



**HAL**  
open science

## Novel thienopyrimidones targeting hepatic and erythrocytic stages of Plasmodium parasites with increased microsomal stability

Prisca Lagardère, Romain Mustière, Nadia Amanzougaghene, Sébastien Hutter, Marion Casanova, Jean-François Franetich, Shahin Tajeri, Aurélie Malzert-Fréon, Sophie Corvaisier, Marc Since, et al.

### ► To cite this version:

Prisca Lagardère, Romain Mustière, Nadia Amanzougaghene, Sébastien Hutter, Marion Casanova, et al.. Novel thienopyrimidones targeting hepatic and erythrocytic stages of Plasmodium parasites with increased microsomal stability. *European Journal of Medicinal Chemistry*, 2023, 261, pp.115873. 10.1016/j.ejmech.2023.115873 . hal-04503282

**HAL Id: hal-04503282**

**<https://amu.hal.science/hal-04503282v1>**

Submitted on 13 Mar 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Novel Thienopyrimidones Targeting Hepatic and Erythrocytic Stages of *Plasmodium* Parasites with Increased Microsomal Stability.

Prisca Lagardère,<sup>a</sup> Romain Mustière,<sup>b</sup> Nadia Amanzougaghene,<sup>c</sup> Sébastien Hutter,<sup>d</sup> Marion Casanova,<sup>d</sup> Jean-François Franetich,<sup>c</sup> Shahin Tajeri,<sup>c</sup> Aurélie Malzert-Fréon,<sup>e</sup> Sophie Corvaisier,<sup>e</sup> Marc Since,<sup>e</sup> Nadine Azas,<sup>d</sup> Patrice Vanelle,<sup>b, f</sup> Pierre Verhaeghe,<sup>g, h, i</sup> Nicolas Primas,<sup>b, f</sup> Dominique Mazier,<sup>c</sup> Nicolas Masurier<sup>a\*</sup> and Vincent Lisowski<sup>a\*</sup>

- a. Institut des Biomolécules Max Mousseron, UMR 5247, CNRS, Université de Montpellier, ENSCM, UFR des Sciences Pharmaceutiques et Biologiques, Montpellier, France.
- b. Aix Marseille Univ, CNRS, ICR UMR 7273, Equipe Pharmaco-Chimie Radicalaire, Faculté de Pharmacie, 13385 Marseille cedex 05, France.
- c. Centre d'Immunologie et des Maladies Infectieuses (CIMI), INSERM, CNRS, Sorbonne Université, Paris, France.
- d. Aix Marseille Université, IRD, AP-HM, SSA, VITROME, Marseille, France.
- e. CERMN, Université de Caen Normandie, UNICAEN.
- f. AP-HM, Hôpital Conception, Service Central de la Qualité et de l'Information Pharmaceutiques, 13005 Marseille, France.
- g. Univ. Grenoble Alpes, CNRS, DPM UMR 5063, F-38041, Grenoble, France
- h. LCC-CNRS Université de Toulouse, CNRS, UPS, Toulouse, France
- i. CHU de Nîmes, Service de Pharmacie, Nîmes, France

\*Correspondence: nicolas.masurier@umontpellier.fr; Tel.: +33-4117-59642;  
vincent.lisowski@umontpellier.fr; Tel.: +33-411 7-59599

## Abstract

Based on the structure of a previously identified hit, Gamhepathiopine **1**, which showed promising antiplasmodial activity, but poor microsomal stability, several strategies were investigated to improve the metabolic stability of the compounds. This included the introduction of fluorine or deuterium atoms, as well as carbocyclic groups. Among the new compounds, the 2-aminocyclobutyl derivative **5g** demonstrated enhanced microsomal stability compared to compound **1**, while retaining antiplasmodial activity against erythrocytic and hepatic stages of *Plasmodium*, without significant cytotoxicity against primary hepatocytes.

37

## 1. Introduction

38

39

40

41

42

43

44

45

46

47

48

49

50

Malaria is a potentially life-threatening disease caused by the *Plasmodium* parasite, which is transmitted to humans through the bites of infected *Anopheles* mosquitoes. Among the species that can infect humans, *Plasmodium falciparum* is responsible for the majority of malaria cases and deaths, especially in sub-Saharan Africa. In 2021, an estimated 247 million cases of malaria and 619,000 deaths occurred worldwide, with the highest burden in children under the age of five.[1] Over the past 20 years, tremendous progress has been made in the diagnosis (rapid diagnostic test (RDT), microscopy-based analysis, thick and thin layer blood smear analysis...),[2] prevention (vector control,[3] vaccines[4]) and effective treatment of malaria.[5] However, despite the availability of several effective drugs for malaria treatment, the emergence and spread of drug-resistant strains of the parasite,[6,7] as well as the high cost, limited efficacy, and potential side effects of some existing drugs, highlight the need for the discovery and development of new antimalarial compounds, particularly those active against multiple stages of parasite development.

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

66

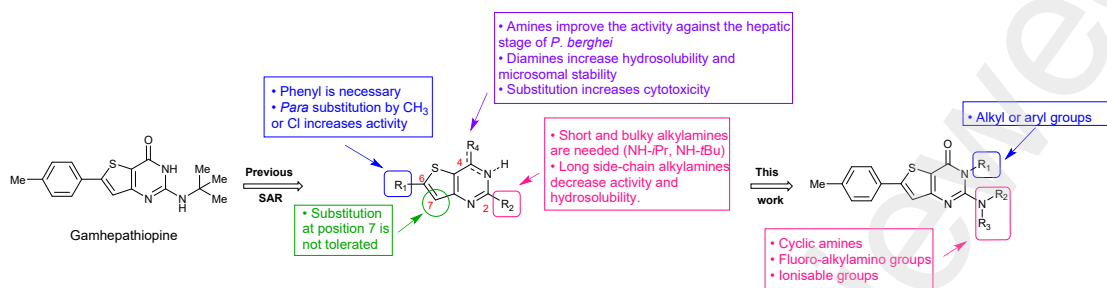
67

68

69

In this context, Gamhepathiopine (Fig. 1) has been identified as a hit compound, with potent antiplasmodial activity against both the sexual and asexual stages of *Plasmodium falciparum*. [8,9] However, the evaluation of the *in vivo* activity of this compound is limited by its low aqueous solubility and high metabolism by cytochromes P450. In order to improve these parameters, while maintaining or enhancing its antiplasmodial activity, extensive structure-activity relationship (SAR) studies have been carried out, based on the structure of Gamhepathiopine **1** [9–14]. Previous studies have shown a preference for a *p*-tolyl group at R<sub>1</sub> and a hydrogen at position 7 (Figure 1).[9] Some improvements in the antiplasmodial activity against the hepatic stage of *P. berghei* were achieved by introducing aminoalkyl chains at position 4 of the thienopyrimidine core. Additionally, the introduction of diamines at this position increased the hydrosolubility and the microsomal stability of the compounds. [10,14] However, it was observed that this led to increased cytotoxicity against mammalian cells, resulting in a low selectivity index. This strategy has therefore not been explored further, and a carbonyl group at position 4 appears to be the best compromise between antiplasmodial activity and safety. In position 2, short and bulky aminoalkyl moieties are needed, while long aminoalkyl chains decrease activity and aqueous solubility. To complete the SAR and enhance the microsomal stability of this series of compounds, we present the synthesis and biological activity of new 2-aminosubstituted thienopyrimidone derivatives. Specifically, the introduction of cyclic amines, fluoroalkyl chains and ionizable functions were studied. We also

70 considered the modulation of position 3 of the pyrimidine moiety with alkyl or aryl groups  
71 (Figure 1).



72

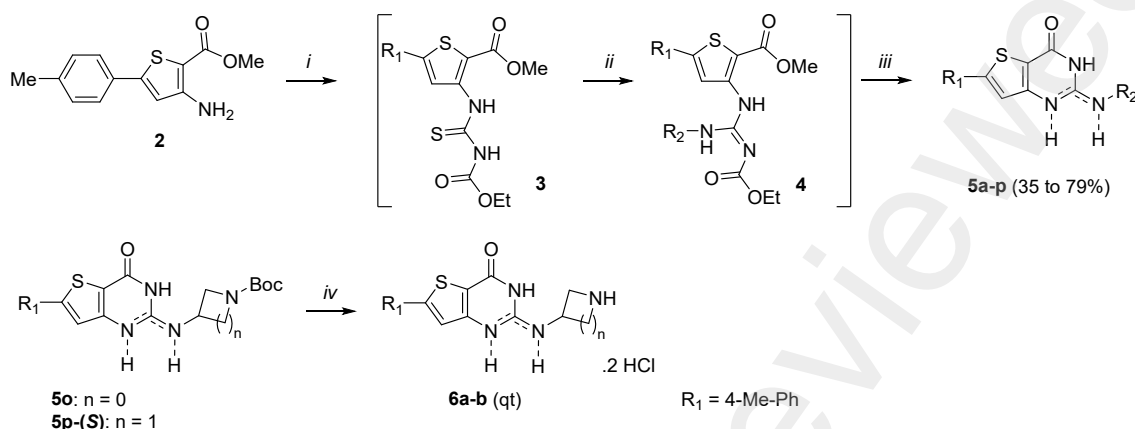
73 **Figure 1.** Gamhepathiopine structure and previous SAR studies.

## 74 2. Results and Discussion

### 75 2.1. Chemistry

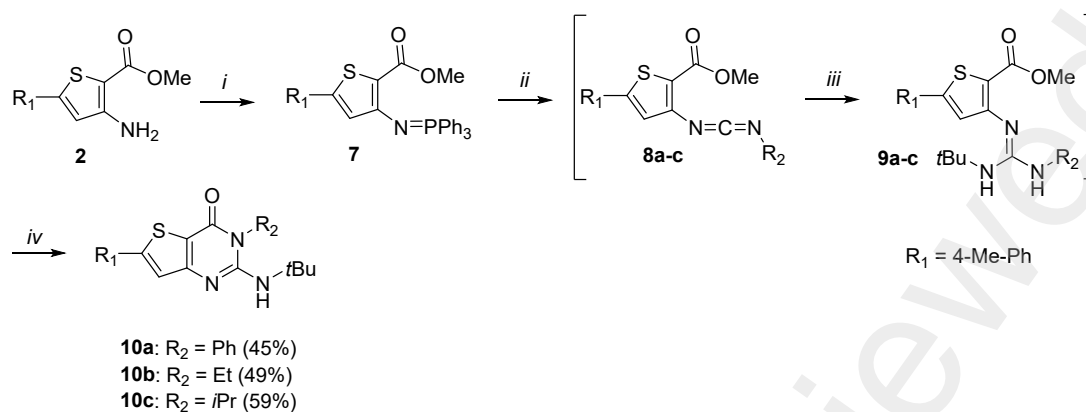
76 All 2-aminosubstituted derivatives were prepared according to Scheme 1. Methyl 3-amino-5-  
77 (*p*-tolyl)thiophene-2-carboxylate **2**[9] reacted with ethoxycarbonyl isothiocyanate in  
78 dimethylformamide (DMF) to yield intermediate **3**. The latter reacted with a set of primary and  
79 secondary amines in presence of EDCI and triethylamine to afford the corresponding guanidine  
80 derivatives **4**. The targeted compounds **5a-p** were obtained through final cyclization at 170°C  
81 and subsequently purified by chromatography, with yields ranging from 35% to 79%. For  
82 compounds containing an additional nitrogen (azetidine or pyrrolidine moiety),  
83 thienopyrimidones **5o** and **5p**, protected by a Boc group, were synthesized. Final acidic  
84 deprotection led to compounds **6a-b**, isolated as their corresponding hydrochloric salts. The  
85 structures of all final compounds were confirmed with different spectral analyses, such as <sup>1</sup>H-  
86 NMR, <sup>13</sup>C-NMR, mass spectroscopy and high-resolution mass spectrometry. It should be noted  
87 that in the case of the insertion of a 3-aminopropyl dimethylamine (compound **5e**) or a 3-  
88 aminoazetidine (compound **6a**), the <sup>1</sup>H NMR spectrum of these two compounds suggested the  
89 presence of an exocyclic double bond. Indeed, the signal around 10 ppm, which is attributed  
90 to the NH in position 3, and which is present in the spectra of all other analogues has  
91 disappeared. Furthermore, the presence of a triplet at 3.95 ppm for compound **5e** and a  
92 quintuplet at 4.95 ppm for compound **6a** was observed; these signals were attributed to the  
93 methylene in the alpha position of the guanidine function, suggesting the absence of a  
94 neighbouring NH in these two compounds. In contrast, for all other compounds, this

95 methylene consistently displayed a multiplicity compatible with the presence of an exocyclic  
96 NH.



