<b>H<sup>f</sup></b>	<b>3k</b>	<b>71</b>	<b>0.32 <math>\pm</math> 0.07</b>	<b>56.0 <math>\pm</math> 11.0</b>	<b>175.0</b>	<b>4.68</b>
<b>4-Br</b>	3l	44	0.46 $\pm$ 0.10	42.2 $\pm$ 8.6	93.0	5.25
<b>4-CF<sub>3</sub></b>	3m	80	0.50 $\pm$ 0.10	29.9 $\pm$ 5.1	59.8	5.68
<b>4-OCF<sub>3</sub></b>	3n	68	0.36 $\pm$ 0.10	21.0 $\pm$ 3.1	58.3	5.55
<b>4-NO<sub>2</sub></b>	3o	64	0.25 $\pm$ 0.05	19.9 $\pm$ 3.0	79.6	3.92



<b>4-CH<sub>3</sub></b>	3p	40	1.50 ± 0.20	69.7 ± 14.6	46.5	5.01
<b>Hit B</b>			0.50	38.6	77.2	5.17
<b>Chloroquine<sup>a</sup></b>			0.80	30.0	37.5	3.82
<b>Atovaquone<sup>a</sup></b>			0.001	>15.6	15600	4.23
<b>Doxycycline<sup>a</sup></b>			6.00	20.0	3.3	-
<b>Doxorubicin<sup>b</sup></b>			-	0.20	-	-

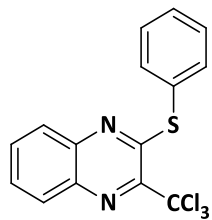
234  
 235 <sup>a</sup> Chloroquine, Atovaquone and doxycycline were used as antimalarial reference-drugs; <sup>b</sup> Doxorubicin  
 236 was used as a cytotoxic reference-drug; <sup>c</sup> Highest concentration tested due to a lack of solubility; <sup>d</sup>  
 237 Selectivity index (SI) was calculated according to the formula: SI = CC<sub>50</sub>/EC<sub>50</sub>, <sup>e</sup> Weighted clogP was  
 238 computed by swissADME; <sup>f</sup> The best antiplasmodial molecule.

239  
 240 *Toxicity data*

241 In order to complete the cytotoxicity data of the best molecule (**3k**) in this series, we tested it  
 242 on CHO Chinese Hamster Ovary cells and Vero cells (**Table 3**). After 72 h of treatment, low  
 243 cytotoxicities was observed both on cell lines (58.5 μM and 63.5 μM respectively), similar to that  
 244 measured on HepG2 cell line (56.0 μM).

245 To complete the *in vitro* toxicological evaluation of compound (**3k**), its genotoxic potential  
 246 was studied, using the comet assay on the HepG2 cell line (**Table 3**). Compound (**3k**) did not induce  
 247 DNA strand breaks or alkali labile sites after either short (2 h) or long (72 h) exposure at two  
 248 concentrations (5.6 and 28 μM; CC<sub>50</sub>/2 and CC<sub>50</sub>/10 after 72 h of treatment) ( see supplementary  
 249 material). The positive control (i.e., cells treated with 1 mM Methyl MethaneSulphonate, MMS)  
 250 showed the expected results.

251  
 252 **Table 3.** Cytotoxic and genotoxic data for hit compound (**3k**).

Entry	CC <sub>50</sub> HepG2 (μM)	CC <sub>50</sub> CHO (μM)	CC <sub>50</sub> Vero (μM)	Comet assay <sup>a</sup>
	56.0 ± 11.0	58.5 ± 3.9	63.5 ± 8.6	Negative
<b>(3k)</b>				

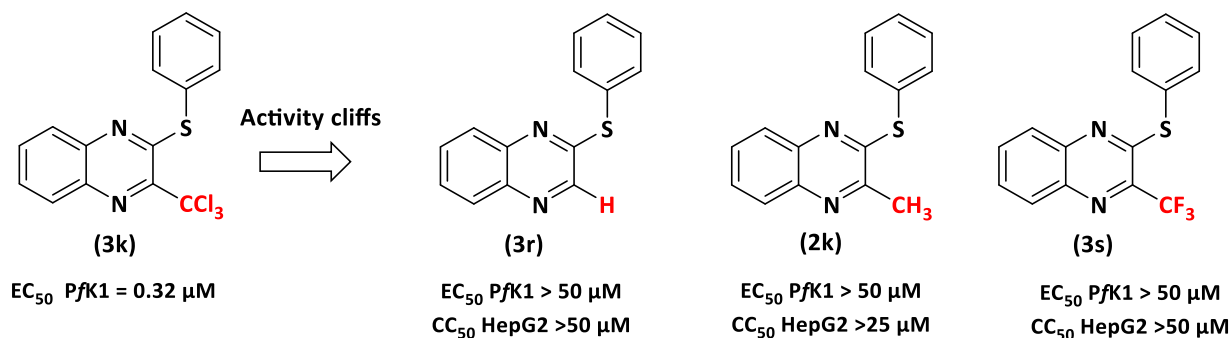
254 <sup>a</sup> Comet assay was performed on HepG2 cell line at 5.6 and 28 μM for 2 or 72 h.

255  
 256 *Role of the 2-CCl<sub>3</sub> group*

257 We have previously shown that the -CCl<sub>3</sub> group is mandatory to provide antiplasmodial  
 258 activity [17, 21, 41]. To confirm the key role of the CCl<sub>3</sub> group in the activity of **3k**, we compared the  
 259 antiplasmodial activity of (**3k**) with that of three analogs bearing a proton (**3r**), a methyl group (**2k**) or  
 260 a trifluoromethyl group (**3s**) at position 3 of the quinoxaline ring respectively. None of these  
 261 analogues lacking a CCl<sub>3</sub> group was active against *P. falciparum* (EC<sub>50</sub> Pfk1 > 50 μM). These activity

262 cliffs revealed once again the key role of the CCl<sub>3</sub> group in antiplasmodial activity in the studied series  
263 (**Figure 4**) [42].

264



265

266

267 **Figure 4.** Comparison of the *in vitro* antiplasmodial activities of hit molecule **3k** with analogs **3r**, **2k**  
268 and **3s**.

269

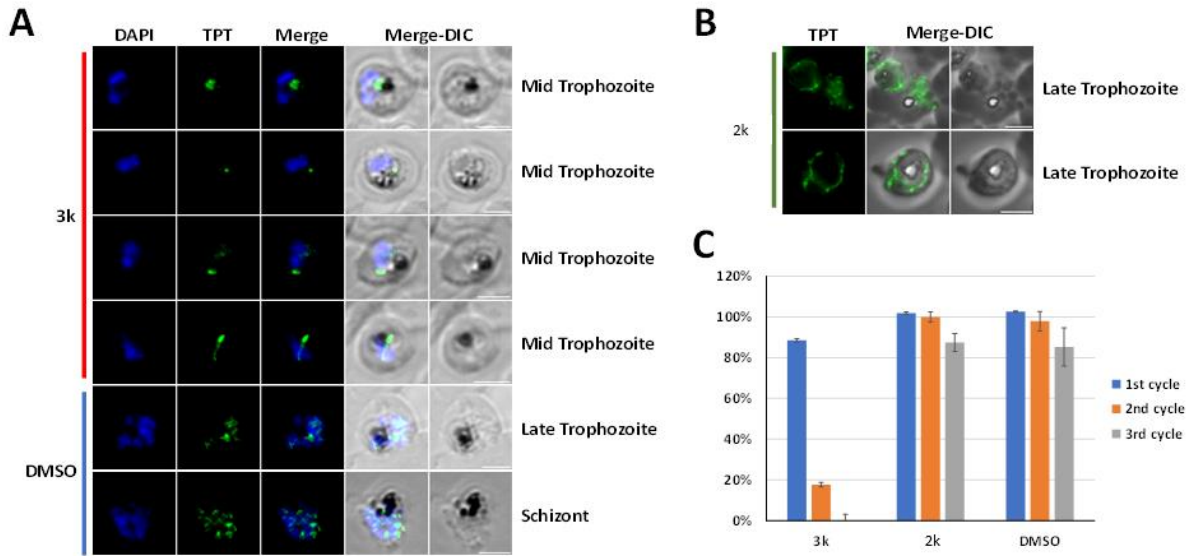
270 *Evaluation on P. falciparum apicoplast*

- 271 • *Treatment with molecule 3k affects parasites growth and apicoplast development*

272 Using an epifluorescence microscope, we monitored the effect of molecule (**3k**) on different  
273 intraerythrocytic stages of *P. falciparum* parasite in order to identify the mechanism by which the  
274 molecule could affect parasite survival and whether this was directed against the apicoplast. The loss  
275 of apicoplast by molecule (**3k**)-treatment can then be visualized via fluorescence microscopy. The  
276  $EC_{50}$  treatment was performed on synchronized cultures of *P. falciparum* 3D7 strain during a 48-hour  
277 parasite life cycle (**Figure 5A**).

278 Parasites treated with 0.3  $\mu$ M of molecule (**3k**) shows a severe defect in apicoplast biogenesis  
279 when compared to the DMSO control. The apicoplast development, elongation and branching  
280 typically happening during asexual blood stage intracellular development is highly affected by the  
281 treatment and strongly suggest a direct effect on *Plasmodium* apicoplast (**Figure 5A**). Furthermore,  
282 overall parasite development is also slightly reduced, as the nucleus staining shows a defect in  
283 replication and the culture is enriched in mid trophozoite parasites (**Figure 5A**), suggesting either that  
284 apicoplast disruption induces parasite death or that the drug could have some sort of off target  
285 mechanism. If the apicoplast is the primary target of the (**3k**) molecule, one could expect a so-called  
286 delayed death phenotype, which is characteristic of some apicoplast targeting drugs such as  
287 chloramphenicol, doxycycline or ciprofloxacin. To confirm this, we performed a growth assay on  
288 three life cycles (3x 48 h) by measuring the development of treated parasite with 0.3  $\mu$ M of (**3k**) and  
289 0.3  $\mu$ M of (**2k**). The replacement of CCl<sub>3</sub> by a methyl group in (**2k**) analog shows to have no effect on  
290 the apicoplast development (**Figure 5B**) compared to untreated parasites (DMSO control). Results  
291 show a slight reduction of the parasites development at the first cycle (48 h), and a drastic reduction  
292 of the growth at the second and third cycles, confirming the delayed effect of apicoplast targeting  
293 drugs, compared to inactive (**2k**) molecule, which has no significant difference with DMSO (**Figure**  
294 **5C**). Nevertheless, the slight decrease of the growth at the first cycle confirms the potential off target  
295 of (**3k**) molecule, outside of the apicoplast, coherent with the reduction of nucleus replication shown  
296 by IFA at 48 h treatment (**Figure 5A**).