98 **Scheme 1.** Synthesis of 2-aminosubstituted thienopyrimidones **5** and **6**. Reagents and conditions: (i)  
99  $\text{SCNCO}_2\text{Et}$ , DMF,  $30^\circ\text{C}$ , 2 h; (ii)  $\text{R}_2\text{NH}_2$ ,  $\text{NEt}_3$ ,  $\text{EDCI}\cdot\text{HCl}$ , DMF, RT, 18 h; (iii)  $170^\circ\text{C}$  and 2 h; (iv) 1,4-  
100 dioxane/HCl 37% 4:1 v:v, RT.

101 Then, we turned our attention to the preparation of 3-substituted derivatives (Scheme 2).  
102 Initially, the direct alkylation of gamhepathiopine **1** was studied, using 1.1 equivalents of  
103 sodium hydride and benzyl bromide as alkylating agent, in dry THF. Under these conditions,  
104 only traces of several mono-benzylated compounds were detected by LC-MS analysis, even  
105 after 6 days of reaction. Due to the low reactivity of the starting material and the lack of  
106 selectivity in alkylation, an alternative approach using an aza-Wittig reaction was explored,  
107 starting from compound **2**. Thus, the reaction between triphenylphosphine and **2** in the  
108 presence of hexachloroethane in dry acetonitrile at  $0^\circ\text{C}$  afforded the corresponding  
109 iminophosphorane **7** in 65% yield, after recrystallization in ethanol. Carbodiimides **8** were then  
110 prepared by reacting compound **7** with three different isocyanates in dichloromethane at  
111 room temperature. An aryl (phenyl), a linear (ethyl) and a ramified alkyl (isopropyl) isocyanate  
112 were chosen to introduce diversity at position 3 of the final thienopyrimidones **10a-c**.  
113 Depending on the nature of the isocyanate substituent, adjustments to the operating  
114 conditions were applied. Phenylisocyanate reacted smoothly at  $0^\circ\text{C}$ , resulting in complete  
115 conversion of **7** after 15 hours (followed by HPLC), whereas ethyl- and isopropylisocyanate  
116 proved to be less reactive under these conditions. With ethylisocyanate, total conversion was  
117 achieved after 48 hours at  $35^\circ\text{C}$ , while the isopropyl derivative required 48 hours at  $65^\circ\text{C}$ .  
118 Carbodiimides **8a-c** were not isolated and reacted directly with *tert*-butylamine to generate  
119 substituted guanidine derivatives **9a-c**. The latter were cyclized in the presence of sodium  
120 ethylate in ethanol to afford the corresponding 3-substituted thienopyrimidones **10a-c** in 3-  
121 step overall yields of 45% to 59%, after recrystallization.



122

123 **Scheme 2.** Synthesis of 3-substituted thienopyrimidones **10**. Reagents and conditions: (i) PPh<sub>3</sub>, C<sub>2</sub>Cl<sub>6</sub>, dry  
 124 ACN, 0°C, and 2 h 30; (ii) R<sub>2</sub>NCO, DCM, 0°C, 15 h for **8a**, DCM, 35°C, 48h for **8b**, THF, reflux, and 48 h for  
 125 **8c**; (iii) *t*BuNH<sub>2</sub>, DCM, RT; (iv) EtONa, EtOH, RT.

126

## 127 2.2. Biological evaluation

### 128 2.2.1. Blood-stage Antiplasmodial Activity and Cytotoxic Activity against HepG2 Human Cells

129 All newly synthesized 2-aminosubstituted and 3-substituted thieno[3,2-*d*]pyrimidines were  
 130 screened for their activity against asexual erythrocytic stage parasites of a multidrug-resistant  
 131 *P. falciparum* K1 strain (In order to investigate the impact of the substitution of position 3 in  
 132 this series of compounds, compounds **10a-c** were evaluated on the erythrocytic stage of *P.*  
 133 *falciparum*. Compounds **10a** and **10b** were inactive (EC<sub>50</sub> > 12.5 μM). On the other hand, **10c**  
 134 showed very low activity on *P. falciparum* compared to Gamhepathiopine **1** (EC<sub>50</sub> of 4.2 μM vs.  
 135 0.2 μM). Furthermore, this substitution led to an increase in the cytotoxicity of the compounds,  
 136 particularly for compound **10b**, which exhibited a CC<sub>50</sub> value of 6.3 μM against the HepG2 cell  
 137 line. Hence, the presence of an NH group at position 3 appears to be essential for the  
 138 antiplasmodial activity of this series of compounds and was consistently maintained in all other  
 139 synthesized derivatives.

140 Then, we focused on the influence of modulating position 2 of the thienopyrimidone scaffold.  
 141 Initially, the introduction of a methylamine group in position 2 was considered in view of the  
 142 activity of different series of thieno[3,2-*d*]pyrimidine analogues proposed by González Cabrera  
 143 *et al.*, which showed strong activity on several strains of *P. falciparum*. [15,16] However, in our  
 144 hands, the 2-aminomethyl analogue **5a** was found to be inactive. This result demonstrates  
 145 that, despite the structural similarity with other reported antiplasmodial compounds,  
 146 Gamhepathiopine derivatives do not appear to share the same mechanism of action.

147 Then, as gamhepathiopine **1** was highly metabolized in the presence of female mouse  
148 microsomes (half-life,  $t_{1/2}$  = 11 min) and the *tert*-butyl group at position 2 proved to be highly  
149 sensitive to oxidation, the introduction of more stable groups was considered in order to  
150 decrease metabolism at this position. In drug design, a common strategy involves replacing  
151 chemical groups that are substrates of cytochromes with bioisosteres called metabolic  
152 blockers, such as fluorine or deuterium atoms. Deuterium, a heavy and non-radioactive  
153 isotope of hydrogen, has been officially recognized in medicinal chemistry since 2017,  
154 following the FDA approval of the first deuterated drug (Deutetrabenazine), for the treatment  
155 of Huntington's disease. [17] Deuterium is mainly used for its kinetic isotope effect. It  
156 expresses twice the mass of hydrogen; therefore, the carbon-deuterium bond is more stable  
157 and resistant to oxidative processes that may occur during phase I reactions of metabolism.  
158 Additionally, deuterium exhibits steric and electronic properties similar to hydrogen.[18] This  
159 alternative isostere effectively limits oxidation and improves the pharmacokinetic properties  
160 of compounds, such as their half-life and clearance. On the other hand, the introduction of a  
161 fluorine atom or fluorinated groups is a widely employed approach to address metabolic  
162 stability liabilities. Fluorine possesses a Van der Waals radius similar close to hydrogen (1.47 Å  
163 vs. 1.20 Å), and the strength of the carbon-fluorine bond exceeds that of the carbon-hydrogen  
164 bond.[19] Consequently, phase I oxidation reactions are more difficult, without significantly  
165 altering the structure of the compound. Therefore, to enhance the metabolic stability of  
166 gamhepathiopine, introduction of a fluorine or deuterium atom(s) at position 2 was  
167 considered. Thus, the deuterated analogues (**5b** and **5c**), respective bioisosteres of compounds  
168 **11**[9] and **1**, as well as the fluorinated derivative **5d** were synthesized and evaluated. While  
169 the monotrifluoromethyl analogue of **1** was inactive (compound **5d**), the deuterated  
170 compounds **5b** and **5c** showed similar activity to the reference compounds **1** and **11** on Pf. K1  
171 ( $EC_{50}$  = 0.2 and 0.12  $\mu$ M vs. 0.2  $\mu$ M). Moreover, their cytotoxicity on HepG2 was acceptable  
172 ( $CC_{50}$  > 10  $\mu$ M). Thus, the selectivity index of **5c** is higher than that of its isotopic analogue **1** (SI  
173 = 149 vs. 128).

174 Then, a second strategy was explored to increase the metabolic stability of the compounds. It  
175 is well-known that the lipophilicity of a drug has a strong impact on various pharmacokinetic  
176 parameters, including its biotransformation.[17] The introduction of a linear or a cyclic  
177 alkylamine substituent at position 2 was explored in order to reduce their LogD, increase their  
178 aqueous solubility and potentially enhance their metabolic stability. This approach has been  
179 successfully employed in this series by introducing a diaminoalkyl chain at position 4, resulting  
180 in an 100-fold improvement in aqueous solubility at pH 7.4, and a 6-fold enhancement in

181 microsomal stability, compared to compound **1**.<sup>[14]</sup> Thus, compounds bearing a diamine  
182 (compound **5e**), a 4-aminoazetidine (compound **6a**) and a 3-aminopyrrolidine group  
183 (compound **6b**) at position 2 were synthesized and evaluated. These compounds exhibited  
184 calculated LogD<sub>7.4</sub> values lower than compound **1** (between -0.4 and 1.55, vs. 3.73 for **1**).<sup>[20]</sup>  
185 However, these modifications resulted in a loss of antiplasmodial activity (EC<sub>50</sub> > 9 μM). These  
186 results suggest that the presence of a polar group at position 2 is unfavourable and that this  
187 substituent occupies a hydrophobic pocket within the target. In addition, these modifications  
188 led to some cytotoxicity against HepG2 cells. For example, compound **6a** showed high  
189 cytotoxicity, with a CC<sub>50</sub> around 1 μM.

190 Finally, as the *tert*-butyl group at position 2 was previously identified as a sensitive site for  
191 oxidation, the substitution of this substituent with a cyclic group was explored. Indeed, it has  
192 been postulated that the reduction in the number of available conformations in a cyclic  
193 template could potentially attenuate the rate of metabolism, compared to the acyclic  
194 form.<sup>[21]</sup> A series of cycloalkylamines (compounds **5f** to **5i**) and cyclic amines (compounds **5j**  
195 to **5n**) were introduced at position 2. Compounds with an aminocyclopropyl (**5f**),  
196 aminocyclobutyl (**5g**) and aminocyclopentyl moiety (**5h**) showed an activity on *Pf.* K1 in the  
197 micromolar range (IC<sub>50</sub> between 0.9 and 1.9 μM), only slightly lower than compound **1**.  
198 Compound **5g** displayed a moderate selectivity index (SI = 12.7), whereas the two other  
199 compounds showed some cytotoxicity against HepG2, particularly compound **5f** displaying a  
200 CC<sub>50</sub> of 4 μM. The introduction of an aminocyclohexyl group (**5i**) resulted in a loss of activity,  
201 suggesting that the size of this substituent is too large to interact with the target and a 5-  
202 member ring is the maximum tolerated size.

203 The introduction of tertiary cyclic amines (compound **5j** to **5n**) was then studied, and the  
204 influence of steric hindrance caused by the ring size was evaluated on the antiparasitic activity.  
205 Compound **5j** bearing an azetidiny group could not be tested, due to its lack of solubility in  
206 DMSO. A pyrrolidinyl group (compound **5k**) was tolerated in position 2, giving a moderately  
207 active compound (IC<sub>50</sub> = 1.4 μM). Interestingly, the presence of an exocyclic NH group is not  
208 necessary for activity against the erythrocytic stage of *P. falciparum*. Finally, as observed with  
209 compound **5i**, introduction of a 6-membered cycle was not tolerated, compounds **5l** to **5n** were  
210 inactive.

211



212 Table 1), resistant to chloroquine, pyrimethamine and sulfadoxine. All derivatives were  
213 compared to Gamhepathiopine **1**. Chloroquine and doxycycline were included as reference  
214 drugs.

215 In order to investigate the impact of the substitution of position 3 in this series of compounds,  
216 compounds **10a-c** were evaluated on the erythrocytic stage of *P. falciparum*. Compounds **10a**  
217 and **10b** were inactive ( $EC_{50} > 12.5 \mu\text{M}$ ). On the other hand, **10c** showed very low activity on *P.*  
218 *falciparum* compared to Gamhepathiopine **1** ( $EC_{50}$  of  $4.2 \mu\text{M}$  vs.  $0.2 \mu\text{M}$ ). Furthermore, this  
219 substitution led to an increase in the cytotoxicity of the compounds, particularly for compound  
220 **10b**, which exhibited a  $CC_{50}$  value of  $6.3 \mu\text{M}$  against the HepG2 cell line. Hence, the presence  
221 of an NH group at position 3 appears to be essential for the antiplasmodial activity of this series  
222 of compounds and was consistently maintained in all other synthesized derivatives.