297



298

299 **Figure 5. (3k) molecule treatment affects apicoplast biogenesis.**

300 **(A, B)** Triose Phosphate Transporter (TPT) fluorescent signal is shown in green (TPT labeled with an  
 301 3xHA tag). DAPI, as a marker for the nucleus, is shown in blue. Apicoplasts of treated parasites with  
 302 0.3  $\mu$ M of **(3k)** molecule or 0.3  $\mu$ M of **(2k)** molecule are compared to normal plastid visualized in  
 303 DMSO-treated control. The microscopic images were obtained by epifluorescence microscope. DIC,  
 304 differential interference contrast. **(C)** Plastid associated with the delayed death of *P. falciparum*  
 305 trophozoite was observed after treatment with molecule **(3k)** on three life cycles (n = 3, error bar =  
 306 standard deviation, SD).

307 It should be noted out that all derivatives **(3a-p)** including **(3k)** have a high lipophilicity ( $3.92 <$   
 308  $\text{clogP} < 5.69$ ). This may be necessary property to cross the four membranes that envelop the  
 309 apicoplast.

310

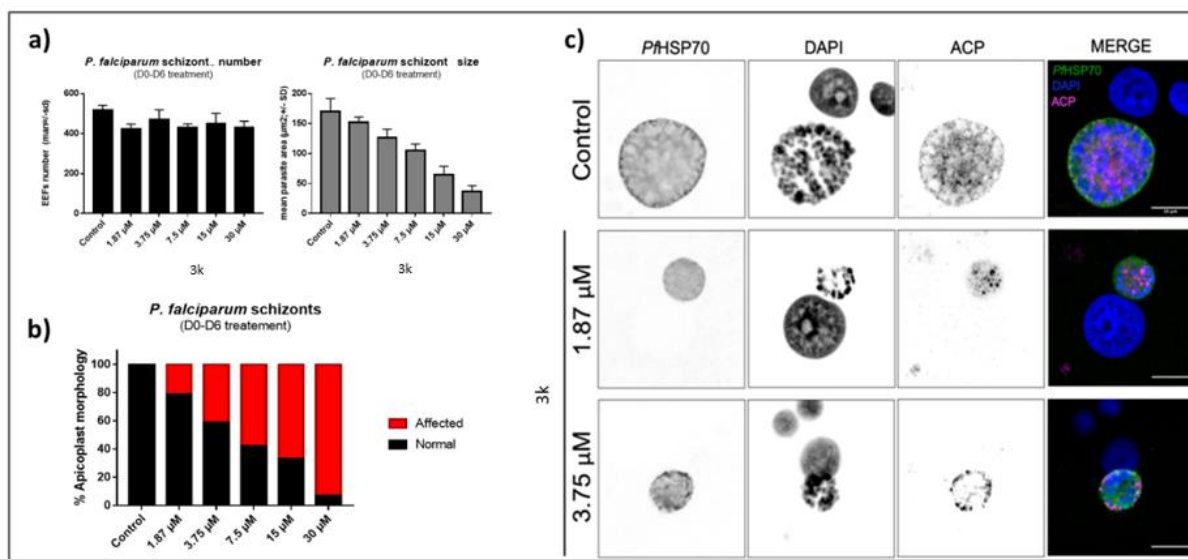
### 311 *Evaluation on human hepatocyte infection*

312 In order to further explore the potency of molecule **(3k)**, an evaluation was made on the  
 313 hepatic stage of *P. falciparum* infection. Cryopreserved primary human hepatocyte cultures were  
 314 infected with *P. falciparum* NF135 strain. Treatment with **(3k)** at various doses was applied  
 315 simultaneously with the sporozoites addition to hepatocytes and contact was maintained until post-  
 316 infection day 6 (pi). **(3k)** treatment significantly reduced parasite size without affecting parasite  
 317 numbers in a dose-dependent manner. Atovaquone (ATQ), a potent hepatic schizonticide was used  
 318 as positive control.

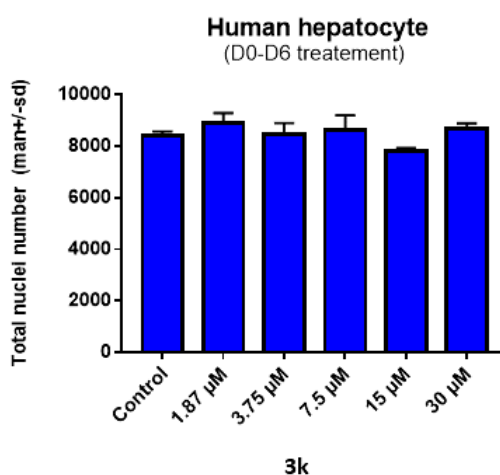
319 We then tried to understand if apicoplast of *P. falciparum* schizonts was targeted by **(3k)**. In  
 320 untreated control wells fixed at day 6 pi, the parasitic apicoplast has a uniform appearance. The  
 321 apicoplast is in its terminal stage of development (considering the 5-6 days schizogony of *P.*  
 322 *falciparum*) and the Apicoplast protein acyl Carrier Protein (ACP) signal looks like small dots densely  
 323 decorating the periphery of the organelle. In **(3k)** treated cultures, the integrity of small dots shown  
 324 in the control parasites appears to be affected and the ACP signal is condensed leading to the  
 325 appearance of larger aggregates. At the high dose of 30  $\mu$ M approximately 93% of *P. falciparum*  
 326 schizonts showed partial or complete alteration of the apicoplast signal **(Figure 6)**. Interestingly, no  
 327 cytotoxicity was observed on human hepatocytes up to 30  $\mu$ M **(Figure 7)**.

328

329  
330



331  
332 **Figure 6.** The effect of (3k) on liver stage *P. falciparum* growth. a) D0 pi-D6 (3k) exposure of human  
333 hepatocytes infected with *P. falciparum* and its impact on parasite numbers expressed as mean +/-  
334 SD and parasite size expressed as surface area expressed as µm<sup>2</sup> mean +/- SD. Parasites were  
335 immunostained with anti-HSP70 antibody and plates were scanned. b) Quantification of different  
336 apicoplast morphologies (normal and affected) in 100 parasites with percentage distribution of each  
337 defined category. c) Confocal microscopic images of control and treated *P. falciparum* schizonts. The  
338 schizonts were immunolabelled with anti-HSP70 antibody. Host and parasite DNA were visualized by  
339 4',6-diamidino-2-phenylindole (DAPI) dye. The apicoplast was labelled by Anti-PyACP (Scale bar = 10  
340 µm).  
341



342  
343 **Figure 7.** Toxicity of (3k) on primary human hepatocytes. The number of hepatocyte nuclei quantified  
344 from a certain number of microscopic scanning fields from control and (3k) treated groups.  
345

346 **2. Conclusion**

347 An antiplasmodial SAR study was conducted through the synthesis and evaluation of 16 original  
348 thiophenoxyquinoxaline derivatives. All of these molecules displayed good *in vitro* antiplasmodial

349 activity. Among these compounds, (**3k**) showed the best antiplasmodial activity ( $EC_{50} = 0.32 \mu\text{M}$ ) and  
350 low cytotoxicity towards three cell-lines and no cytotoxicity on human hepatocytes. Moreover,  
351 compound (**3k**) presented no genotoxic effect in the comet assay. The activity cliffs showed that the  
352  $\text{CCl}_3$  group was essential to provide antiplasmodial activity. Although no effect was demonstrated on  
353 hepatic stage, at high concentration, the size of parasite was affected. Interestingly, the molecule  
354 (**3k**) shows a delayed death inhibition and apicoplast biogenesis branching defect, confirming that it  
355 affects growth of *P. falciparum* by targeting the apicoplast, and probably another target outside of  
356 the apicoplast which affects the cell division. Essential metabolic pathways reside within the  
357 apicoplast which provide promising new drug targets for the development of new antimalarials, with  
358 a novel mechanism of action. Further investigations will be necessary in order to decipher the action  
359 of trichloromethylquinoxaline derivatives on the apicoplast.

360

## 361 **Acknowledgments**

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364 DCM20181039565].

365

## 366 **Experimental section**

### 367 **2.1. Chemistry**

#### 368 **2.1.1. Generality**

369 Melting points were determined on a Köfler melting point apparatus (Wagner & Munz GmbH,  
370 München, Germany) and are uncorrected. Elemental analyses were carried out at the Spectropole,  
371 Faculté des Sciences de Saint-Jérôme (Marseille) with a Thermo Finnigan EA1112 analyzer (Thermo  
372 Finnigan, San Jose, CA, USA). NMR spectra were recorded on a Bruker AV (Billerica, MA, USA) 200 or  
373 AV 250 spectrometers or on a Bruker Avance NEO 400MHz NanoBay spectrometer at the Faculté de  
374 Pharmacie of Marseille or on a Bruker Avance III nanobay 400 MHz spectrometer at the Spectropole,  
375 Faculté des Sciences de Saint-Jérôme (Marseille). ( $^1\text{H}$  NMR: reference  $\text{CDCl}_3$   $\delta = 7.26$  ppm, reference  
376  $\text{DMSO-d}_6$   $\delta = 2.50$  ppm and  $^{13}\text{C}$  NMR: reference  $\text{CHCl}_3$   $\delta = 76.9$  ppm, reference  $\text{DMSO-d}_6$   $\delta = 39.52$   
377 ppm). The following adsorbent was used for column chromatography: silica gel 60 (Merck KGaA,  
378 Darmstadt, Germany, particle size 0.063–0.200 mm, 70–230 mesh ASTM). TLC was performed on 5  
379 cm  $\times$  10 cm aluminum plates coated with silica gel 60F-254 (Merck) in an appropriate eluent.  
380 Visualization was performed with ultraviolet light (234 nm). The purity of synthesized compounds  
381 was checked by LC/MS analyses, which were performed at the Faculté de Pharmacie of Marseille  
382 with a Thermo Scientific Accela High Speed LC System<sup>®</sup> (Waltham, MA, USA) coupled to a single  
383 quadrupole mass spectrometer Thermo MSQ Plus<sup>®</sup>. The RP-HPLC column is a Thermo Hypersil Gold<sup>®</sup>  
384 50  $\times$  2.1 mm (C18 bounded), with particles of a diameter of 1.9 mm. The volume of sample injected  
385 on the column is 1  $\mu\text{L}$ . The chromatographic analysis with a total duration of 8 min, is performed on  
386 the following solvents gradient:  $t = 0$  min, methanol/water 50:50;  $0 < t < 4$  min, linear increase in the  
387 proportion of methanol to a methanol/water ratio of 95:5;  $4 < t < 6$  min, methanol/water 95:5;  $6 < t$   
388  $< 7$  min, linear decrease in the proportion of methanol to return to a methanol/water ratio of 50:50;  
389  $6 < t < 7$  min, methanol/water 50:50. The water used was buffered with ammonium acetate 5 mM.  
390 The flow rate of the mobile phase was 0.3 mL/min. The retention times ( $t_R$ ) of the molecules analyzed  
391 are indicated in min. The microwave reactions were performed using multimode reactors: ETHOS  
392 Synth Lab station and MicroSYNTH<sup>®</sup> Lab terminal 1024 (Ethos start, MLS GmbH, Leutkirch,  
393 Germany.); or monomode reactors: Biotage Initiator<sup>®</sup> classic in sealed vials with a power output of 0