223 Then, we focused on the influence of modulating position 2 of the thienopyrimidone scaffold.  
224 Initially, the introduction of a methylamine group in position 2 was considered in view of the  
225 activity of different series of thieno[3,2-*d*]pyrimidine analogues proposed by González Cabrera  
226 *et al.*, which showed strong activity on several strains of *P. falciparum*. [15,16] However, in our  
227 hands, the 2-aminomethyl analogue **5a** was found to be inactive. This result demonstrates  
228 that, despite the structural similarity with other reported antiplasmodial compounds,  
229 Gamhepathiopine derivatives do not appear to share the same mechanism of action.

230 Then, as gamhepathiopine **1** was highly metabolized in the presence of female mouse  
231 microsomes (half-life,  $t_{1/2} = 11 \text{ min}$ ) and the *tert*-butyl group at position 2 proved to be highly  
232 sensitive to oxidation, the introduction of more stable groups was considered in order to  
233 decrease metabolism at this position. In drug design, a common strategy involves replacing  
234 chemical groups that are substrates of cytochromes with bioisosteres called metabolic  
235 blockers, such as fluorine or deuterium atoms. Deuterium, a heavy and non-radioactive  
236 isotope of hydrogen, has been officially recognized in medicinal chemistry since 2017,  
237 following the FDA approval of the first deuterated drug (Deutetrabenazine), for the treatment  
238 of Huntington's disease. [17] Deuterium is mainly used for its kinetic isotope effect. It  
239 expresses twice the mass of hydrogen; therefore, the carbon-deuterium bond is more stable  
240 and resistant to oxidative processes that may occur during phase I reactions of metabolism.  
241 Additionally, deuterium exhibits steric and electronic properties similar to hydrogen.[18] This  
242 alternative isostere effectively limits oxidation and improves the pharmacokinetic properties  
243 of compounds, such as their half-life and clearance. On the other hand, the introduction of a  
244 fluorine atom or fluorinated groups is a widely employed approach to address metabolic

245 stability liabilities. Fluorine possesses a Van der Waals radius similar close to hydrogen (1.47 Å  
246 vs. 1.20 Å), and the strength of the carbon-fluorine bond exceeds that of the carbon-hydrogen  
247 bond.[19] Consequently, phase I oxidation reactions are more difficult, without significantly  
248 altering the structure of the compound. Therefore, to enhance the metabolic stability of  
249 gamhepathiopine, introduction of a fluorine or deuterium atom(s) at position 2 was  
250 considered. Thus, the deuterated analogues (**5b** and **5c**), respective bioisosteres of compounds  
251 **11**[9] and **1**, as well as the fluorinated derivative **5d** were synthesized and evaluated. While  
252 the monotrifluoromethyl analogue of **1** was inactive (compound **5d**), the deuterated  
253 compounds **5b** and **5c** showed similar activity to the reference compounds **1** and **11** on Pf. K1  
254 ( $EC_{50} = 0.2$  and  $0.12 \mu\text{M}$  vs.  $0.2 \mu\text{M}$ ). Moreover, their cytotoxicity on HepG2 was acceptable  
255 ( $CC_{50} > 10 \mu\text{M}$ ). Thus, the selectivity index of **5c** is higher than that of its isotopic analogue **1** ( $SI$   
256  $= 149$  vs.  $128$ ).

257 Then, a second strategy was explored to increase the metabolic stability of the compounds. It  
258 is well-known that the lipophilicity of a drug has a strong impact on various pharmacokinetic  
259 parameters, including its biotransformation.[17] The introduction of a linear or a cyclic  
260 alkylamine substituent at position 2 was explored in order to reduce their LogD, increase their  
261 aqueous solubility and potentially enhance their metabolic stability. This approach has been  
262 successfully employed in this series by introducing a diaminoalkyl chain at position 4, resulting  
263 in an 100-fold improvement in aqueous solubility at pH 7.4, and a 6-fold enhancement in  
264 microsomal stability, compared to compound **1**. [14] Thus, compounds bearing a diamine  
265 (compound **5e**), a 4-aminoazetidine (compound **6a**) and a 3-aminopyrrolidine group  
266 (compound **6b**) at position 2 were synthesized and evaluated. These compounds exhibited  
267 calculated  $\text{LogD}_{7.4}$  values lower than compound **1** (between  $-0.4$  and  $1.55$ , vs.  $3.73$  for **1**). [20]  
268 However, these modifications resulted in a loss of antiplasmodial activity ( $EC_{50} > 9 \mu\text{M}$ ). These  
269 results suggest that the presence of a polar group at position 2 is unfavourable and that this  
270 substituent occupies a hydrophobic pocket within the target. In addition, these modifications  
271 led to some cytotoxicity against HepG2 cells. For example, compound **6a** showed high  
272 cytotoxicity, with a  $CC_{50}$  around  $1 \mu\text{M}$ .

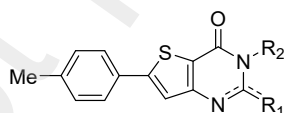
273 Finally, as the *tert*-butyl group at position 2 was previously identified as a sensitive site for  
274 oxidation, the substitution of this substituent with a cyclic group was explored. Indeed, it has  
275 been postulated that the reduction in the number of available conformations in a cyclic  
276 template could potentially attenuate the rate of metabolism, compared to the acyclic  
277 form.[21] A series of cycloalkylamines (compounds **5f** to **5i**) and cyclic amines (compounds **5j**  
278 to **5n**) were introduced at position 2. Compounds with an aminocyclopropyl (**5f**),

aminocyclobutyl (**5g**) and aminocyclopentyl moiety (**5h**) showed an activity on *Pf.* K1 in the micromolar range ( $IC_{50}$  between 0.9 and 1.9  $\mu\text{M}$ ), only slightly lower than compound **1**. Compound **5g** displayed a moderate selectivity index ( $SI = 12.7$ ), whereas the two other compounds showed some cytotoxicity against HepG2, particularly compound **5f** displaying a  $CC_{50}$  of 4  $\mu\text{M}$ . The introduction of an aminocyclohexyl group (**5i**) resulted in a loss of activity, suggesting that the size of this substituent is too large to interact with the target and a 5-member ring is the maximum tolerated size.

The introduction of tertiary cyclic amines (compound **5j** to **5n**) was then studied, and the influence of steric hindrance caused by the ring size was evaluated on the antiparasitic activity. Compound **5j** bearing an azetidiny group could not be tested, due to its lack of solubility in DMSO. A pyrrolidinyl group (compound **5k**) was tolerated in position 2, giving a moderately active compound ( $IC_{50} = 1.4 \mu\text{M}$ ). Interestingly, the presence of an exocyclic NH group is not necessary for activity against the erythrocytic stage of *P. falciparum*. Finally, as observed with compound **5i**, introduction of a 6-membered cycle was not tolerated, compounds **5l** to **5n** were inactive.

294

**Table 1.** *In vitro* antiplasmodial activity ( $EC_{50}$ ) of thienopyrimidones on the erythrocytic stage of a K1 *P. falciparum* strain and corresponding cytotoxicity ( $CC_{50}$ ) on the human HepG2 cell line.



297

Compound	R <sub>1</sub>	R <sub>2</sub>	<i>P. falciparum</i> K1 EC <sub>50</sub> ( $\mu\text{M}$ ) <sup>a</sup>	HepG2 CC <sub>50</sub> ( $\mu\text{M}$ ) <sup>b</sup>	SI <sup>c</sup>
<b>1</b> (Gamhepathiopine)	NHtBu	H	0.2 ± 0.02[9]	25.6 ± 3.1[9]	128
<b>11</b> [9]	NH/Pr	H	0.8 ± 0.5[9]	49.4 ± 1.0[9]	62
<b>5a</b>	NHCH <sub>3</sub>	H	> 25	> 6.2	-
<b>5b</b>	NHCH(CD <sub>3</sub> ) <sub>2</sub>	H	0.2 ± 0.07	> 10	> 50
<b>5c</b>	NHC(CD <sub>3</sub> ) <sub>3</sub>	H	0.1 ± 0.07	17.9 ± 5.3	149
<b>5d</b>	NHC(CF <sub>3</sub> )(CH <sub>3</sub> ) <sub>2</sub>	H	> 10	23.9 ± 1.4	> 10
<b>5e</b>	N=(CH <sub>2</sub> ) <sub>3</sub> N(CH <sub>3</sub> ) <sub>2</sub>	H	9.8 ± 3.1	4.1 ± 1.8	0.42
<b>5f</b>		H	0.9 ± 0.1	4.0 ± 0.1	4.4
<b>5g</b>		H	1.1 ± 0.2	14.0 ± 2.8	12.7
<b>5h</b>		H	1.4 ± 0.2	> 7.8	> 5.6

5i		H	> 12.5	> 12.5	-
5j		H	NT <sup>d</sup>	NT <sup>d</sup>	-
5k		H	1.4 ± 0.5	> 3.1	> 2.1
5l		H	> 25	> 3.1	-
5m		H	> 10	3.6 ± 1.5	< 0.4
5n		H	> 25	8.7 ± 2.8	< 0.3
6a <sup>e</sup>		H	17.9 ± 0.1	1.3 ± 0.1	0.1
6b <sup>e</sup>		H	> 31.25	13.2 ± 2.1	< 0.4
10a	NHtBu	Ph	> 12.5	> 3.1	-
10b	NHtBu	Et	> 12.5	6.3	< 0.5
10c	NHtBu	iPr	4.2 ± 0.9	> 3.9	> 0.9
<b>Chloroquine</b>			0.8 ± 0.1[10]	30[9]	37.5
<b>Doxycycline</b>			5.0[9]	20[9]	4

<sup>a</sup> EC<sub>50</sub>: half-maximal effective concentration. The values are mean ± SD of three independent experiments.

<sup>b</sup> CC<sub>50</sub>: half-maximal cytotoxic concentration. The values are mean ± SD of three independent experiments.

<sup>c</sup> Selectivity index (SI) was calculated according to the following formula: SI = CC<sub>50</sub> (HepG2)/EC<sub>50</sub>.

<sup>d</sup> NT: not tested due to low solubility in DMSO.

<sup>e</sup> Isolated as a trifluoroacetate salt.

298  
299  
300  
301  
302  
303

### 304 2.2.2. Hepatic-stage Antiplasmodial Activity and Cytotoxicity against Primary Simian 305 Hepatocytes

306 Then, seven compounds with an EC<sub>50</sub> below 1.5 μM on the erythrocytic stage of *P. falciparum*  
307 were selected for further studies. Thus, thienopyrimidines were evaluated for their ability to  
308 inhibit hepatic infection caused by the rodent malaria parasite *P. berghei* in simian  
309 hepatocytes. Their cytotoxicity was also determined on primary simian hepatocytes. The  
310 results are presented in Table .

311 **Table 2.** *In vitro* activity (EC<sub>50</sub>) against the hepatic stage of *P. berghei* parasites and cytotoxicity (CC<sub>50</sub>)  
312 against primary simian hepatocytes of selected thieno[3,2-*d*]pyrimidones.

Compound	<i>P. berghei</i> EC <sub>50</sub> (μM) <sup>a</sup>	Primary simian hepatocytes CC <sub>50</sub> (μM) <sup>b</sup>
----------	---	---

<b>1 (Gamhepathiopine)</b>	24.3 ± 1.6[10]	> 200[10]
<b>11</b>	55.6 ± 10.4	> 200
<b>5b</b>	15.9 ± 2.3	> 100
<b>5c</b>	13.7 ± 1.3	> 100
<b>5f</b>	5.5 ± 0.8	> 100
<b>5g</b>	11.2 ± 1.2	> 100
<b>5h</b>	10.5 ± 1.3	> 100
<b>5k</b>	> 100	> 200

<sup>a</sup> EC<sub>50</sub>: half-maximal effective concentration. The values are mean ± SD of three independent experiments.

<sup>b</sup> CC<sub>50</sub>: half-maximal cytotoxic concentration. The values are mean ± SD of three independent experiments.

Except compounds **11** and **5k**, all other compounds showed higher activity against the hepatic stage of *P. berghei* than Gamhepathiopine **1**. Thus, the activity of deuterated analogues of **1** and **11**, namely compound **5b** and **5c**, was increased by a factor of 1.5 to 4, indicating that the introduction of deuterium atoms is favourable for activity. Interestingly, the introduction of a 3- to 5-membered carbocycle at position 2 increased the activity against the hepatic stage. Among this series, compound **5f** with an aminocyclopropyl substituent, showed the highest activity, with an EC<sub>50</sub> of 5.56 μM. Finally, while the presence of a NH group was not necessary to maintain erythrocytic activity, its removal was deleterious for the hepatic activity. These results suggest that this compound may have a different mechanism of action than compound **1**. Lastly, none of the selected compounds showed cytotoxicity against simian hepatocytes at the highest dose tested (EC<sub>50</sub> > 100 μM).