394 to 400 W. Reagents were purchased and used without further purifications from Sigma-Aldrich or  
395 Fluorochem.

396

### 397 **3.1.2. 2-Chloro-3-methylquinoxaline (1)**

398 **Step 1:** To a solution of *o*-phenylenediamine (6 g, 1.0 equiv) in H<sub>2</sub>O was added ethyl pyruvate (1.0  
399 equiv) dissolved in H<sub>2</sub>O (80 mL). The reaction mixture was heated at 50 °C for 15 to 30 min. After  
400 cooling, the precipitate product of 3-methylquinoxalin-2(1*H*)-one was recrystallized from ethanol as a  
401 white solid, 7.92 g, yield 89%. This was used directly in step 2.

402 **Step 2:** To a solution of 3-methyl-2(1*H*)-quinoxalinone (12.5 g, 121.8 mmol, 1.0 equiv) was added  
403 cold POCl<sub>3</sub> (65 mL) in portions to get a slurry. The mixture was refluxed for 2 h. Then, the reaction  
404 was added to ice cold water (500 mL) and basified slowly under cooling with Na<sub>2</sub>CO<sub>3</sub> to pH 8. The  
405 organic phase was separated and washed with brine, dried over anhydrous MgSO<sub>4</sub>, filtered, and  
406 concentrated in vacuum to afford the crude product, which was purified by silica gel flash  
407 chromatography (using dichloromethane) to afford (1) as an off-white solid, yield 80% (7g). Mp 84 °C.  
408 <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.08 – 7.84 (m, 2H), 7.79 – 7.62 (m, 2H), 2.82 (s, 3H). <sup>13</sup>C NMR (63 MHz,  
409 CDCl<sub>3</sub>) δ 152.9, 147.9, 141.0, 141.0, 130.2, 130.1, 128.5, 128.2, 23.4. Spectral data matched with  
410 literature [43].

411

### 412 **3.1.3. General procedure for 3-methyl-2-substituted-quinoxaline (2a-2p)**

413 To a solution of 2-chloro-3-methylquinoxaline (1) (500 mg, 2.8 mmol) and the appropriate thiophenol  
414 (2.8 mmol, 1.0 equiv) in anhydrous DMF (10 mL), Cs<sub>2</sub>CO<sub>3</sub> (912 mg, 2.8 mmol, 1.0 equiv) was added  
415 under inert atmosphere. The mixture was stirred at 70 °C overnight. After completion of the reaction,  
416 water was added, leading to a precipitate which was separated by filtration. The resulting precipitate  
417 was then thoroughly washed with water. The precipitate was dissolved in CH<sub>2</sub>Cl<sub>2</sub> and dried with  
418 Na<sub>2</sub>SO<sub>4</sub>. After filtration and evaporation, the resulting solid was purified by silica gel column  
419 chromatography (using the appropriate eluant) to afford the desired compound.

420

#### **3.1.3.1. 2-(2-Chlorophenylthio)-3-methylquinoxaline (2a)**

421 Yield 100% (803 mg). White solid. Mp 155 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.97 – 7.93 (m, 1H), 7.70 –  
422 7.51 (m, 5H), 7.46 – 7.31 (m, 2H), 2.82 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 154.6, 151.5, 141.6, 139.6,  
423 139.6, 137.6, 131.0, 130.4, 129.2, 128.7, 128.3, 128.2, 128.1, 127.5, 22.4. LC-MS (ESI+) *t*<sub>R</sub> 6.54 min;  
424 m/z [M+H]<sup>+</sup> 286.36/289.00. MW: 286.78 g.mol<sup>-1</sup>. HRMS (ESI): m/z calcd. for C<sub>15</sub>H<sub>11</sub>ClN<sub>2</sub>S [M+H]<sup>+</sup>  
425 287.0404. Found: 287.0402.

426

427

#### **3.1.3.2. 2-(3-Chlorophenylthio)-3-methylquinoxaline (2b)**

428 Yield 94% (755 mg). White solid. Mp 97 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.98 – 7.94 (m, 1H), 7.74 –  
429 7.69 (m, 1H), 7.65 – 7.55 (m, 3H), 7.52 – 7.33 (m, 3H), 2.78 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 155.0,  
430 151.4, 141.4, 139.8, 134.9, 134.8, 133.3, 130.5, 130.3, 130.2, 129.5, 129.3, 128.8, 128.2, 22.4. LC-MS  
431 (ESI+) *t*<sub>R</sub> 6.83 min; m/z [M+H]<sup>+</sup> 286.36/289.04. MW: 286.78 g.mol<sup>-1</sup>. HRMS (ESI): m/z calcd. for  
432 C<sub>15</sub>H<sub>11</sub>ClN<sub>2</sub>S [M+H]<sup>+</sup> 287.0404. Found: 287.0402.

433

#### **3.1.3.3. 2-(2,3-Dichlorophenylthio)-3-methylquinoxaline (2c)**

434 Yield 100% (899 mg). Brown solid. Mp 142 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.92 – 7.88 (m, 1H), 7.65 –  
435 7.49 (m, 5H), 7.23 – 7.20 (m, 1H), 2.76 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 154.0, 151.5, 141.4, 140.0,  
436 137.7, 135.6, 134.1, 131.7, 130.9, 129.2, 128.9, 128.3, 128.2, 127.6, 22.5. LC-MS (ESI+) *t*<sub>R</sub> 4.69 min;

437 m/z [M+H]<sup>+</sup> 319.75/322.54. MW: 321.22 g.mol<sup>-1</sup>. **HRMS (ESI)** : m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>S;  
438 321.0015 Found: 321.0013.

#### 439 **3.1.3.4. 2-(2,4-Dichlorophenylthio)-3-methylquinoxaline (2d)**

440 Yield 80% (719 mg). Brown solid. Mp 116 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.97 – 7.91 (m, 1H), 7.70 –  
441 7.66 (m, 1H), 7.65 – 7.54 (m, 4H), 7.34 (dd, *J* = 8.3, 2.2 Hz, 1H), 2.80 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)  
442 δ 154.0, 151.4, 141.5, 140.5, 140.0, 138.4, 136.5, 130.3, 129.2, 128.8, 128.4, 128.2, 127.8, 127.0,  
443 22.5. **LC-MS (ESI+)** *t<sub>R</sub>* 0.74 min; m/z [M+H]<sup>+</sup> 320.94/322.85/325.10. MW: 321.22 g.mol<sup>-1</sup>. **HRMS (ESI)**:  
444 m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>S: 321.0015, Found: 321.0013.

#### 445 **3.1.3.5. 2-(2-Fluorophenylthio)-3-methylquinoxaline (2e)**

446 Yield 78% (590 mg). Brown solid. Mp 159 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.03 – 7.85 (m, 1H), 7.69 –  
447 7.47 (m, 4H), 7.28 – 7.20 (m, *J* = 19.4 Hz, 3H), 2.82 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 163.3 (d, *J* =  
448 249.6 Hz), 154.3, 151.3, 141.5, 139.9, 137.3, 132.01 (d, *J* = 8.2 Hz), 129.1, 128.6, 128.3, 128.2, 124.8  
449 (d, *J* = 3.8 Hz), 116.3 (d, *J* = 22.9 Hz), 115.8, 22.5. **LC-MS (ESI+)** *t<sub>R</sub>* 3.90 min; m/z [M+H]<sup>+</sup> 271.10. MW:  
450 270.32 g.mol<sup>-1</sup>. **HRMS (ESI)** : m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>11</sub>FN<sub>2</sub>S: 271.0700, Found: 271.0699.

#### 451 **3.1.3.6. 2-(3-Fluorophenylthio)-3-methylquinoxaline (2f)**

452 Yield 95% (719 mg). Brown solid. Mp 92 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.92 – 7.89 (m, 1H), 7.74 –  
453 7.70 (m, 1H), 7.63 – 7.53 (m, 2H), 7.48 – 7.36 (m, 3H), 7.20 – 7.12 (m, 1H), 2.77 (s, 3H). <sup>13</sup>C NMR (63  
454 MHz, CDCl<sub>3</sub>) δ 162.8 (d, *J* = 248.5 Hz), 155.0, 151.5, 141.4, 139.9, 130.8, 130.7 (d, *J* = 3.2 Hz), 130.4 (d,  
455 *J* = 8.3 Hz), 129.3, 128.8, 128.3, 128.2, 122.1 (d, *J* = 22.6 Hz), 116.4 (d, *J* = 21.1 Hz), 22.5. **LC-MS (ESI+)**  
456 *t<sub>R</sub>* 4.24 min; m/z [M+H]<sup>+</sup> 271.26. MW: 270.32 g.mol<sup>-1</sup>. **HRMS (ESI)**: m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>11</sub>FN<sub>2</sub>S:  
457 271.0700, Found: 271.0698.

#### 458 **3.1.3.7. 2-(4-Fluorophenylthio)-3-methylquinoxaline (2g)**

459 Yield 98% (742 mg). orange solid. Mp 115 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.93 (dd, *J* = 6.5, 3.2 Hz,  
460 1H), 7.69 – 7.52 (m, 5H), 7.17 (t, *J* = 8.7 Hz, 2H), 2.77 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 163.6 (d, *J* =  
461 249.5 Hz), 155.8, 151.3, 141.4, 139.8, 137.7 (d, *J* = 8.5 Hz), 129.2, 128.6, 128.2, 128.1, 123.7 (d, *J* = 3.4  
462 Hz), 116.5 (d, *J* = 22.1 Hz), 22.4. **LC-MS (ESI+)** *t<sub>R</sub>* 4.20 min; m/z [M+H]<sup>+</sup> 271.24. MW: 270.32 g.mol<sup>-1</sup>.  
463 **HRMS (ESI)**: m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>11</sub>FN<sub>2</sub>S: 271.0700, Found: 271.0699.

464

#### 465 **3.1.3.8. 2-(2-Methoxyphenylthio)-3-methylquinoxaline (2h)**

466 Yield 72% (569 mg). Brown solid. Mp 138 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.93 (dd, *J* = 8.1, 1.5 Hz, 1H),  
467 7.67 – 7.62 (m, 1H), 7.58 (dd, *J* = 7.5, 1.6 Hz, 1H), 7.56 – 7.50 (m, 2H), 7.51 – 7.43 (m, 1H), 7.08 – 6.99  
468 (m, 2H), 3.76 (s, 3H), 2.81 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 160.3, 155.3, 151.9, 141.6, 139.9,  
469 137.0, 131.4, 128.8, 128.3, 128.1, 128.1, 121.3, 116.9, 111.8, 56.2, 22.6. **LC-MS (ESI+)** *t<sub>R</sub>* 3.61 min; m/z  
470 [M+H]<sup>+</sup> 283.19. MW: 282.36 g.mol<sup>-1</sup>. **HRMS (ESI)**: m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>OS: 283.0900, Found:  
471 283.0899.

#### 472 **3.1.3.9. 2-(3-Methoxyphenylthio)-3-methylquinoxaline (2i)**

473 Yield 100% (791 mg). orange solid. Mp 103 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.95 – 7.91 (m, 1H), 7.74 –  
474 7.72 (m, 1H), 7.61 – 7.53 (m, 2H), 7.38 – 7.34 (m, 1H), 7.21 – 7.19 (m, 2H), 7.00 (m, 1H), 3.83 (s, 3H),  
475 2.77 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 160.0, 155.7, 151.8, 141.5, 139.9, 130.0, 129.8, 129.1,  
476 128.6, 128.2, 128.2, 127.2, 120.0, 115.5, 55.6, 22.6. **LC-MS (ESI+)** *t<sub>R</sub>* 4.12 min; m/z [M+H]<sup>+</sup> 283.28.  
477 MW: 282,36 g.mol<sup>-1</sup>. **HRMS (ESI)** : m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>OS: 283.0900, Found: 283.0898.

#### 478 **3.1.3.10. 2-(4-Methoxyphenylthio)-3-methylquinoxaline (2j)**

479 Yield 39% (308 mg). White solid. Mp 113 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.96 – 7.88 (m, 1H), 7.71 –  
480 7.65 (m, 1H), 7.60 – 7.50 (m, 4H), 7.03 – 6.97 (m, 2H), 3.88 (s, 3H), 2.77 (s, 3H). <sup>13</sup>C NMR (63 MHz,  
481 CDCl<sub>3</sub>) δ 160.7, 156.7, 151.5, 141.5, 139.8, 137.2 (2C), 129.0, 128.3, 128.2, 128.1, 119.0, 114.9 (2C),  
482 55.5, 22.5. LC-MS (ESI+) *t<sub>R</sub>* 4.12 min; m/z [M+H]<sup>+</sup> 283.20. MW: 282.36 g.mol<sup>-1</sup>. HRMS (ESI): m/z  
483 [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>OS: 283.0900, Found: 283.0901.

#### 484 **3.1.3.11. 2-Methyl-3-(phenylthio)quinoxaline (2k)**

485 Yield 87% (615 mg). Red solid. Mp 134 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.95 – 7.89 (m, 1H), 7.71 –  
486 7.66 (m, 1H), 7.65 – 7.60 (m, 2H), 7.60 – 7.51 (m, 2H), 7.49 – 7.04 (m, 3H), 2.78 (s, 3H). <sup>13</sup>C NMR (101  
487 MHz, CDCl<sub>3</sub>) δ 155.8, 151.7, 141.5, 140.1, 135.3(2C), 129.3(2C), 129.2, 129.0, 128.9, 128.4, 128.3,  
488 128.2, 22.6. LC-MS (ESI+) *t<sub>R</sub>* 4.05 min; m/z [M+H]<sup>+</sup> 253.28. MW: 252.33 g.mol<sup>-1</sup>. Chemical Formula:  
489 C<sub>15</sub>H<sub>12</sub>N<sub>2</sub>S. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>12</sub>N<sub>2</sub>S: 253.0794, Found: 253.0789.

#### 490 **3.1.3.12. 2-(4-Bromophenylthio)-3-methylquinoxaline (2l)**

491 Yield 99% (918 mg). White solid. Mp 121 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.95 – 7.91 (m, 1H), 7.72 –  
492 7.68 (m, 1H), 7.61 – 7.54 (m, 4H), 7.49 – 7.46 (m, 2H), 2.77 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 155.1,  
493 151.5, 141.4, 140.1, 136.9 (2C), 132.4 (2C), 129.2, 128.6, 128.3, 128.1, 127.8, 123.8, 22.5. LC-MS  
494 (ESI+) *t<sub>R</sub>* 4.81 min; m/z [M+H]<sup>+</sup> 331.00/333.08/334.19. MW: 331.23 g.mol<sup>-1</sup>. HRMS (ESI): m/z [M+H]<sup>+</sup>  
495 calcd for C<sub>15</sub>H<sub>11</sub>BrN<sub>2</sub>S: 332.9879, Found: 332.9879.

#### 496 **3.1.3.13. 2-Methyl-3-(4-trifluoromethylphenylthio)quinoxaline (2m)**

497 Yield 87% (780 mg). Beige solid. Mp 99 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.98 – 7.94 (m, 1H), 7.77 –  
498 7.69 (m, 5H), 7.66 – 7.54 (m, 2H), 2.79 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 154.5, 151.6, 141.3, 140.1,  
499 135.1, 133.8 (q, *J* = 1.5), 131.1 (q, *J* = 32.7 Hz), 130.8, 129.3, 128.9, 128.4, 128.1, 126.2, 126.0 (q, *J* =  
500 3.7 Hz), 124.2 (q, *J* = 272.36 Hz), 22.5. LC-MS (ESI+) *t<sub>R</sub>* 4.70 min; m/z [M+H]<sup>+</sup> 319.78. MW: 320.33  
501 g.mol<sup>-1</sup>. HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>11</sub>F<sub>3</sub>N<sub>2</sub>S: 321.0668, Found: 321.0663.

#### 502 **3.1.3.14. 2-Methyl-3-(4-trifluoromethoxyphenylthio)quinoxaline (2n)**

503 Yield 89% (838 mg). Yellow solid. Mp 81 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 7.94 (m, 1H), 7.71 – 7.62 (m,  
504 3H), 7.62 – 7.54 (m, 2H), 7.31 (d, *J* = 8.0 Hz, 2H), 2.78 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ 155.2, 151.4,  
505 150.0 (q, *J* = 1.8 Hz), 141.4, 140.1, 137.0 (2C), 129.2, 128.7, 128.4, 128.1, 127.2, 121.5 (2C), 120.6 (q, *J*  
506 = 257.9 Hz), 22.5. LC-MS (ESI+) *t<sub>R</sub>* 4.97 min; m/z [M+H]<sup>+</sup> no ionization. MW: 336.33 g.mol<sup>-1</sup>. HRMS  
507 (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>11</sub>F<sub>3</sub>N<sub>2</sub>OS: 337.0617, Found: 337.0615.

#### 508 **3.1.3.15. 2-Methyl-3-(4-nitrophenylthio)quinoxaline (2o)**

509 Yield 86% (716 mg). Brown solid. Mp 163 °C. <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.35 – 8.25 (m, 2H), 8.00 –  
510 7.93 (m, 1H), 7.84 – 7.76 (m, 2H), 7.75 – 7.70 (m, 1H), 7.68 – 7.59 (m, 2H), 2.80 (s, 3H). <sup>13</sup>C NMR (101  
511 MHz, CDCl<sub>3</sub>) δ 153.5, 151.7, 148.0, 141.2, 140.4, 138.2, 134.9 (2C), 129.6, 129.3, 128.5, 128.1, 124.0  
512 (2C), 22.5. LC-MS (ESI+) *t<sub>R</sub>* 4.26 min; m/z [M+H]<sup>+</sup> 298.16. MW: 297.33 g.mol<sup>-1</sup>. HRMS (ESI): m/z  
513 [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>11</sub>N<sub>3</sub>O<sub>2</sub>S: 298.0645, Found: 298.0645.

#### 514 **3.1.3.16. 2-Methyl-3-(4-tolylthio)quinoxaline (2p)**

515 Yield 74% (552 mg). Brown solid. Mp 92 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.96 – 7.89 (m, 1H), 7.73 –  
516 7.67 (m, 1H), 7.59 – 7.52 (m, 2H), 7.52 – 7.47 (m, 2H), 7.30 – 7.26 (m, 2H), 2.77 (s, 3H), 2.43 (s, 3H).  
517 <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ 156.2, 151.7, 141.5, 139.9, 139.5, 135.3 (2C), 130.1 (2C), 128.9, 128.3,  
518 128.3, 128.2, 125.1, 22.5, 21.5. LC-MS (ESI+) *t<sub>R</sub>* 0.71 min; m/z [M+H]<sup>+</sup> 266.66. MW: 266.36 g.mol<sup>-1</sup>.  
519 HRMS (ESI): m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>S: 267.0950, Found: 267.0948.