### 2.2.3. Characterization of Drug-like Properties of Compounds **5b-c** and **5f-h**

Deuterated compounds **5b** and **5c**, as well as 3-, 4- and 5-membered aminocarbocyclic derivatives **5f**, **5g** and **5h** were selected to assess their stability in the presence of female mouse microsomes. Results are reported in Table 3. Deuterated analogues (compounds **5b** and **5c**, respectively) showed weak microsomal stability, similar to the reference compounds **1** and **11** (T<sub>1/2</sub> close to 10 minutes). Thus, this strategy to increase the metabolic stability of the thienopyrimidones proved to be inefficient for this series of compounds. However, the rigidification of the alkyl substituent by a carbocycle increased the metabolic stability by a factor of 1.5 to 3.8, compared to Gamhepathiopine **1**. Notably, compounds **5g** and **5h** showed half-lives of 29 and 38 minutes, respectively. Consequently, the intrinsic clearance of these two compounds decreased (Cl<sub>int</sub> > 58 μL/min/mg vs. Cl<sub>int</sub> > 240 for compound **1**). Considering both the antiplasmodial activities, cytotoxicity and microsomal stability, compound **5g** was selected for further characterizations. Indeed, the aminocyclobutyl derivative was preferred

343 because it displayed a better activity against the erythrocytic stage of *P. falciparum* and lower  
 344 cytotoxicity on HepG2 compared to the aminocyclopentyl-substituted derivative ( $EC_{50} = 1.1$   
 345  $\mu\text{M}$  and  $CC_{50} = 14.0 \mu\text{M}$  vs.  $EC_{50} = 1.4 \mu\text{M}$  and  $CC_{50} > 7.8 \mu\text{M}$ ).

346 Since the *in-vivo* activity of Gamhepathiopine **1** was studied in its hydrochloride salt form,[8]  
 347 compound **5g** was converted into this form after treatment with a hydrochloric acid solution  
 348 in dioxane. The activity of the hydrochloride salt of **5g** was first evaluated toward both the  
 349 erythrocytic and hepatic parasite stages, along with its potential cytotoxicity against the HepG2  
 350 cell line and primary simian hepatocytes. As expected, no significant differences were found  
 351 (data not shown). However, the introduction of an aminocyclobutyl moiety decreased the  
 352 aqueous solubility of the thienopyrimidone ( $1.4 \mu\text{M}$  vs.  $7.9 \mu\text{M}$  for the reference compound),  
 353 even if **5g.HCl** was less hydrophobic than compound **1.HCl**, as indicated by its lower  $\text{LogD}_{7.4}$   
 354 ( $3.16$  vs.  $4.10$ ). Permeability at different pH was then further evaluated to test the ability of  
 355 thieno[3,2-*d*]pyrimidinones to cross membrane barriers to be readily distributed. While no  
 356 value could be measured at pH 6.0 because compound **5g** precipitated in these conditions, this  
 357 compound diffused at pH 5.0 and 7.4, with an effective permeability coefficient of 180.5 and  
 358  $43.4 \text{ nm/s}$ , respectively, better values than those obtained with the reference compound **1**  
 359 (Table 4). Finally, the affinity for hERG potassium channels was determined to assess the  
 360 cardiac toxicity of these thienopyrimidinones. Gamhepathiopine **1.HCl**, previously studied, did  
 361 not show affinity for hERG channels. Similarly, its aminocyclobutyl analog **5g.HCl** did not cause  
 362 significant inhibition of these channels ( $IC_{50} > 100 \mu\text{M}$ , Table 5).

363 **Table 3.** Microsomal stability in presence of female mouse liver microsomes of selected compounds.

Compound	Microsomal stability	
	$T_{1/2}$ (min)	$Cl_{int}$ ( $\mu\text{L}/\text{min}/\text{mg}$ )
<b>1</b>	10[8]	240[14]
<b>11</b>	11	214
<b>5b</b>	11	214
<b>5c</b>	8.5	209
<b>5f</b>	15	136
<b>5g</b>	29	78
<b>5h</b>	38	58

364

365 **Table 4.** Physicochemical profile, permeability in the PAMPA gastrointestinal assay and hERG affinity of  
 366 selected thieno[3,2-*d*]pyrimidines.

Cpd	Solubility <sup>a</sup> ( $\mu\text{M}$ )	CHI Log D <sup>b</sup>	Pe (nm/s) <sup>c</sup>			hERG $IC_{50}$ ( $\mu\text{M}$ )
			pH			
			5.0	6.0	7.4	
<b>1.HCl</b>	$7.9 \pm 0.8$ [13]	$4.10$ [14]	0[13]	0[13]	$4.6 \pm 7.2$ [13]	> 100
<b>5g.HCl</b>	$1.4 \pm 0.9$	3.16	$180.5 \pm 61.8$	ND <sup>d</sup>	$43.6 \pm 17.8$	> 100

367 <sup>a</sup> Solubility was determined in phosphate-buffered saline. Values are the mean  $\pm$  SD of three independent  
368 experiments.  
369 <sup>b</sup> CHI: Chromatographic hydrophobicity index at pH = 7.4.  
370 <sup>c</sup> Determined using a parallel artificial membrane permeability of gastrointestinal tract (PAMPA-GIT) assay.  
371 <sup>d</sup> Not determined due to a too low solubility of the tested compound.  
372  
373

### 374 3. Conclusions

375 In conclusion, this study aimed to explore different substitutions at positions 2 and 3 of the  
376 thienopyrimidinone scaffold, to improve the understanding of the antiplasmodial SAR of this  
377 compound series and to enhance its metabolic stability. While substitutions at position 3 led  
378 to inactive compounds, indicating the essential role of an NH group at this position, several  
379 modifications at position 2 were found to be tolerated. The introduction of deuterated  
380 moieties did not alter activity against the erythrocytic stage of *P. falciparum* and has even  
381 slightly increased activity against the liver stage of *P. berghei*, resulting in active compounds  
382 **5b** and **5c**, with favourable selectivity indices. On the other hand, the introduction of a fluorine  
383 atom had a detrimental effect, while the introduction of cyclic groups, such as  
384 aminocyclopropyl, aminocyclobutyl, and aminocyclopentyl moieties, resulted in compounds  
385 **5f**, **5g**, and **5h** with activity against *P. falciparum* K1 in the micromolar range. The stability of  
386 the compounds in the presence of microsomes revealed that deuterated analogues did not  
387 exhibit improved metabolic stability, while the introduction of carbocycles increased the  
388 metabolic stability, compared to Gamhepathiopine **1**. Notably, compound **5g** displayed a 3-  
389 fold increased half-life compared to **1**, a promising activity against both the erythrocytic and  
390 hepatic stages of *Plasmodium*, and no affinity for hERG cardiac channels. Further studies and  
391 evaluations are warranted to explore its potential as a candidate for antiparasitic drug  
392 development.

393

### 394 4. Experimental part

#### 395 4.1. Chemistry

##### 396 4.1.1. Material and Methods

397 Commercial reagents and solvents were used without further purification. The synthesized  
398 products were purified by chromatography on silica gel (40–63  $\mu$ m) or by preparative HPLC on  
399 an apparatus equipped with a Delta Pack C18 radial compression column (100 mm x 40 mm,  
400 15  $\mu$ m, 100 Å) at a wavelength of 214 nm, at a flow rate of 28 mL/min with a variable elution  
401 gradient from X % A (H<sub>2</sub>O + 0.1% TFA) to X + 30% B (ACN + 0.1% TFA) in 30 min. The purest  
402 fractions are pooled, freeze-dried, and lyophilized to give the final compounds. LC/MS analyses

403 were recorded on a Quattro microTMESI triple quadrupole mass spectrometer (ESI+  
404 electrospray ionization mode) or on a Micromass ZQ spectrometer (ESI+ electrospray  
405 ionization mode), coupled to an Alliance HPLC system (Waters, Milford, CT, USA) equipped  
406 with a Chromolith High Resolution RP-18e column (25 x 4.6mm), with the samples being  
407 previously separated using a gradient from 100% (H<sub>2</sub>O + 0.1% HCO<sub>2</sub>H) to 100% (ACN + 0.1%  
408 HCO<sub>2</sub>H) in 3 min at a flow rate of 3 mL/min and with UV detection at 214 nm. UPLC/MS analyses  
409 were recorded with an Acquity H-Class UPLC system, coupled to a Waters SQ Detector-2 mass  
410 spectrometer (Waters, Milford, CT, USA). Chromatographic separation was carried out under  
411 the same conditions as above using a Waters Acquity UPLC BEC C18 column (100 x 2.1 mm, 1.5  
412 μm). High-resolution mass spectrometry (HRMS) analyses were performed with a time-of-  
413 flight (TOF) spectrometer coupled to a positive electrospray ionization (ESI) source. The  
414 elemental analysis was realized with a Elementar Vario Micro Cube. The NMR spectra were  
415 recorded on a Brüker 400, 500, or 600 spectrometers. Chemical shifts are expressed in parts  
416 per million (ppm) relative to the residual signal of the deuterated solvent used (CDCl<sub>3</sub>, DMSO-  
417 d<sub>6</sub>, MeOH-d<sub>3</sub>, ACN-d<sub>3</sub>), and the coupling constants J are expressed in Hertz. The multiplicities  
418 are designated as singlet (s), broad singlet (bs) doublet (d), doublet of doublet (dd), triplet (t),  
419 quadruplet (q), quintuplet (qt), sextuplet (st), or multiplet (m). Compounds **1**, **2** and **11** were  
420 synthesized according to the previous reported procedure and its physical characteristics  
421 agreed with the published data.[9]

422

#### 423 4.1.2. General Procedure for the Synthesis of 2-Substituted-thieno[3,2-*d*]pyrimidines **5a-** 424 **p.**

425 To a solution of aminothiophene **2** in DMF was added ethoxycarbonyl isothiocyanate (1 eq.).  
426 The solution was stirring at 30°C for 2h. During this reaction, the thiourea carbamate  
427 intermediate **3** was formed and was not isolated. Then, triethylamine (3 eq.), the appropriate  
428 amine (2 eq.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDCI, HCl)  
429 (1.2 eq.) were added. The reaction mixture was stirred overnight at room temperature to  
430 generate the guanidine intermediate **4**. The solution was then heated under reflux (170°C) for  
431 1 to 2h. The solvent was removed under reduced pressure and the crude was purified by the  
432 appropriate method.

433

434 4.1.2.1. 2-Methylamino-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5a**). Following the  
435 general procedure, using **2** (500 mg, 2.022 mmol), ethoxycarbonyl isothiocyanate (265 mg,



436 2.022 mmol, 0.24 mL), triethylamine (614 mg, 6.065 mmol, 0.84 mL), methylamine (314 mg,  
437 4.044 mmol, 0.35 mL), EDCI.HCl (465 mg, 2.426 mmol). After addition of water in the reaction  
438 mixture, the obtained precipitate was filtered and washed with Et<sub>2</sub>O. The desired compound  
439 was obtained as a green powder (422 mg, 77%). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 7.67 (d, 2H,  
440 CH<sub>ar</sub>, J = 8.1 Hz), 7.35 (s, 1H H-thiophene), 7.27 (d, 2H, CH<sub>ar</sub>, J = 8.0 Hz), 7.03 (s, 2H, NH), 3.36  
441 (s, 3H, CH<sub>3</sub>N), 2.34 (s, 3H, CH<sub>3</sub>); <sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 158.7, 157.4, 155.0, 150.4,  
442 139.1, 130.3, 129.8, 125.8, 118.9, 110.1, 28.2, 20.9; LC/MS: tr-LC/MS = 1.18 min, [M+H]<sup>+</sup> =  
443 272.0; HR-MS (ESI) C<sub>14</sub>H<sub>14</sub>N<sub>3</sub>OS calculated: 272.0852 [M+H]<sup>+</sup>, found: 272.0860.