520

### 521 **3.1.4. General procedure for the preparation of 2-thiophenoxy-3-trichloromethylquinoxaline** 522 **derivatives**

523 To a solution of 2-methyl-3-substituted quinoxaline (**2a-2p**) (624 mg, 2.8 mmol) and PCl<sub>5</sub> (2.88 g, 16.8  
524 mmol, 1.0 equiv), POCl<sub>3</sub> was added to make a slurry (ca.5 mL). The mixture was then heated in a  
525 multimode microwave oven at 100 °C, 800 W for 20 – 30 min. After completion of the reaction, the  
526 mixture was poured into ice and was then neutralized with Na<sub>2</sub>CO<sub>3</sub>. The resulting solution was  
527 extracted with CH<sub>2</sub>Cl<sub>2</sub> and dried with Na<sub>2</sub>SO<sub>4</sub>. After filtration and evaporation, the resulting solid was  
528 purified by silica gel column chromatography (eluent: Petroleum Ether/CH<sub>2</sub>Cl<sub>2</sub>, 9:1) to afford the  
529 desired compound.

530

#### 531 **3.1.4.1. 2-(2-Chlorophenylthio)-3-trichloromethylquinoxaline (3a)**

532 Yield 62% (675 mg). White solid. Mp 127 °C (isopropanol). <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.14 – 8.07  
533 (m, 1H), 7.75 – 7.62 (m, 4H), 7.59 – 7.55 (m, 1H), 7.44 (dt, *J* = 7.6, 1.9 Hz, 1H), 7.33 (dt, *J* = 7.4, 1.6 Hz,  
534 1H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ = 152.1, 147.4, 141.9, 139.9, 138.1, 136.8, 132.0, 131.2, 130.4,  
535 129.8, 129.7, 129.5, 127.9, 127.5, 96.5. LC-MS (ESI+) *t*<sub>R</sub> 5.4 min, *m/z* [M+H]<sup>+</sup> no ionization, purity 99%.  
536 MW: 390.11 g.mol<sup>-1</sup>. HRMS *m/z* [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>8</sub>Cl<sub>4</sub>N<sub>2</sub>S: 388.9235, Found: 388.9240.

537

#### 538 **3.1.4.2. 2-(3-Chlorophenylthio)-3-trichloromethylquinoxaline (3b)**

539 Yield 74% (437 mg). White solid. Mp 81 °C (isopropanol). <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.13 – 8.07 (m,  
540 1H), 7.73 – 7.65 (m, 4H), 7.52 (dt, *J* = 7.2, 1.6 Hz, 1H), 7.47 – 7.36 (m, 2H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ  
541 = 152.4, 147.3, 141.8, 136.8, 135.4, 134.7, 133.8, 132.1, 131.6, 130.2, 129.9, 129.7, 129.6, 127.8,  
542 96.5. LC-MS (ESI+) *t*<sub>R</sub> 5.7 min, *m/z* [M+H]<sup>+</sup> 378.96/380.90/386.38, purity 99%. MW: 390.11 g.mol<sup>-1</sup>.  
543 HRMS *m/z* [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>8</sub>Cl<sub>4</sub>N<sub>2</sub>S: 388.9235, Found: 388.9235.

544

#### 545 **3.1.4.3. 2-(2,3-Dichlorophenylthio)-3-(trichloromethyl)quinoxaline (3c)**

546 Yield 61% (725 mg). Beige solid. Mp 91 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.13 – 8.09 (m,  
547 1H), 7.74 – 7.62 (m, 4H), 7.60 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.30 (t, *J* = 7.9 Hz, 1H). <sup>13</sup>C NMR (101 MHz,  
548 CDCl<sub>3</sub>) δ = 151.5, 147.4, 141.9, 138.1, 136.8, 136.2, 134.2, 132.2, 132.0, 131.9, 130.0, 129.8, 127.9,  
549 127.6, 96.4. LC-MS (ESI+) *t*<sub>R</sub> 5.7 min, *m/z* [M+H]<sup>+</sup> no ionization, purity 99%. MW: 424.56 g.mol<sup>-1</sup>.  
550 HRMS *m/z* [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>7</sub>N<sub>2</sub>SCl<sub>5</sub>: 424.8816, Found: 424.8808.

551

#### 552 **3.1.4.4. 2-(2,4-Dichlorophenylthio)-3-trichloromethylquinoxaline (3d)**

553 Yield 46% (547 mg). Yellow solid. Mp 97 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, DMSO) δ 8.13 – 8.04 (m,  
554 1H), 7.77 – 7.63 (m, 4H), 7.58 (d, *J* = 2.2 Hz, 1H), 7.40 – 7.32 (m, 1H). <sup>13</sup>C NMR (101 MHz, DMSO) δ  
555 151.8, 147.7, 142.1, 141.2, 139.1, 137.1, 137.0, 132.5, 130.6, 130.3, 130.1, 128.4, 128.1 (2C), 96.7. LC-  
556 MS (ESI+) *t*<sub>R</sub> 6.07 min, *m/z* [M+H]<sup>+</sup> no ionization, purity 98%. MW: 424.56 g.mol<sup>-1</sup>. HRMS *m/z* [M+H]<sup>+</sup>  
557 calcd C<sub>15</sub>H<sub>7</sub>N<sub>2</sub>SCl<sub>5</sub>: 424.8816, Found: 424.8808.

558

#### 559 **3.1.4.5. 2-(2-Fluorophenylthio)-3-trichloromethylquinoxaline (3e)**

560 Yield 56% (555 mg). Beige solid. Mp 104 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.11 – 8.06 (m,  
561 1H), 7.70 – 7.61 (m, 4H), 7.53 – 7.47 (m, 1H), 7.26 – 7.19 (m, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ =  
562 163.3 (d, *J* = 250.0 Hz), 151.7, 147.3, 141.9, 137.4, 136.8, 132.2 (d, *J* = 8.2 Hz), 132.0, 129.8, 129.7,  
563 127.8, 124.8 (d, *J* = 3.8 Hz), 117.3 (d, *J* = 18.8 Hz), 116.2 (d, *J* = 22.8 Hz), 96.5. LC-MS (ESI+) *t*<sub>R</sub> 5.2 min,

564 m/z [M+H]<sup>+</sup> no ionization, purity 99%. MW: 353.66 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>8</sub>N<sub>2</sub>FSCl<sub>3</sub>:  
565 374.9502, Found: 374.9492.

566

#### 567 **3.1.4.6. 2-(3-Fluorophenylthio)-3-trichloromethylquinoxaline (3f)**

568 Yield 63% (659 mg). Beige solid. Mp 57 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.12 – 8.06 (m,  
569 1H), 7.73 – 7.64 (m, 3H), 7.45 – 7.40 (m, 3H), 7.22 – 7.15 (m, 1H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ =  
570 162.6 (d, J = 248.9 Hz), 152.3, 147.2, 141.6, 136.6, 131.9, 131.7 (d, J = 8.0 Hz), 131.2 (d, J = 3.0 Hz),  
571 130.3 (d, J = 8.1 Hz), 129.8, 129.6, 127.65, 122.5 (d, J = 22.3 Hz), 116.5 (d, J = 21.0 Hz), 96.5. LC-MS  
572 (ESI+) t<sub>R</sub> 5.4 min, m/z [M+H]<sup>+</sup> no ionization, purity 99%. MW: 373.66 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd  
573 for C<sub>15</sub>H<sub>8</sub>N<sub>2</sub>SFCl<sub>3</sub>: 374.9502, Found: 374.9495.

574

#### 575 **3.1.4.7. 2-(4-Fluorophenylthio)-3-trichloromethylquinoxaline (3g)**

576 Yield 51% (534 mg). Yellow solid. Mp 76 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.09 – 8.08 (m,  
577 1H), 7.69 – 7.61 (m, 5H), 7.18 (t, J = 8.6 Hz, 2H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ = 163.6 (d, J = 250.0 Hz),  
578 153.1, 147.1, 141.7, 138.1 (d, J = 8.6 Hz) (2C), 136.6, 131.9, 129.6, 127.6, 124.8 (d, J = 3.4 Hz), 116.4  
579 (d, J = 22.0 Hz) (2C), 96.6. LC-MS (ESI+) t<sub>R</sub> 5.4 min, m/z [M+H]<sup>+</sup> no ionization, purity 99%. MW: 373.66  
580 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>8</sub>N<sub>2</sub>SFCl<sub>3</sub>: 374.9502, Found: 374.9494.

581

#### 582 **3.1.4.8. 2-(2-Methoxyphenylthio)-3-trichloromethylquinoxaline (3h)**

583 Yield 64% (691 mg). Yellow solid. Mp 96 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.10 – 8.06 (m,  
584 1H), 7.65 – 7.61 (m, 4H), 7.48 (td, J = 8.2, 1.6 Hz, 1H), 7.05 (td, J = 7.5, 0.9 Hz, 1H), 7.00 (d, J = 8.2 Hz,  
585 1H), 3.69 (s, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ = 160.1, 152.9, 147.6, 142.0, 137.1, 136.6, 131.8,  
586 131.6, 129.7, 129.4, 127.7, 121.2, 118.4, 111.6, 96.7, 56.1. LC-MS (ESI+) t<sub>R</sub> 5.1 min, m/z [M+H]<sup>+</sup> no  
587 ionization, purity 99%. MW: 385.70 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>11</sub>N<sub>2</sub>OSCl<sub>3</sub>: 386.9702,  
588 Found: 386.9698.

589

#### 590 **3.1.4.9. 2-(2-Chloro-5-methoxyphenylthio)-3-trichloromethylquinoxaline (3i)**

591 Yield 15% (176 mg). Yellow solid. Mp 144 °C (isopropanol). <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.16 – 8.06  
592 (m, 1H), 7.77 – 7.63 (m, 3H), 7.51 – 7.40 (m, 1H), 7.27 (d, J = 3.0 Hz, 1H), 6.98 (dd, J = 8.8, 3.0 Hz, 1H),  
593 3.84 (s, 3H). <sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>) δ = 158.51, 151.94, 147.40, 141.97, 136.80, 132.06, 131.06,  
594 130.74, 130.07, 129.84, 129.77, 127.93, 122.66, 117.23, 96.54, 55.89. LC-MS (ESI+) t<sub>R</sub> 7.4 min, m/z  
595 [M+H]<sup>+</sup> no ionization, purity = 99%. MW: 420.14 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>10</sub>Cl<sub>4</sub>N<sub>2</sub>OS:  
596 420.9312, Found: 420.9312.

597

#### 598 **3.1.4.10. 2-(4-Methoxyphenylthio)-3-trichloromethylquinoxaline (3j)**

599 Yield 59% (637 mg). Yellow solid. Mp 149 °C (isopropanol). <sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>) δ 8.10 – 8.05  
600 (m, 1H), 7.72 – 7.63 (m, 3H), 7.55 (dt, J = 2.5; 9.0 Hz, 2H), 6.99 (dt, J = 2.6; 8.8 Hz, 2H), 3.89 (s, 3H). <sup>13</sup>C  
601 NMR (63 MHz, CDCl<sub>3</sub>) δ = 160.8, 154.1, 147.2, 141.9, 137.7 (2 C), 136.7, 131.9, 129.7, 129.5, 127.8,  
602 120.2, 114.9 (2 C), 96.7, 55.5. LC-MS (ESI+) t<sub>R</sub> 5.4 min, m/z [M+H]<sup>+</sup> no ionization, purity = 99%. MW:  
603 385.70 g.mol<sup>-1</sup>. HRMS m/z [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>11</sub>N<sub>2</sub>OSCl<sub>3</sub>: 386.9702, Found: 386.9699.

604

#### 605 **3.1.4.11. 2-(Phenylthio)-3-trichloromethylquinoxaline (3k)**

606 Yield 71% (707 mg). Beige solid. Mp 66 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.12 – 8.07 (m,  
607 1H), 7.72 – 7.67 (m, 3H), 7.66 – 7.62 (m, 2H), 7.48 – 7.46 (m, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ =

608 153.4, 147.4, 141.9, 136.8, 135.8 (3 C), 131.9, 129.9, 129.7, 129.4, 129.3 (2 C), 127.8, 96.7. **LC-MS**  
609 (ESI+)  $t_R$  5.3 min,  $m/z$  [M+H]<sup>+</sup> no ionization, purity 98%. MW: 355.67 g.mol<sup>-1</sup>. **HRMS  $m/z$  [M+H]<sup>+</sup> calcd**  
610 for C<sub>15</sub>H<sub>9</sub>N<sub>2</sub>SCl<sub>3</sub>: 356.9596, Found: 356.9594.

611

#### 612 **3.1.4.12. 2-(4-Bromophenylthio)-3-trichloromethylquinoxaline (3l)**

613 Yield 44% (535 mg). Beige solid. Mp 96 °C (isopropanol). **<sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)** δ 8.14 – 8.06 (m,  
614 1H), 7.74 – 7.66 (m, 3H), 7.62 – 7.56 (m, 2H), 7.51 – 7.46 (m, 2H). **<sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>)** δ =  
615 152.6, 147.3, 141.8, 137.4 (2 C), 136.8, 132.4 (2 C), 132.1, 129.9, 129.7, 128.9, 127.8, 124.1, 96.5. **LC-**  
616 **MS (ESI+)**  $t_R$  5.8 min,  $m/z$  [M+H]<sup>+</sup> no ionization, purity 99%. MW: 434.57 g.mol<sup>-1</sup>. **HRMS  $m/z$  [M+H]<sup>+</sup>**  
617 **calcd for C<sub>15</sub>H<sub>8</sub>N<sub>2</sub>SCl<sub>3</sub>Br: 434.8705, Found: 434.8701.**

618

#### 619 **3.1.4.13. 2-Trichloromethyl-3-(4-trifluoromethylphenylthio)quinoxaline (3m)**

620 Yield 80% (949 mg). Yellow solid. Mp 102 °C (isopropanol). **<sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)** δ 8.23 – 7.96  
621 (m, 1H), 7.86 – 7.54 (m, 7H). **<sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)** δ = 152.0, 147.5, 141.8, 136.9, 135.6, 134.9 (d,  
622  $J$  = 1.4 Hz), 132.3, 131.3 (q,  $J$  = 32.7 Hz), 130.2, 129.8, 127.8, 126.0 (q,  $J$  = 3.7 Hz), 124.1 (q,  $J$  = 272.4  
623 Hz), 96.5. **LC-MS (ESI+)**  $t_R$  5.38 min,  $m/z$  [M+H]<sup>+</sup> no ionization. MW: 423.67 g.mol<sup>-1</sup>, purity 99%. **HRMS**  
624  **$m/z$  [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>8</sub>Cl<sub>3</sub>F<sub>3</sub>N<sub>2</sub>S: 424.9470, Found: 424.9468.**

625

#### 626 **3.1.4.14. 2-Trichloromethyl-3-(4-trifluoromethoxyphenylthio)quinoxaline (3n)**

627 Yield 68% (837 mg). Yellow oil. **<sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)** δ 8.16 – 8.06 (m, 1H), 7.76 – 7.62 (m, 5H),  
628 7.36 – 7.28 (m, 2H). **<sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)** δ = 152.7, 150.2 (q,  $J$  = 1.7 Hz), 147.4, 141.8, 137.5  
629 (2C), 136.8, 132.2, 129.9, 129.8, 128.3, 127.8, 121.5 (2C), 120.6 (q,  $J$  = 258.0 Hz), 96.5. **LC-MS (ESI+)**  $t_R$   
630 6.1 min,  $m/z$  [M+H]<sup>+</sup> no ionization. MW: 439.67 g.mol<sup>-1</sup>, purity 99%. **HRMS  $m/z$  [M+H]<sup>+</sup> calcd for**  
631 **C<sub>16</sub>H<sub>8</sub>Cl<sub>3</sub>F<sub>3</sub>N<sub>2</sub>OS: 438.9448, Found: 438.9447.**

632

#### 633 **3.1.4.15. 2-(4-Nitrophenylthio)-3-trichloromethylquinoxaline (3o)**

634 Yield 64% (718 mg). White solid. Mp 161 °C (isopropanol). **<sup>1</sup>H NMR (250 MHz, CDCl<sub>3</sub>)** δ 8.34 – 8.23  
635 (m, 2H), 8.20 – 8.10 (m, 1H), 7.88 – 7.67 (m, 5H). **<sup>13</sup>C NMR (63 MHz, CDCl<sub>3</sub>)** δ = 151.1, 148.1, 147.7,  
636 141.8, 139.2, 137.1, 135.4 (2C), 132.6, 130.6, 129.9, 127.7, 124.1 (2C), 96.3. **LC-MS (ESI+)**  $t_R$  4.9 min,  
637  $m/z$  [M+H]<sup>+</sup> no ionization. MW: 400.67 g.mol<sup>-1</sup>, purity 99%. **HRMS  $m/z$  [M+H]<sup>+</sup> calcd for**  
638 **C<sub>15</sub>H<sub>8</sub>Cl<sub>3</sub>N<sub>3</sub>O<sub>2</sub>S: 401.9447, Found: 401.9447.**

639

#### 640 **3.1.4.16. 2-(4-Tolylthio)-3-trichloromethylquinoxaline (3p)**

641 Yield 40% (414 mg). Yellow solid. Mp 99 °C (isopropanol). **<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)** δ 8.13 – 8.04 (m,  
642 1H), 7.76 – 7.64 (m, 3H), 7.56 – 7.46 (m, 2H), 7.30 – 7.23 (m, 2H), 2.44 (s, 3H). **<sup>13</sup>C NMR (101 MHz,**  
643 **CDCl<sub>3</sub>)** δ = 153.7, 147.4, 142.0, 139.7, 136.7, 135.8 (2C), 131.9, 130.1 (2C), 129.7, 129.6, 127.8, 126.2,  
644 96.7, 21.6. **LC-MS (ESI+)**  $t_R$  5.67 min,  $m/z$  [M+H]<sup>+</sup> 368.18/371.01/373.05. MW: 369.70 g.mol<sup>-1</sup>, purity  
645 99%. **HRMS  $m/z$  [M+H]<sup>+</sup> calcd for C<sub>16</sub>H<sub>11</sub>Cl<sub>3</sub>N<sub>2</sub>S: 370.9753, Found: 370.9752.**

646

### 647 **3.1.5. General procedure for compounds 3q-3s**

#### 648 **3.1.5.1. Preparation of 2-chloro-3-(trifluoromethyl)quinoxaline (3q)**

649 **Step 1:** To a solution of *o*-phenylenediamine (2g, 18.5 mmol, 1.0 equiv) in H<sub>2</sub>O was added ethyl  
650 trifluoropyruvate (3g 146, 18.5 mmol, 1.0 equiv) dissolved in H<sub>2</sub>O (30 mL). The reaction mixture was  
651 heated at 50 °C for 15 min. After cooling, the precipitate was filtered off and washed with H<sub>2</sub>O. 3-

652 (Trifluoromethyl)quinoxalin-2(1*H*)-one was recrystallized from ethanol, precipitating as a white solid  
653 and engaged directly in step 2. Yield 93%.

654 **Step 2:** 3-(Trifluoromethyl)quinoxalin-2-ol (3.5 g, 16.8 mmol, 1.0 equiv) was heated to reflux in  
655 phosphorus oxychloride (30 mL) for 2 h. After the starting material was consumed, the reaction  
656 mixture was cooled to r.t. and then quenched with ice at 0 °C. The precipitate was purified by flash  
657 chromatography, to afford 2-chloro-3-(trifluoromethyl)quinoxaline (3q) as a white solid, yield 96%.  
658 Mp 140 °C. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.25 – 8.20 (m, 1H), 8.15 – 8.09 (m, 1H), 7.99 – 7.86 (m, 2H).  
659 NMR was consistent with description [44].

### 660 3.1.5.2. Preparation of compounds (3r, 3s)

661 To a solution of 2-chloroquinoxaline or 2-chloro-3-(trifluoromethyl)quinoxaline (300 mg, 1.3 mmol,  
662 1.0 equiv.) and thiophenol reagent (143 mg, 1.3 mmol, 1.0 equiv.) in anhydrous DMF (5 mL), Cs<sub>2</sub>CO<sub>3</sub>  
663 (1.0 equiv.) was added under inert atmosphere. The mixture was stirred at 70 °C overnight. After  
664 completion of the reaction, water was added, leading to a precipitate which was separated by  
665 filtration. The resulting precipitate was then thoroughly washed with water. The resulting solid was  
666 purified by silica gel column chromatography (eluent: Cyclohexane/CH<sub>2</sub>Cl<sub>2</sub>, 4:6) to afford the desired  
667 compound.