444  
445 4.1.2.2. 2-(Isopropylamino-*d*<sub>6</sub>)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5b**). Following the  
446 general procedure, using **2** (400 mg, 1.617 mmol), ethoxycarbonyl isothiocyanate (212 mg,  
447 1.617 mmol, 0.19 mL), triethylamine (491 mg, 4.852 mmol, 0.67 mL), isopropylamine-*d*<sub>6</sub> (211  
448 mg, 3.235 mmol, 0.28 mL), EDCI.HCl (372 mg, 1.941 mmol). After addition of water in the  
449 reaction mixture, the obtained precipitate was filtered, washed with Et<sub>2</sub>O and recrystallized  
450 with ACN. The desired compound was obtained as an off-white powder (323 mg, 65%). <sup>1</sup>H NMR  
451 (500 MHz, DMSO-*d*<sub>6</sub>): δ 10.60 (s, 1H, NH), 7.66 (d, 2H, CH<sub>ar</sub>, J = 8.1 Hz), 7.47 (s, 1H, H-  
452 thiophene), 7.27 (d, 2H, CH<sub>ar</sub>, J = 8.0 Hz), 6.11 (d, 1H, NHCH, J = 7.6 Hz), 3.99 (d, 1H, CHNH, J =  
453 7.5 Hz), 2.33 (s, 3H, CH<sub>3</sub>-tolyl); <sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 160.5, 157.6, 152.7, 150.2,  
454 139.0, 130.3, 129.7, 125.7, 119.7, 111.0 41.8, 20.8; LC/MS: tr-LC/MS = 1.60 min, [M+H]<sup>+</sup> =  
455 306.2; HR-MS (ESI) C<sub>16</sub>H<sub>12</sub>D<sub>6</sub>N<sub>3</sub>OS calculated: 306.1542 [M+H]<sup>+</sup>, found: 306.1557.

456  
457 4.1.2.3. 2-(*Tert*-butylamino-*d*<sub>9</sub>)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5c**). Following the  
458 general procedure, using **2** (250 mg, 1.011 mmol), ethoxycarbonyl isothiocyanate (133 mg,  
459 1.011 mmol, 0.12 mL), triethylamine (307 mg, 3.033 mmol, 0.42 mL), *tert*-butylamine-*d*<sub>9</sub> (166  
460 mg, 2.022 mmol, 0.18 mL), EDCI.HCl (233 mg, 1.213 mmol). After addition of water in the  
461 reaction mixture, the obtained precipitate was filtered, washed with Et<sub>2</sub>O and recrystallized  
462 with ACN. The desired compound was obtained as a white powder (200 mg, 61%). <sup>1</sup>H NMR  
463 (500 MHz, DMSO-*d*<sub>6</sub>): δ 10.46 (s, 1H, NH), 7.68 (d, 2H, CH<sub>ar</sub>, J = 7.0 Hz), 7.46 (s, 1H, H-  
464 thiophene), 7.26 (d, 2H, CH<sub>ar</sub>, J = 7.3 Hz), 6.07 (s, 1H, NH), 2.33 (s, 3H, CH<sub>3</sub>-tolyl); <sup>13</sup>C NMR (500  
465 MHz, DMSO-*d*<sub>6</sub>): δ 160.3, 157.6, 152.3, 150.2, 139.0, 130.3, 129.8, 125.8, 120.0, 111.0, 50.5,  
466 20.9; LC/MS: tr-LC/MS = 1.80 min, [M+H]<sup>+</sup> = 323.2; HR-MS (ESI) C<sub>17</sub>H<sub>11</sub>D<sub>9</sub>N<sub>3</sub>OS calculated:  
467 323.1887 [M+H]<sup>+</sup>, found: 323.1898.

468

469 4.1.2.4. 2-(1,1,1-Trifluoro-2-methylpropan-2-yl)amino)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-  
470 4(3*H*)-one (**5d**). Following the general procedure, using **2** (300 mg, 1.213 mmol),  
471 ethoxycarbonyl isothiocyanate (164 mg, 1.213 mmol, 0.15 mL), triethylamine (368 mg, 3.639  
472 mmol, 0.51 mL), 2,2,2-trifluoro-1,1-dimethyl-ethylamine (315 mg, 2.426 mmol, 0.28 mL),  
473 EDCI.HCl (279 mg, 1.456 mmol). The crude was partitioned in water and DCM. Organic layer  
474 was washed with brine, dried with MgSO<sub>4</sub>, filtered and concentrated under reduce pressure.  
475 The crude was purified by chromatography on silica gel (eluent: 99.5/0.5 DCM/MeOH to  
476 97/03). The solid collected was recrystallized with EtOH to afford the desired compound as an  
477 off-white solid (213 mg, 48%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 10.62 (s, 1H, NH), 7.69 (d, 2H,  
478 CH<sub>ar</sub>, *J* = 8.2 Hz), 7.49 (s, 1H, H-thiophene), 7.28 (d, 2H, CH<sub>ar</sub>, *J* = 8.0 Hz), 6.56 (s, 1H, NH), 2.34  
479 (s, 3H, CH<sub>3</sub>-tolyl), 1.68 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>); <sup>13</sup>C NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 159.4, 157.2, 151.6,  
480 150.6, 139.2, 130.1, 129.8, 127.0 (q, 1J<sub>CF</sub> = 285.9) Hz), 125.8, 120.0, 112.5, 56.8 (q, 2J<sub>CF</sub> = 27.6  
481 Hz), 21.0, 20.9; <sup>19</sup>F NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ -79.35; LC/MS: tr-LC/MS = 2.07 min, [M+H]<sup>+</sup>  
482 = 368.2; HR-MS (ESI) C<sub>17</sub>H<sub>17</sub>F<sub>3</sub>N<sub>3</sub>OS calculated: 368.1039 [M+H]<sup>+</sup>, found: 368.1037.

483  
484 4.1.2.5. 2-((3-Dimethylamino)propyl)imino)-6-(*p*-tolyl)-2,3-dihydrothieno[3,2-*d*]pyrimidin-  
485 4(3*H*)-one (**5e**). Following the general procedure, using **2** (250 mg, 1.011 mmol),  
486 ethoxycarbonyl isothiocyanate (133 mg, 1.011 mmol, 0.12 mL), triethylamine (307 mg, 3.033  
487 mmol, 0.42 mL), 3-dimethylaminopropylamine (207 mg, 2.022 mmol, 0.26 mL), EDCI.HCl (233  
488 mg, 1.213 mmol). After addition of water in the reaction mixture, the obtained precipitate was  
489 filtered, washed with ACN. The desired compound was obtained off-white solid (206 mg, 60%)  
490 ; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 7.66 (d, 2H, CH<sub>ar</sub>, *J* = 8.1 Hz), 7.34 (s, 1H, H-thiophene), 7.29-  
491 7.26 (m, 4H, CH<sub>ar</sub> + NH), 3.96-3.93 (m, 2H, CH<sub>2</sub>N), 2.34 (s, 3H, CH<sub>3</sub>-tolyl), 2.27-2.24 (m, 2H,  
492 CH<sub>2</sub>N), 2.17 (s, 6H, (CH<sub>3</sub>)<sub>2</sub>), 1.81-1.76 (m, 2H, CH<sub>2</sub>); <sup>13</sup>C NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ 158.9,  
493 157.6, 155.1, 150.4, 139.1, 130.3, 129.8, 125.9, 119.0, 110.4, 55.1, 44.6, 39.3, 25.1, 20.9; HR-  
494 MS (ESI) C<sub>18</sub>H<sub>22</sub>N<sub>4</sub>OS calculated: 343.1601 [M+H]<sup>+</sup>, found: 343.1594.

495  
496 4.1.2.6. 2-(Cyclopropylamino)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5f**). Following the  
497 general procedure, using **2** (300 mg, 1.213 mmol), ethoxycarbonyl isothiocyanate (164 mg,  
498 1.213 mmol, 0.15 mL), triethylamine (368 mg, 3.639 mmol, 0.51 mL), cyclopropylamine (139  
499 mg, 2.426 mmol, 0.17 mL), EDCI.HCl (279 mg, 1.456 mmol). After addition of water in the  
500 reaction mixture, the obtained precipitate was filtered, washed with Et<sub>2</sub>O to afford a green  
501 powder (229 mg, 63%); <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>, T = 373 K): δ 7.65 (d, 2H, CH<sub>ar</sub>, *J* = 7.8 Hz),

502 7.39 (s, 1H, H-thiophene), 7.28 (d, 2H, CH<sub>ar</sub>, *J* = 7.6 Hz), 6.60 (s, 1H, NH), 2.76-2.75 (m, 1H, CH),  
503 2.34 (s, 3H, CH<sub>3</sub>-tolyl), 0.77-0.76 (m, 2H, CH<sub>2</sub>), 0.55-0.52 (m, 2H, CH<sub>2</sub>); <sup>13</sup>C NMR (500 MHz,  
504 DMSO-d<sub>6</sub>, T = 373 K): δ 159.4, 157.2, 154.0, 150.0, 138.5, 130.0, 129.2, 125.4, 119.0, 111.5,  
505 22.9, 20.2, 6.2; UPLC/MS: t<sub>r</sub> = 2.76 min, [M+H]<sup>+</sup> = 298.1; HR-MS (ESI) C<sub>16</sub>H<sub>16</sub>N<sub>3</sub>OS calculated:  
506 298.1009 [M+H]<sup>+</sup>, found: 298.1021.

507  
508 4.1.2.7. 2-(Cyclobutylamino)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5g**). Following the  
509 general procedure, using **2** (300 mg, 1.213 mmol), ethoxycarbonyl isothiocyanate (164 mg,  
510 1.213 mmol, 0.15 mL), triethylamine (368 mg, 3.639 mmol, 0.51 mL), cyclobutylamine (173  
511 mg, 2.426 mmol, 0.21 mL), EDCI.HCl (279 mg, 1.456 mmol). After addition of water in the  
512 reaction mixture, the obtained precipitate was filtered, washed with Et<sub>2</sub>O and recrystallized  
513 with ACN. A white powder was obtained (150 mg, 40%); <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>): δ 10.72  
514 (s, 1H, NH), 7.66 (d, 2H, CH<sub>ar</sub>, *J* = 8.0 Hz), 7.45 (s, 1H, H-thiophene), 7.26 (d, 2H, CH<sub>ar</sub>, *J* = 7.9 Hz),  
515 6.58 (s, 1H, NH), 4.36-4.29 (m, 1H, CHNH), 2.33 (s, 3H, CH<sub>3</sub>-tolyl), 2.30-2.26 (m, 2H, CH<sub>2</sub>), 1.94-  
516 1.86 (m, 2H, CH<sub>2</sub>), 1.70-1.62 (m, 2H, CH<sub>2</sub>); <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>): δ 160.4, 157.7, 152.4,  
517 150.3, 139.1, 130.3, 129.8, 125.8, 119.6, 111.3, 45.5, 30.6, 20.9, 14.7; LC/MS: t<sub>r</sub> = 1.65 min,  
518 [M+H]<sup>+</sup> = 312.1; HR-MS (ESI) C<sub>17</sub>H<sub>18</sub>N<sub>3</sub>OS calculated: 312.1165 [M+H]<sup>+</sup>, found: 312.1182.

519  
520 4.1.2.8. 2-(Cyclopentylamino)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5h**). Following the  
521 general procedure, using **2** (300 mg, 1.213 mmol), ethoxycarbonyl isothiocyanate (164 mg,  
522 1.213 mmol, 0.15 mL), triethylamine (368 mg, 3.639 mmol, 0.51 mL), cyclopentylamine (207  
523 mg, 2.426 mmol, 0.24 mL), EDCI.HCl (279 mg, 1.456 mmol). After addition of water in the  
524 reaction mixture, the obtained precipitate was filtered and washed with Et<sub>2</sub>O. An off-white  
525 powder was obtained (224 mg, 57%); <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 10.53 (s, 1H, NH), 7.67  
526 (d, 2H, CH<sub>ar</sub>, *J* = 8.2 Hz), 7.47 (s, 1H, H-thiophene), 7.27 (d, 2H, CH<sub>ar</sub>, *J* = 8.2 Hz), 6.30 (d, 1H,  
527 NHCH, *J* = 7.0 Hz), 4.15 (sx, 1H, CH, *J* = 6.6 Hz), 2.34 (s, 3H, CH<sub>3</sub>-tolyl), 1.98-1.90 (m, 2H, CH<sub>2</sub>),  
528 1.70-1.52 (m, 4H, CH<sub>2</sub>), 1.48-1.41 (m, 2H, CH<sub>2</sub>); <sup>13</sup>C NMR (400 MHz, DMSO-d<sub>6</sub>): δ 160.5, 157.5,  
529 153.0, 150.2, 139.0, 130.2, 129.7, 125.7, 119.7, 111.0, 52.0, 32.5, 23.1, 20.8; LC/MS: t<sub>r</sub> = 1.75  
530 min, [M+H]<sup>+</sup> = 326.1; HR-MS (ESI) C<sub>18</sub>H<sub>20</sub>N<sub>3</sub>OS calculated: 326.1322 [M+H]<sup>+</sup>, found: 326.1334.