#### 668 3.1.5.2.1. 2-(Phenylthio)quinoxaline (3r)

669 Yield 90% (279 mg). orange solid. Mp 88 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.44 (s, 1H),  
670 7.99 (dd, *J* = 8.2, 1.4 Hz, 1H), 7.90 (dd, *J* = 8.3, 1.3 Hz, 1H), 7.73 – 7.60 (m, 4H), 7.50 – 7.44 (m, 3H). <sup>13</sup>C  
671 NMR (101 MHz, CDCl<sub>3</sub>) δ = 157.3, 143.6, 142.3, 140.0, 135.1 (2C), 130.6, 129.9 (2C), 129.8, 129.3,  
672 129.1, 128.9, 128.4. LC-MS (ESI+) *t*<sub>R</sub> 3.4 min, *m/z* [M+H]<sup>+</sup> 239.16. MW: 238.31 g.mol<sup>-1</sup>, purity 99%.  
673 HRMS *m/z* [M+H]<sup>+</sup> calcd for C<sub>14</sub>H<sub>10</sub>N<sub>2</sub>S: 239.0637, Found: 239.0638.

674

#### 675 3.1.5.2.2. 2-(Phenylthio)-3-(trifluoromethyl)quinoxaline (3s)

676 Yield 89% (354 mg). white solid. Mp 93 °C (isopropanol). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.14 – 8.07 (m,  
677 1H), 7.75 – 7.67 (m, 3H), 7.66 – 7.61 (m, 2H), 7.51 – 7.45 (m, 3H). <sup>13</sup>C NMR (101 MHz, CDCl<sub>3</sub>) δ =  
678 153.5, 143.0, 139.8 (q, *J* = 35.8 Hz), 137.9, 136.0 (2C), 132.5, 129.8, 129.8, 129.4 (2C), 129.2, 128.3,  
679 127.9 (d, *J* = 1.9 Hz), 121.2 (q, *J* = 276.0 Hz). LC-MS (ESI+) *t*<sub>R</sub> 6.8 min, *m/z* [M+H]<sup>+</sup> 307.07. MW: 306.31  
680 g.mol<sup>-1</sup>, purity 99%. HRMS *m/z* [M+H]<sup>+</sup> calcd for C<sub>15</sub>H<sub>9</sub>F<sub>3</sub>N<sub>2</sub>S: 307.0511, Found: 307.0511.

681

## 682 3.2. Biology

### 683 3.2.1. In Vitro Cytotoxicity Evaluation HepG2

684 HepG2 cell line was maintained at 37 °C, 5% CO<sub>2</sub>, at 90% humidity in MEM supplemented with 10%  
685 fetal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL) / streptomycin (100 µg/mL)  
686 (complete RPMI medium). The cytotoxicity of the tested molecules on the HepG2 (hepatocarcinoma  
687 cell line purchased from ATCC, ref HB-8065) cell line was assessed according to the method of  
688 Mosmann [45] with slight modifications. Briefly, 5.10<sup>3</sup> cells in 100 µL of complete medium were  
689 inoculated into each well of 96-well plates and incubated at 37 °C in humidified 5% CO<sub>2</sub>. After 24 h  
690 incubation, 100 µL of medium with various product concentrations dissolved in DMSO (final  
691 concentration less than 0.5% v/v) were added and the plates were incubated for 72 h at 37 °C.  
692 Triplicate assays were performed for each sample. Each plate-well was then microscope examined  
693 for possible precipitate formation before the medium was aspirated from the wells. 100 µL of MTT (3-  
694 (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (0.5 mg/mL in medium  
695 without FBS) were then added to each well. Cells were incubated for 2 h at 37 °C. After this time, the

696 MTT solution was removed and DMSO (100  $\mu$ L) was added to dissolve the resulting blue formazan  
697 crystals. Plates were shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570  
698 nm with 630 nm as reference wavelength using a BIO-TEK ELx808 Absorbance Microplate Reader  
699 (LabX, Midland, ON, Canada). DMSO was used as blank and doxorubicin (purchased from Sigma  
700 Aldrich) as positive control. Cell viability was calculated as percentage of control (cells incubated  
701 without compound). The 50% cytotoxic concentration ( $CC_{50}$ ) was determined from the dose–  
702 response curve, using TableCurve software 2D v.5.0.  $CC_{50}$  values represent the mean value calculated  
703 from three independent experiments.

704

### 705 **3.2.2. *In vitro* Cytotoxicity evaluation CHO**

706 CHO cell line was maintained at 37 °C, 5% CO<sub>2</sub> with 90% humidity in RPMI supplemented with 10%  
707 foetal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL) / streptomycin (100  $\mu$ g/mL)  
708 (complete RPMI medium). The evaluation of the tested molecules cytotoxicity on the CHO cell line  
709 (purchased from ATCC, ref CCL-61) was performed according to the method of Mosmann with slight  
710 modifications. Briefly,  $5 \cdot 10^3$  cells in 100  $\mu$ L of complete medium were inoculated into each well of 96-  
711 well plates and incubated at 37 °C in a humidified 5% CO<sub>2</sub>. After 24 h incubation, 100  $\mu$ L of medium  
712 with various product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were  
713 added and the plates were incubated for 24 h at 37 °C. Triplicate assays were performed for each  
714 sample. Each plate-well was then microscope-examined for detecting possible precipitate formation  
715 before the medium was aspirated from the wells. 100  $\mu$ L of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-  
716 diphenyl-2H-tetrazolium bromide) solution (0.5 mg/mL in medium without FCS) were then added to  
717 each well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was removed and  
718 DMSO (100  $\mu$ L) was added to dissolve the resulting blue formazan crystals. Plates were shaken  
719 vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as reference  
720 wavelength using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as blank and  
721 doxorubicin (purchased from Sigma Aldrich) as positive control. Cell viability was calculated as  
722 percentage of control (cells incubated without compound). The 50% cytotoxic concentration ( $CC_{50}$ )  
723 was determined from the dose–response curve by using the TableCurve software 2D v.5.0.  $CC_{50}$   
724 values represent the mean value calculated from three independent experiments.

725

### 726 **3.2.3. *In vitro* Cytotoxicity evaluation VERO**

727 VERO cell line was maintained at 37 °C, 5% CO<sub>2</sub> with 90% humidity in MEM supplemented with 10%  
728 foetal bovine serum, 1% L-glutamine (200 mM) and penicillin (100 U/mL) / streptomycin (100  $\mu$ g/mL)  
729 (complete MEM medium). The evaluation of the tested molecules cytotoxicity on the VERO cell line  
730 (purchased from ATCC, ref CRL-1586) was performed according to the method of Mosmann with  
731 slight modifications. Briefly,  $5 \cdot 10^3$  cells in 100  $\mu$ L of complete medium were inoculated into each well  
732 of 96-well plates and incubated at 37 °C in a humidified 5% CO<sub>2</sub>. After 24 h incubation, 100  $\mu$ L of  
733 medium with various product concentrations dissolved in DMSO (final concentration less than 0.5%  
734 v/v) were added and the plates were incubated for 48 h at 37 °C. Triplicate assays were performed  
735 for each sample. Each plate-well was then microscope-examined for detecting possible precipitate  
736 formation before the medium was aspirated from the wells. 100  $\mu$ L of MTT (3-(4,5-dimethyl-2-  
737 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (0.5 mg/mL in medium without FCS) were  
738 then added to each well. Cells were incubated for 2 h at 37 °C. After this time, the MTT solution was  
739 removed and DMSO (100  $\mu$ L) was added to dissolve the resulting blue formazan crystals. Plates were  
740 shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm with 630 nm as

741 reference wavelength using a BIO-TEK ELx808 Absorbance Microplate Reader. DMSO was used as  
742 blank and doxorubicin (purchased from Sigma Aldrich) as positive control. Cell viability was calculated  
743 as percentage of control (cells incubated without compound). The 50% cytotoxic concentration (CC<sub>50</sub>)  
744 was determined from the dose–response curve by using the TableCurve software 2D v.5.0. CC<sub>50</sub>  
745 values represent the mean value calculated from three independent experiments.

746

747

### 748 **3.2.4. In vitro Antiplasmodial Evaluation**

749 In this study, a K1 culture-adapted *P. falciparum* strain resistant to chloroquine, pyrimethamine, and  
750 proguanil was used in an in vitro culture. It was maintained in continuous culture as described  
751 previously by Trager and Jensen [46]. Cultures were maintained in fresh A+ human erythrocytes at  
752 2.5% hematocrit in complete medium (RPMI 1640 with 25 mM HEPES, 25 mM NaHCO<sub>3</sub>, 10% of A+  
753 human serum) at 37 °C under reduced O<sub>2</sub> atmosphere (gas mixture 10% O<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>).  
754 Parasitemia was maintained daily at between 1 and 3%. The *P. falciparum* drug susceptibility test was  
755 carried out by comparing quantities of DNA in treated and control cultures of parasite in human  
756 erythrocytes according to an SYBR Green I fluorescence-based method [47] using a 96-well  
757 fluorescence plate reader. Compounds, previously dissolved in DMSO (final concentration less than  
758 0.5% v/v), were incubated in a total assay volume of 200 µL (RPMI, 2% hematocrit and 0.4%  
759 parasitemia) for 72 h in a humidified atmosphere (10% O<sub>2</sub> and 5% CO<sub>2</sub>) at 37 °C, in 96-well flat  
760 bottom plates. Duplicate assays were performed for each sample. After incubation, plates were  
761 frozen at -20 °C for 24 h. Then, the frozen plates were thawed for 1 h at 37 °C. Fifteen µL of each  
762 sample were transferred to 96-well flat bottom non-sterile black plates (Greiner Bio-one) already  
763 containing 15 µL of the SYBR Green I lysis buffer (2X SYBR Green I, 20 mM Tris base pH 7.5, 20 mM  
764 EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). Negative control treated by solvents (DMSO or  
765 H<sub>2</sub>O) and positive controls (chloroquine and doxycycline) were added to each set of experiments.  
766 Plates were incubated for 15 min at 37 °C and then read on a TECAN Infinite F-200  
767 spectrophotometer with excitation and emission wavelengths at 485 and 535 nm, respectively. The  
768 concentrations of compounds required to induce a 50% decrease of parasite growth (EC<sub>50</sub> K1) were  
769 calculated from three independent experiments.

770

### 771 **3.2.5 Comet assay**

#### 772 **3.2.5.1. Cell culture and treatment**

773 The human hepatocarcinoma cell line HepG2 was obtained from the American Type Culture  
774 Collection (ATCC, ref. HB-8065). Cells were cultured in Eagle's Minimum Essential Medium (EMEM,  
775 ref. ATCC® 30-2003TM) supplemented with 10% heat-inactivated foetal bovine serum, 100 U/mL  
776 penicillin and 0.1 mg/mL streptomycin (all from Gibco). Cells were maintained at 37 °C in a  
777 humidified atmosphere with 5% CO<sub>2</sub>. Cells were used in passage number 11 to 15. Two  
778 concentrations of the compound **3k** (5.6 and 28 µM) were tested for 2 different times of incubation  
779 (2 and 72 h). Briefly, HepG2 cells were seeded at 1.13 x 10<sup>5</sup> cells/mL in 6-well plates (3 mL of cell  
780 suspension per well) and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. After 24 and 94  
781 h of incubation, cells were treated with different concentrations of the compound or the vehicle  
782 (0.5% dimethylsulfoxide, DMSO) for 72 and 2 h, respectively. Additionally, in the 2 h treatment plate,  
783 cells in an additional well were treated with 1 mM MMS as positive control for the comet assay. After  
784 treatment, medium was removed from the wells and cells were washed with phosphate buffered

785 saline (PBS). Finally, cells were trypsinized and trypsin was neutralized with fresh medium. From this  
786 point, cells were kept ice-cold to avoid DNA repair.

787

### 788 **3.2.5.2. Comet assay**

789 The standard alkaline comet assay was employed for the detection of DNA strand breaks (SBs) and  
790 alkali-labile sites (ALS) in cells treated with molecule **3k**. Trypsinized HepG2 cells were centrifuged at  
791 125 g for 5 min at 4 °C and resuspended in cold PBS at  $1 \times 10^6$  cells/mL. For the preparation of the  
792 agarose gels, 30  $\mu$ L of cell suspension were mixed with 140  $\mu$ L of 1% low melting point agarose in PBS  
793 at 37 °C and 2 aliquots of 70  $\mu$ L of cell/agarose mixture were placed on agarose-precoated  
794 microscope slides. Each droplet was covered with a 20 x 20 mm coverslip and after 2-3 min on a cold  
795 metal plate, the coverslips were removed. Then, slides were immersed in lysis solution (2.5 M NaCl,  
796 0.1 M Na<sub>2</sub>EDTA, 0.01 M Tris base, pH 10, and 1% Triton X-100) at 4 °C for 1 h. After lysis, slides were  
797 transferred to the electrophoresis tank and incubated for 40 min at 4 °C in the electrophoresis  
798 solution (0.3 M NaOH, 1 mM Na<sub>2</sub>EDTA, pH > 13) to allow DNA unwinding. After that, electrophoresis  
799 was carried out at 1 V/cm for 20 min (4 °C). Then, gels were neutralized and washed by immersing  
800 the slides in PBS for 10 min and distilled water for another 10 min (both at 4 °C). Gels were then air  
801 dried at room temperature. Comets were stained by adding 30  $\mu$ L of 1 mg/mL of 4,6- diamidino-2-  
802 phenylindole (DAPI) on top of each gel and placing 22 x 22 mm coverslips on top. Slides were  
803 incubated with DAPI at room temperature for 30 min before the analysis. The semiautomated image  
804 analysis system Comet Assay IV (Instem) was used to evaluate 50 comets per gel (i.e., 100/condition).  
805 The percentage of DNA in tail was the descriptor used for each comet.

806

### 807 **3.2.5.3. Statistics**

808 The median percentage of DNA in tail for 50 comets was calculated for each of the duplicate gels in  
809 each experiment, and the mean of the two medians was then calculated. The mean percentage of  
810 DNA in tail of 3 independent experiments and the standard deviation (SD) were calculated.

811

## 812 **3.2.6. APICOPLAST**

### 813 **3.2.6.1 Culturing *Plasmodium*-infected red blood cells**

814 *Plasmodium falciparum* blood stage parasites were maintained at 2% hematocrit in 1640 RPMI-  
815 HEPES supplemented with 5% AlbuMAX II (GIBCO) and 0.25% gentamycin. Parasites were grown  
816 sealed Perspex chambers gassed with beta mix gas (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>) at 37 °C and  
817 maintained on 48-hour cycles. Cultures were tightly synchronized at ring stage using sorbitol  
818 treatment (5% v/v) as previously described [31].

819

### 820 **3.2.6.2 IFA on treated parasites**

821 Prior to the treatment parasites are synchronized using 5% sorbitol. After 48h treatment (0.3  $\mu$ M of  
822 **(3k)** molecule, 0.3  $\mu$ M of **(2a)** molecule or DMSO) parasites are fixed using 4% paraformaldehyde  
823 (PFA) and 0.0075% glutaraldehyde for 30 minutes at room temperature. Fixing solution is washed 3x  
824 times with PBS and cells are permeabilized with 0.1% TX-100 for 10 minutes at room temperature.  
825 Permeabilization solution is washed 3x times with PBS and cells are blocked with 3% fetal bovine  
826 serum (FBS) for 1 h. Primary antibody (Rat IgG anti-HA, Roche, 1/500 in 3% FBS) is incubated 1h at  
827 room temperature. Primary antibody is washed out 3x times with PBS and cells are incubated with  
828 secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, Invitrogen, 1/1000 in 3% FBS) for 1h at  
829 room temperature. Secondary antibody is washed out 3x times with PBS and cells are incubated with

830 DAPI, 1/25000 in PBS. Samples are fixed between a slide and a coverslip with fluorogel and observed  
831 by epi-fluorescent microscopy.

832

### 833 **3.2.6.3 Growth assay**

834 To observe a potential effect of delayed death of the molecule on the parasite, *Plasmodium* is  
835 maintained on Three life cycles (48h). At 48 h, 96 h and 144 h, 100 µL of cultures are transferred into  
836 a 96 wells black wall flat bottom plate and mixed with 100 µL SYBR Green lysis buffer (20 mM Tris, pH  
837 7.5; 5 mM EDTA; 0.008 % (w/v) saponin; 0.08% (v/v) Triton X-100) with freshly added SYBR Green I  
838 (10000X), and incubated 1h at room temperature protected from the light. Fluorescence from each  
839 well is measured with TECAN infinite M200 plate reader (excitation: 485 nm, emission: 538 nm and  
840 integration time: 1000 µs). The rest of the cultures are diluted 1/10 as the untreated cultures. Graph  
841 is obtain by doing the ration the treated culture fluorescence intensity on the untreated culture  
842 fluorescence intensity (n= 3).

843

### 844 **3.2.7. Study on the liver stage**

#### 845 **3.2.7.1. Parasite strain and sporozoites isolation**

846 *P. falciparum* (NF135 strain) sporozoites were isolated by dissection of the salivary glands of  
847 infected *A. stephensi* 14-21 days after an infective blood meal (Department of medical microbiology,  
848 University Medical Centre, St Radboud, Nijmegen Netherland). All infected salivary glands were  
849 removed by hand dissection and crushed in a potter for sporozoites isolation and filtrated through a  
850 40 µm filter to remove mosquito debris (Cell Strainer, BD BioSciences, USA). The sporozoites were  
851 counted using a disposable Glasstic microscope slide (KOVA, USA).

#### 852 **3.2.7.2. Primary hepatocytes culture**

853 Conserved primary human hepatocytes were purchased from Biopredic International (France). Cells  
854 were seeded in 96-well plates (Falcon by Becton Dickinson Labware Europe, France) coated with  
855 collagen I (BD Bioscience, USA), at a density of 80 000 cells per well. They are cultured at 37 °C in 5%  
856 CO<sub>2</sub> in William's E medium (Gibco, Life Technologies, Saint Aubin, France) supplemented with 10% of  
857 Foetal Bovine Serum FCIII, 5x10<sup>-5</sup> M hydrocortisone hemisuccinate (Upjohn Laboratoires SERB,  
858 France), 5 µg per ml Insulin (Sigma Aldrich, USA), 2 mM L-glutamine, and 0.02 U per ml – 0.02 µg per  
859 ml penicillin – streptomycin (Life Technologies) until infection with sporozoites.

#### 860 **3.2.7.3. In vitro infection and drug assays**

861 *P. falciparum* sporozoites were re-suspended in the above complete medium used for hepatocytes  
862 culture. Cultured human hepatocytes were inoculated with *P. falciparum* sporozoites (30,000  
863 sporozoites/well of 96 well plates each) in 50 µl of complete media. The infected culture plate was  
864 centrifuged 10 min at 2000 rpm to allow fast parasite sedimentation onto the target cells and further  
865 incubated with serial dilution of drugs that were prepared in advance. After 3 hours incubation,  
866 cultures were washed and further incubated in fresh medium containing the appropriate drug  
867 concentration which were changed daily until cells fixation at day 6 post infection with 4%PFA for 20  
868 min at room temperature.

869 The exo-erythrocytic forms (EEFs) were stained using the anti-HSP70 serum for *P. falciparum* and  
870 revealed with an Alexa 488-conjugated goat anti-mouse immunoglobulin (Molecular Probes). Host  
871 cell and parasite nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI). The EEFs were  
872 counted under a fluorescence microscope (Leica DMI4000B) or by using a CellInsight High Content  
873 Screening platform equipped with the Studio HCS software (Thermo Fisher, Scientific). To investigate  
874 the effect of drugs inhibition on the apicoplast maturation during liver-stage development, the EEFs



875 were stained using Acyl carrier protein (ACP) antibody and revealed with an Alexa Fluor 680-  
876 conjugated goat anti-rabbit immunoglobulin (Molecular Probes), and then imaged using a confocal  
877 microscope (Olympus FV1200). The images were analyzed using ImageJ software.

#### 878 **3.2.7.4. Data analysis**

879 GraphPad Prism 7 statistical Software (GraphPad. Software, San Diego, CA, USA) were used for data  
880 analysis and graphing. All values were expressed as means and standard deviations (SD).

881

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