531  
532 4.1.2.9. 2-(Cyclohexylamino)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5h**). Following the  
533 general procedure, using **2** (300 mg, 1.213 mmol), ethoxycarbonyl isothiocyanate (164 mg,  
534 1.213 mmol, 0.15 mL), triethylamine (368 mg, 3.639 mmol, 0.51 mL), cyclohexylamine (241

535 mg, 2.426 mmol, 0.28 mL), EDCI.HCl (279 mg, 1.456 mmol). After addition of water in the  
536 reaction mixture, the obtained precipitate was filtered and washed with Et<sub>2</sub>O. The crude was  
537 then purified by chromatography on silica (eluent: 98/02 DCM/MeOH to 95/05). The desired  
538 compound was a white solid (326 mg, 79%); <sup>1</sup>H NMR (400 MHz, DMSO-d<sub>6</sub>): δ 10.56 (s, 1H, NH),  
539 7.67 (d, 2H, CH<sub>ar</sub>, *J* = 8.1 Hz), 7.48 (s, 1H, H-thiophene), 7.27 (d, 2H, CH<sub>ar</sub>, *J* = 8.0 Hz), 6.20 (d,  
540 1H, NHCH, *J* = 6.9 Hz), 3.77-3.72 (m, 1H, CH), 2.34 (s, 3H, CH<sub>3</sub>-tolyl), 1.93-1.90 (m, 2H, CH<sub>2</sub>),  
541 1.69-1.67 (m, 2H, CH<sub>2</sub>), 1.57-1.55 (m, 1H, CH<sub>2</sub>), 1.38-1.31 (m, 2H, CH<sub>2</sub>), 1.27-1.21 (m, 3H, CH<sub>2</sub>);  
542 <sup>13</sup>C NMR (400 MHz, DMSO-d<sub>6</sub>): δ 160.5, 157.6, 152.6, 150.2, 139.0, 130.3, 129.7, 125.7, 119.7,  
543 110.9, 48.6, 32.3, 25.2, 24.2, 20.8; UPLC/MS: t<sub>r</sub> = 3.66 min, [M+H]<sup>+</sup> = 340.1; HR-MS (ESI) C<sub>19</sub>H<sub>22</sub>  
544 N<sub>3</sub>OS calculated: 340.1478 [M+H]<sup>+</sup>, found: 340.1495.

545  
546 4.1.2.10. 2-(Azetidin-1-yl)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5j**). Following the  
547 general procedure, using **2** (1 g, 4.044 mmol), ethoxycarbonyl isothiocyanate (0.530 g, 4.044  
548 mmol, 0.48 mL), triethylamine (1.228 g, 12.13 mmol, 1.69 mL), azetidine (0.462 g, 8.087 mmol,  
549 0.54 mL), EDCI.HCl (0.930 g, 4.852 mmol). After addition of water in the reaction mixture, the  
550 obtained precipitate was filtered, washed with Et<sub>2</sub>O and recrystallized with ACN. The desired  
551 compound was obtained as a green powder (0.473 g, 39%); <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>, T =  
552 373 K) δ 11.04 (s, 1H, NH), 7.63 (d, 2H, CH<sub>ar</sub>, *J* = 8.2 Hz), 7.36 (s, 1H, H-thiophene), 7.28 (d, 2H,  
553 CH<sub>ar</sub>, *J* = 7.9 Hz), 4.11-4.08 (m, 4H, CH<sub>2</sub>N), 2.36 (s, 3H, CH<sub>3</sub>-tolyl), 2.34-2.28 (m, 2H, CH<sub>2</sub>); <sup>13</sup>C  
554 NMR (500 MHz, DMSO-d<sub>6</sub>, T = 373 K): δ 159.5, 157.9, 154.4, 150.0, 138.5, 130.1, 129.2, 125.4,  
555 119.0, 111.2, 50.3, 20.2, 15.4; LC/MS: t<sub>r</sub> = 1.19 min, [M+H]<sup>+</sup> = 298.0; HR-MS (ESI) C<sub>16</sub>H<sub>16</sub>N<sub>3</sub>OS  
556 calculated: 298.1 [M+H]<sup>+</sup>, found: 298.1.

557  
558 4.1.2.12. 2-(Pyrrolidin-1-yl)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5k**). Following the  
559 general procedure, using **2** (1 g, 4.044 mmol), ethoxycarbonyl isothiocyanate (0.530 g, 4.044  
560 mmol, 0.48 mL), triethylamine (1.228 g, 12.13 mmol, 1.69 mL), pyrrolidine (0.575 g, 8.087  
561 mmol, 0.66 mL), EDCI.HCl (0.930 g, 4.852 mmol). After addition of water in the reaction  
562 mixture, the obtained precipitate was filtered, washed with ACN. The desired compound was  
563 obtained as a green powder (0.732 g, 58%); <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>): δ 10.60 (s, 1H, NH),  
564 7.63 (d, 2H, CH<sub>ar</sub>, *J* = 8.2 Hz), 7.33 (s, 1H, H-thiophene), 7.28 (d, 2H, CH<sub>ar</sub>, *J* = 8.0 Hz), 3.53-3.50  
565 (m, 4H, CH<sub>2</sub>NH), 2.36 (s, 3H, CH<sub>3</sub>-tolyl), 1.96-1.93 (m, 4H, CH<sub>2</sub>); <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>):  
566 δ 160.1, 158.1, 151.8, 149.9, 138.4, 130.1, 129.2, 125.3, 119.0, 110.2, 46.3, 24.2, 20.2; LC/MS:

567  $t_r = 1.23$  min,  $[M+H]^+ = 312.1$ ; HR-MS (ESI)  $C_{17}H_{18}N_3OS$  calculated: 312.1171  $[M+H]^+$ , found:  
568 312.1169.

569

570 4.1.2.13. 2-(Piperidin-1-yl)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5l**). Following the  
571 general procedure, using **2** (1 g, 4.044 mmol), ethoxycarbonyl isothiocyanate (0.530 g, 4.044  
572 mmol, 0.48 mL), triethylamine (1.228 g, 12.13 mmol, 1.69 mL), piperidine (0.689 g, 8.087  
573 mmol, 0.799 mL), EDCI.HCl (0.930 g, 4.852 mmol). After addition of water in the reaction  
574 mixture, the obtained precipitate was filtered, washed with  $Et_2O$  to afford a green powder  
575 (0.743 g, 56%);  $^1H$  NMR (500 MHz,  $DMSO-d_6$ ,  $T = 373$  K):  $\delta$  10.91 (s, 1H, NH), 7.64 (d, 2H,  $CH_{ar}$ ,  
576  $J = 8.0$  Hz), 7.35 (s, 1H, H-thiophene), 7.28 (d, 2H,  $CH_{ar}$ ,  $J = 8.0$  Hz), 3.62-3.60 (m, 4H,  $CH_2$ ), 2.36  
577 (s, 3H,  $CH_3$ -tolyl), 1.64-1.32 (m, 2H,  $CH_2$ ), 1.59-1.56 (m, 4H,  $CH_2$ );  $^{13}C$  NMR (500 MHz,  $DMSO-d_6$ ,  
578  $T = 373$  K):  $\delta$  159.8, 158.2, 153.3, 150.1, 138.5, 130.1, 129.2, 125.4, 119.2, 110.6, 45.6, 24.6,  
579 23.4, 20.2; LC/MS:  $t_r = 1.77$  min,  $[M+H]^+ = 326.1$ ; HR-MS (ESI)  $C_{18}H_{20}N_3OS$  calculated: 326.1327  
580  $[M+H]^+$ , found: 326.1324.

581

582 4.1.2.14. 2-Morpholino-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5m**). Following the  
583 general procedure, using **2** (250 mg, 1.011 mmol), ethoxycarbonyl isothiocyanate (133 mg,  
584 1.011 mmol, 0.12 mL), triethylamine (307 mg, 3.033 mmol, 0.42 mL), morpholine (176 mg,  
585 2.022 mmol, 0.18 mL), EDCI.HCl (233 mg, 1.213 mmol). After addition of water in the reaction  
586 mixture, the obtained precipitate was filtered, washed with  $Et_2O$ . The desired compound was  
587 obtained as a green powder (242 mg, 73%);  $^1H$  NMR (500 MHz,  $DMSO-d_6$ ,  $T = 373$  K):  $\delta$  11.07  
588 (s, 1H, NH), 7.65 (d, 2H,  $CH_{ar}$ ,  $J = 8.0$  Hz), 7.38 (s, 1H, H-thiophene), 7.29 (d, 2H,  $CH_{ar}$ ,  $J = 8.0$  Hz),  
589 3.70-3.68 (m, 4H,  $CH_2N$ ), 3.61-3.60 (m, 4H,  $CH_2O$ ), 2.36 (s, 3H,  $CH_3$ -tolyl);  $^{13}C$  NMR (500 MHz,  
590  $DMSO-d_6$ ,  $T = 373$  K):  $\delta$  159.0, 158.2, 153.6, 150.3, 138.6, 130.0, 129.3, 125.4, 119.1, 111.6  
591 65.2, 45.2, 20.3; LC/MS:  $t_r = 1.39$  min,  $[M+H]^+ = 328.2$ ; HR-MS (ESI)  $C_{17}H_{18}N_3O_2S$  calculated:  
592 328.1114  $[M+H]^+$ , found: 328.1119.

593

594 4.1.2.15. 2-(4-Methylpiperazin-1-yl)-6-(*p*-tolyl)-thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**5n**).  
595 Following the general procedure, using **2** (250 mg, 1.011 mmol), ethoxycarbonyl  
596 isothiocyanate (133 mg, 1.011 mmol, 0.12 mL), triethylamine (307 mg, 3.033 mmol, 0.42 mL),  
597 1-methylpiperazine (203 mg, 2.022 mmol, 0.22 mL), EDCI.HCl (233 mg, 1.213 mmol). After  
598 addition of water in the reaction mixture, the obtained precipitate was filtered, washed with  
599  $Et_2O$ . The desired compound was obtained as a yellow powder (210 mg, 61%);  $^1H$  NMR (500

600 MHz, DMSO-d<sub>6</sub>): δ 11.38 (s, 1H, NH), 7.67 (d, 2H, CH<sub>ar</sub>, J = 8.1 Hz), 7.48 (s, 1H, H-thiophene),  
601 7.28 (d, 2H, CH<sub>ar</sub>, J = 7.9 Hz), 3.59-3.57 (m, 4H, CH<sub>2</sub>N), 2.37-2.35 (m, 4H, CH<sub>2</sub>NCH<sub>3</sub>), 2.34 (s, 3H,  
602 CH<sub>3</sub>-tolyl), 2.20 (s, 3H, CH<sub>3</sub>N); <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>): δ 159.9, 158.8, 153.8, 150.6,  
603 139.2, 130.2, 129.8, 125.9, 119.6, 111.5, 54.1, 45.7, 45.0, 20.9; UPLC/MS: t<sub>r</sub> = 2.21 min, [M+H]<sup>+</sup>  
604 = 341.2; HR-MS (ESI) C<sub>18</sub>H<sub>21</sub>N<sub>4</sub>OS calculated: 341.1436 [M+H]<sup>+</sup>, found: 341.1438.

605

606 4.1.2.16. *Tert*-butyl 3-((4-oxo-6-(*p*-tolyl)-3,4-dihydrothieno[3,2-*d*]pyrimidin-2(1H)-  
607 ylidene)amino)azetidone-1-carboxylate (**5o**). Following the general procedure, using **2** (600 mg,  
608 2.426 mmol), ethoxycarbonyl isothiocyanate (321 mg, 2.426 mmol, 0.29 mL), triethylamine  
609 (736.5 mg, 7.278 mmol, 1.01 mL), *tert*-butyl 3-aminoazetidone-1-carboxylate (880 mg, 4.852  
610 mmol, 0.80 mL), EDCI.HCl (558 mg, 2.911 mmol). After addition of water in the reaction  
611 mixture, the obtained precipitate was filtered and purified by chromatography on alumina  
612 (eluent: 99.5/0.5 DCM/MeOH to 95/05). The solid collected was a white solid (351 mg, 35%);  
613 <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>): δ 7.67 (d, 2H, CH<sub>ar</sub>, J = 8.1 Hz), 7.35 (s, 1H, H-thiophene), 7.28  
614 (d, 2H, CH<sub>ar</sub>, J = 7.9 Hz), 6.88 (s, 2H, NH), 4.94 (qt, 1H, CH, J = 7.4 Hz), 4.33-4.32 (s, 2H, CH<sub>2</sub>),  
615 4.14 (t, 2H, CH<sub>2</sub>, J = 8.1 Hz), 2.34 (s, 3H, CH<sub>3</sub>-tolyl), 1.39 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>); <sup>13</sup>C NMR (400 MHz,  
616 DMSO-d<sub>6</sub>): δ 158.7, 158.2, 156.0, 154.6, 150.7, 139.2, 130.2, 129.8, 125.9, 118.8, 111.5, 78.8,  
617 55.0, 54.0, 53.4, 44.7, 28.1, 20.9; LC/MS: t<sub>r</sub> = 3.48 min, [M+H]<sup>+</sup> = 413.2; HR-MS (ESI) C<sub>21</sub>H<sub>25</sub>N<sub>4</sub>O<sub>3</sub>S  
618 calculated: 413.1642 [M+H]<sup>+</sup>, found: 413.1644.

619

620 4.1.2.17. (*S*) *Tert*-butyl 3-((4-oxo-6-(*p*-tolyl)-3,4-dihydrothieno[3,2-*d*]pyrimidin-2(1H)-  
621 ylidene)amino)-pyrrolidine-1-carboxylate (**5p**). Following the general procedure, using **2** (331  
622 mg, 1.34 mmol), ethoxycarbonyl isothiocyanate (176 mg, 1.34 mmol, 0.16 mL), triethylamine  
623 (407 mg, 4.02 mmol, 0.56 mL), (*S*)-*tert*-butyl 3-aminopyrrolidine-1-carboxylate (500 mg, 2.68  
624 mmol, 0.47 mL), EDCI.HCl (282 mg, 1.47 mmol). After addition of water in the reaction mixture,  
625 the obtained precipitate was filtered and purified by chromatography on alumina (eluent:  
626 99/01 DCM/MeOH to 96/04). The solid collected was a white solid (282 mg, 49%); <sup>1</sup>H NMR (400  
627 MHz, DMSO-d<sub>6</sub>): δ 10.67 (bs, 1H, NH), 7.68 (d, 2H, CH<sub>ar</sub>, J = 8.2 Hz), 7.51 (s, 1H, H-thiophène),  
628 7.27 (d, 2H, CH<sub>ar</sub>, J = 8.0 Hz), 6.66 (bs, 1H, NH), 4.38-4.32 (m, 1H, CH), 3.57-3.54 (m, 1H, CH<sub>2</sub>N),  
629 3.34-3.33 (m, 2H, CH<sub>2</sub>N), 3.20-3.16 (m, 1H, CH<sub>2</sub>N), 2.34 (s, CH<sub>3</sub>, 3H), 2.18-2.08 (m, 1H, CH), 1.92-  
630 1.84 (m, 1H, CH), 1.40-1.39 (m, 9H, (CH<sub>3</sub>)<sub>3</sub>); <sup>13</sup>C NMR (400 MHz, DMSO-d<sub>6</sub>): δ 160.2, 157.7,  
631 153.6, 153.0, 150.4, 139.1, 130.2, 129.8, 125.8, 119.7, 111.7, 78.5, 51.0 (d, J = 124.8 Hz), 50.4

632 (d,  $J = 176.2$  Hz), 43.8 (d,  $J = 20.2$  Hz), 30.6 (d,  $J = 104.1$  Hz), 28.2, 20.9; LC/MS :  $t_r = 1.87$  min,  
633  $[M+H]^+ = 427.4$ ; HR-MS (ESI)  $C_{22}H_{27}N_4O_3S$  calculated: 427.1806  $[M+H]^+$ , found: 427.1798.

634

#### 635 4.1.3. Synthesis of Compounds **6a** and **6b**

636 Compound **5o** (0.80 mmol) or **5p** (0.16 mmol) was dissolved in a 4/1 v/v solution of dioxane  
637 and 37% aqueous HCl. The reaction mixture was stirred at room temperature for 1h. The  
638 reaction was concentrated under reduce pressure. The solid was dissolved in ACN and water  
639 and freeze-dried. An off-white powder was obtained (quantitative yield).

640

641 4.1.3.1. 2-(Azetidin-3-ylamino)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one dihydrochloride  
642 (**6a**).  $^1H$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  9.59-9.58 (m, 1H, NH), 9.05 (br, 1H, NH), 8.10 (br, 1H, NH),  
643 7.73-7.72 (m, 2H,  $CH_{ar}$ ), 7.50 (s, 1H, H-thiophene), 7.30-7.31 (m, 2H,  $CH_{ar}$ ), 5.28-5.22 (m, 1H,  
644 CH), 4.42-4.49 (m, 2H,  $CH_2$ ), 4.31-4.32 (m, 2H,  $CH_2$ ), 2.34 (s, 3H,  $CH_3$ -tolyl).  $^{13}C$  NMR (400 MHz,  
645 DMSO- $d_6$ ):  $\delta$  157.2, 156.3, 152.8, 151.4, 139.6, 130.0, 129.8, 125.9, 116.1, 111.6, 49.6, 47.1,  
646 20.9. LC/MS :  $t_r = 1.23$  min,  $[M+H]^+ = 313.0$ ; HR-MS (ESI)  $C_{16}H_{17}N_4OS$  calculated: 313.1118  
647  $[M+H]^+$ , found: 313.1116.

648

649 4.1.3.2. (S)-2-(pyrrolidin-3-ylamino)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one  
650 dihydrochloride (**6b**).  $^1H$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  9.33-9.30 (m, 2H, NH), 7.68 (d, 2H,  $CH_{ar}$ ,  
651  $J = 8.2$  Hz), 7.54 (s, 1H, H-thiophene), 7.29 (d, 2H,  $CH_{ar}$ ,  $J = 7.9$  Hz), 4.54-4.49 (m, 1H, CH), 3.50-  
652 3.44 (m, 1H,  $CH_2$ ), 3.38-3.31 (m, 1H,  $CH_2$ ), 3.28-3.14 (m, 2H,  $CH_2$ ), 2.34 (s, 3H,  $CH_3$ -tolyl), 2.31-  
653 2.24 (m, 1H,  $CH_2$ ), 1.97-1.91 (m, 1H,  $CH_2$ );  $^{13}C$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  157.3, 157.1, 152.7,  
654 151.0, 139.4, 130.0, 129.9, 125.9, 118.6, 112.1, 50.1, 49.4, 43.7, 30.2, 20.9; LC/MS :  $t_r = 1.23$   
655 min,  $[M+H]^+ = 327.3$ ; HR-MS (ESI)  $C_{17}H_{19}N_4OS$  calculated: 327.1274  $[M+H]^+$ , found: 327.1283.

656

657 4.1.4. Synthesis of methyl 5-(*p*-tolyl)-3-((triphenylphosphoranylidene)amino)thiophene-  
658 2-carboxylate **7**.

659 A mixture of methyl 3-amino-5-(*p*-tolyl)thiophene-2-carboxylate **2** (1eq., 2 g, 8.087 mmol) in  
660 anhydrous ACN (60 mL) was stirred at 0°C. Then,  $PPh_3$  (2 eq., 3.214 g, 12.13 mmol) and  $C_2Cl_6$   
661 (1.5 éq, 2.872 g, 12.13 mmol) were added. Then,  $Et_3N$  (3eq., 2.455 g, 24.26 mmol) was added  
662 dropwise at 0°C. The color of the reaction mixture quickly turned to be orange. After stirring  
663 for 2h30 at 0°C, the solvent was removed under reduced pressure and the residue was

664 recrystallized from EtOH to give compound **7** as an off-white powder (2.662 g, 65%); <sup>1</sup>H NMR  
665 (500 MHz, DMSO-d<sub>6</sub>): δ 7.86-7.84 (m, 6H, CH<sub>ar</sub>), 7.67-7.58 (m, 9H, CH<sub>ar</sub>), 7.22 (d, 2H, CH<sub>ar</sub>, J =  
666 8.2 Hz), 7.15 (d, 2H, CH<sub>ar</sub>, J = 7.9 Hz), 6.35 (s, 1H, H-thiophene), 3.73 (s, 3H, CH<sub>3</sub>O), 2.27 (s, 3H,  
667 CH<sub>3</sub>-tolyl); <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>): δ 162.5, 156.1, 145.2, 138.2, 132.3-132.2 (m), 130.3-  
668 130.3 (m), 129.6, 128.9 (d, J = 11.9 Hz), 124.9, 120.0 (d, <sup>3</sup>J = 9.4 Hz), 108.7 (d, J = 23.3 Hz), 50.5,  
669 20.6; HR-MS (ESI) C<sub>31</sub>H<sub>27</sub>NO<sub>2</sub>PS calculated: 508.1495 [M+H]<sup>+</sup>, found: 508.1497.

670

671 4.1.5. General Procedure for the Synthesis of 3-substituted thieno[3,2-*d*]pyrimidines  
672 **10a-c.**

673 The appropriate isocyanate (1.5 to 3 eq.) under nitrogen atmosphere was added to a solution  
674 of iminophosphorane **7** (1 eq.) in anhydrous DCM (0.500g/20mL). When the  
675 iminophosphorane was consumed (HPLC monitored), the solvent was removed under reduced  
676 pressure. The carbodiimide obtained was directly used without purification. *Tert*-butylamine  
677 (1 to 3 eq.) was added to the solution of carbodiimide in anhydrous dichloromethane. After  
678 complete consumption of the carbodiimide, the solvent was removed. The crude was  
679 dissolved in anhydrous EtOH and several drops of EtONa (in EtOH) was added to the mixture.  
680 The mixture was stirred at room temperature. After that, the solution was concentrated under  
681 vacuum and the residual was purified by the appropriate purification method.

682

683 4.1.5.1. 2-(*Tert*-butylamino)-3-phenyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**10a**).  
684 Following the general procedure, using **7** (1eq., 600 mg, 1.182 mmol), phenyl isocyanate (1.5  
685 eq., 211 mg, 1.773 mmol, 193 μL). Then, *tert*-butylamine (1 eq., 56 mg, 0.7634 mmol, 81 μL)  
686 was added to the carbodiimide (1 eq., 266 mg, 0.7634 mmol). The crude product was purified  
687 by recrystallization in DCM/EtOH. A white powder was obtained (125 mg, 45%); <sup>1</sup>H NMR (500  
688 MHz, DMSO-d<sub>6</sub>): δ 7.73 (d, 2H, CH<sub>ar</sub>, J = 8.1 Hz), 7.64-7.62 (m, 2H, CH<sub>ar</sub>), 7.59-7.57 (m, 1H, CH<sub>ar</sub>),  
689 7.56 (s, 1H, H-thiophene), 7.43-7.42 (m, 2H, CH<sub>ar</sub>), 7.29 (d, 2H, CH<sub>ar</sub>, J = 8.0 Hz), 4.11 (bs, 1H,  
690 NH), 2.35 (s, 3H, CH<sub>3</sub>-tolyl), 1.33 (s, 9H, (CH<sub>3</sub>)<sub>3</sub>); <sup>13</sup>C NMR (500 MHz, DMSO-d<sub>6</sub>): δ 158.1, 157.2,  
691 151.2, 150.9, 139.2, 134.8, 130.3, 130.1, 129.8, 129.7, 129.2, 125.8, 119.8, 115.5, 52.1, 28.4,  
692 20.9; LC/MS: t<sub>r</sub> = 2.31 min, [M+H]<sup>+</sup> = 390.0; HR-MS (ESI) C<sub>23</sub>H<sub>24</sub>N<sub>3</sub>OS calculated: 390.1640  
693 [M+H]<sup>+</sup>, found: 390.1647.

694

695 4.1.5.2. 2-(*Tert*-butylamino)-3-ethyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**10b**).  
696 Following the general procedure, using **7** (1eq., 500 mg, 0.985 mmol), isocyanatoethane (1.5



697 eq., 105 mg, 1.478 mmol, 117  $\mu$ L) and *tert*-butylamine (1 eq., 72 mg, 0.985 mmol, 104  $\mu$ L). The  
698 crude product was purified by chromatography on silica gel (eluent: 90/10 Hexane/EtOAc to  
699 100% EtOAc). An off-white solid was obtained (166 mg, 49%);  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$   
700 7.68 (d, 2H,  $\text{CH}_{\text{ar}}$ ,  $J = 8.1$  Hz), 7.42 (s, 1H, H-thiophene), 7.27 (d, 2H,  $\text{CH}_{\text{ar}}$ ,  $J = 8.0$  Hz), 5.8(bs, 1H,  
701 NH), 4.16 (q, 2H,  $\text{CH}_2\text{CH}_3$ ,  $J = 7.0$  Hz), 2.34 (s, 3H,  $\text{CH}_3$ -tolyl), 1.49 (s, 9H,  $(\text{CH}_3)_3$ ), 1.15 (t, 3H,  
702  $\text{CH}_3\text{CH}_2$ ,  $(\text{CH}_3)_3$ ,  $J = 7.0$  Hz);  $^{13}\text{C}$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  157.6, 157.3, 151.0, 150.3, 139.0,  
703 130.2, 129.7, 125.8, 119.6, 111.0, 52.2, 35.0, 28.7, 20.8, 12.5; LC/MS:  $t_r = 2.32$  min,  $[\text{M}+\text{H}]^+ =$   
704 342.1; HR-MS (ESI)  $\text{C}_{19}\text{H}_{24}\text{N}_3\text{OS}$  calculated: 342.1640  $[\text{M}+\text{H}]^+$ , found: 342.1642.

705  
706 4.1.5.3. 2-(*Tert*-butylamino)-3-isopropyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4(3*H*)-one (**10c**).  
707 Following the general procedure, using **7** (1eq., 500 mg, 0.985 mmol), 2- isocyanatopropane (3  
708 eq., 252 mg, 2.955 mmol, 290  $\mu$ L) and *tert*-butylamine (3 eq., 216 mg, 2.955 mmol, 312  $\mu$ L).  
709 The crude product was purified by chromatography on silica gel (eluent: 90/10 Hexane/EtOAc  
710 to 100% EtOAc) and with recrystallization in DCM/EtOH. Off-white crystals were obtained (207  
711 mg, 59%);  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  7.68 (d, 2H,  $\text{CH}_{\text{ar}}$ ,  $J = 8.1$  Hz), 7.43 (s, 1H, H-  
712 thiophene), 7.27 (d, 2H,  $\text{CH}_{\text{ar}}$ ,  $J = 8.1$  Hz), 5.69 (bs, 1H, NH), 4.92-4.89 (m, 1H, CH), 2.33 (s, 3H,  
713  $\text{CH}_3$ -tolyl), 1.50-1.48 (m, 15H,  $(\text{CH}_2)_2$  et  $(\text{CH}_3)_3$ );  $^{13}\text{C}$  NMR (500 MHz, DMSO- $d_6$ ):  $\delta$  158.4, 157.3,  
714 151.6, 150.3, 139.0, 130.3, 129.8, 125.8, 119.5, 112.4, 52.4, 46.8, 28.8, 20.9, 19.8; LC/MS:  $t_r =$   
715 2.47 min,  $[\text{M}+\text{H}]^+ = 356.2$ ; HR-MS (ESI)  $\text{C}_{20}\text{H}_{26}\text{N}_3\text{OS}$  calculated: 356.1791  $[\text{M}+\text{H}]^+$ , found :  
716 356.1795.

## 717

### 718 4.2. Biology

719 All the in vitro biological evaluation assays, including blood-stage and hepatic antiplasmodial  
720 evaluation,[14] MTT cell viability assay on HepG2 cells,[13] chromatographic hydrophobicity  
721 index (CHI) Log  $D_{7,4}$ , microsomal stability and hERG inhibition assays[14] were performed as  
722 previously reported.

#### 723 4.2.1. Blood-Stage Antiplasmodial Evaluation

724 A K1 culture-adapted *P. falciparum* strain resistant to chloroquine, pyrimethamine, and  
725 proguanil was used in an in vitro culture. It was maintained in a continuous culture, as  
726 described previously by Trager and Jensen.[22] Cultures were maintained in fresh A+ human  
727 erythrocytes at 2.5% hematocrit in a complete medium (RPMI 1640 with 25 mM HEPES, 25  
728 mM  $\text{NaHCO}_3$ , and 10% of A+ human serum) at 37°C under a reduced  $\text{O}_2$  atmosphere (gas

729 mixture: 10% O<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>). Parasitemia was maintained daily between 1 and 3%.  
730 The *P. falciparum* drug susceptibility test was carried out by comparing quantities of DNA in  
731 treated and control cultures of parasites in human erythrocytes, according to a SYBR Green I  
732 fluorescence-based method[23] using a 96-well fluorescence plate reader. Compounds,  
733 previously dissolved in DMSO (final concentration less than 0.5% v/v), were incubated in a total  
734 assay volume of 200 µL (RPMI, 2% hematocrit and 0.4% parasitemia) for 72 h in a humidified  
735 atmosphere (10% O<sub>2</sub> and 5% CO<sub>2</sub>) at 37°C, in 96-well flat bottom plates. Duplicate assays were  
736 performed for each sample. After incubation, plates were frozen at 20°C for 24 h. Then, the  
737 frozen plates were thawed for 1 h at 37°C. A total of 15 µL of each sample was transferred to  
738 96-well flat-bottom non-sterile black plates (Greiner Bio-One, Kremsmünster, Austria) already  
739 containing 15 µL of the SYBR Green I lysis buffer (2X SYBR Green I, 20 mM Tris base pH 7.5, 20  
740 mM EDTA, 0.008% w/v saponin, 0.08% w/v Triton X-100). Negative control treated by solvents  
741 (DMSO or H<sub>2</sub>O) and positive controls (chloroquine) were added to each set of experiments.  
742 Plates were incubated for 15 min at 37°C and then read on a TECAN Infinite F-200  
743 spectrophotometer with excitation and emission wavelengths at 485 and 535 nm, respectively.  
744 The concentrations of compounds required to induce a 50% decrease in parasite growth (EC<sub>50</sub>  
745 K1) were calculated from three independent experiments.

#### 746 4.2.2. Cytotoxicity Evaluation

747 The HepG2 cell line was maintained at 37°C, 5% CO<sub>2</sub>, at 90% humidity in MEM supplemented  
748 with 10% fetal bovine serum, 1% L-glutamine (200 mM), and penicillin (100  
749 U/mL)/streptomycin (100 µg/mL) (complete RPMI medium). The cytotoxicity of the tested  
750 molecules on the HepG2 (hepatocarcinoma cell line purchased from ATCC, ref HB-8065) cell  
751 line was assessed according to the method of Mosmann[24] with slight modifications. Briefly,  
752 5.103 cells in 100 µL of complete medium were inoculated into each well of 96-well plates and  
753 incubated at 37°C in humidified 5% CO<sub>2</sub>. After 24 h incubation, 100 µL of medium with various  
754 product concentrations dissolved in DMSO (final concentration less than 0.5% v/v) were added  
755 and the plates were incubated for 72 h at 37°C. Triplicate assays were performed for each  
756 sample. Each plate was then microscopically examined for possible precipitate formation  
757 before the medium was aspirated from the wells. Next, 100 µL of MTT (3-(4,5-dimethyl-2-  
758 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) solution (0.5 mg/mL in medium without FBS)  
759 was added to each well. Cells were incubated for 2 h at 37°C. After this time, the MTT solution  
760 was removed and DMSO (100 µL) was added to dissolve the resulting blue formazan crystals.  
761 Plates were shaken vigorously (700 rpm) for 10 min. The absorbance was measured at 570 nm  
762 with 630 nm as the reference wavelength using a BIO-TEK ELx808 Absorbance Microplate

763 Reader (LabX, Midland, ON, Canada). DMSO was used as blank and doxorubicin (purchased  
764 from Sigma Aldrich) as positive control. Cell viability was calculated as a percentage of control  
765 (cells incubated without compounds). The 50% cytotoxic concentration (CC<sub>50</sub>) was determined  
766 from the dose–response curve, using TableCurve software 2D v.5.0. CC<sub>50</sub> values to represent  
767 the mean value calculated from three independent experiments.

#### 768 4.2.3. Hepatic-Stage Antiplasmodial Evaluation

##### 769 4.2.2.1. Parasite Strains and Sporozoite Isolation

770 *P. berghei* sporozoites constitutively expressing green fluorescent protein (*PbGFP*, ANKA  
771 strain)[25] were obtained by the dissection of salivary glands from infected *Anopheles*  
772 *stephensi* mosquitoes bred and infected in the insectary facilities of UMR-S 1135 (CIMI, Paris,  
773 France). Infected salivary glands were removed by hand dissection, crushed in a potter, and  
774 filtered through a 40 µm filter for sporozoite isolation (Cell Strainer, BD BioSciences, San Jose,  
775 CA, USA). The sporozoites were counted using a disposable plastic microscope slide (KOVA).

##### 776 4.2.2.2. Primary Hepatocyte Culture

777 Primary simian hepatocytes were isolated from liver segments collected from healthy *Macaca*  
778 *fascicularis* from the facilities of the Centre d'Energie Atomique (CEA) (Fontenay-aux-Roses,  
779 France). All hepatocytes were obtained using collagenase perfusion, as previously  
780 described[26], and were immediately cryopreserved. One day before infection, the  
781 cryopreserved hepatocytes were thawed at 37°C and were seeded into collagen-coated (5  
782 µg/cm<sup>2</sup> rat tail collagen I, Invitrogen, Carlsbad, CA, USA) 96-well plate, at a density of 80,000  
783 cells per well. They were cultured at 37°C in 5% CO<sub>2</sub> in William's E medium (Gibco, Life  
784 Technologies, Saint Aubin, France) supplemented with 10% of Fetal Bovine Serum FCIII, 5 x  
785 10<sup>-5</sup> M hydrocortisone hemisuccinate (Serb Laboratories, Paris, France), 5 µg per ml bovine  
786 insulin (Sigma Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, and 0.02 U per ml-0.02 µg per  
787 ml penicillinstreptomycin (Life Technologies) until infection with sporozoites.

##### 788 4.2.2.3. *In Vitro* Infection and Drug Assays

789 Sporozoites of *P. berghei* were re-suspended in the above complete medium used for the  
790 hepatocytes culture. Simian hepatocytes were inoculated in 50 µL of complete media with  
791 20,000 *P. berghei*-sporozoites/well in 96-well plates. The infected culture plates were  
792 centrifuged for 6 min at 900x g to allow fast parasite sedimentation onto the target cells and  
793 were further incubated with the serial dilution of drugs that were prepared in advance. After  
794 3 h of incubation, cultures were washed and further incubated in a fresh medium containing

795 the appropriate drug concentration, which were changed every 24 h during the study period.  
796 Cultured hepatocytes were fixed using 4% paraformaldehyde (PFA) for 15 min at room  
797 temperature. Host cell and parasite nuclei were labeled with 4',6-diamidino-2-phenylindole  
798 (DAPI). Upon fixation and immunostaining, cell culture plates were analysed to determine the  
799 number and size of the parasites using a Cell Insight High Content Screening platform equipped  
800 with the Studio HCS software (Thermo Fisher Scientific, Waltham, MA, USA). The parasite size  
801 reduction was calculated on the average object area using the total surface area of each  
802 selected object ( $\mu\text{m}^2$ ). To assess the cell toxicity of drugs for hepatic cultures, fixed plates were  
803 further scanned for the DAPI signal representing host nuclei. The analysis was based on  
804 counting of total cell nuclei. GraphPad Prism 7 statistical software (GraphPad. Software, San  
805 Diego, CA, USA) was used for data analysis and graphing. All values were expressed as means  
806 and standard deviations (SD).

807

808

### 809 4.3. *Physicochemical Properties*

#### 810 4.3.1. Determination of Solubility in PBS

811 1 mg of compound was weighted into an Eppendorf and 1 mL of PBS was added. The mixture  
812 was sonicated for 10 min and then stirred at 25°C overnight. The mixture was centrifuged for  
813 10 min at 3000 rpm. The supernatant was recovered and filtered. 10  $\mu\text{L}$  were injected in  
814 analytical HPLC. The area under the curve corresponding to the product was measured at 214  
815 nm and compared to the calibration curves obtained after injection of a range of the product  
816 previously solubilized in methanol (concentrations 50, 125, 250, 400, 500  $\mu\text{M}$ ). Calibration  
817 curves were linear with  $R^2 > 0.99$ .

#### 818 4.3.2. Chromatographic Hydrophobicity Index (CHI) $\text{Log } D_{7,4}$

819 The determination of Chromatographic Hydrophobicity Index (CHI)[27] at neutral pH was  
820 realized by retention time measurements using a 1290 Agilent chromatographic system  
821 (Agilent Technologies, Santa Clara, California, USA) equipped with a PDA detector 1260  
822 operating at 220, 240, 254, 290 and 350 nm for all compounds using a Ultra C18 column (5  $\mu\text{m}$ ,  
823 3 x 50 mm) from Restek (Lisses, France). Before measurements, compounds were dissolved  
824 (100  $\mu\text{M}$ ) in methanol. In the used HPLC method, the flow rate was 0.3 mL/min, the  
825 temperature was kept constant at 30°C. The mobile phase A was made of 50 mM ammonium  
826 acetate aqueous solution, and the mobile phase B was ACN. A HPLC gradient method was

827 applied, with 0 to 0.2 min: 0% B solvent; 0.2 to 3.5 min: 0 to 100% B solvent; 3.5 to 4.0 min:  
828 100% B solvent; post run: 2 min. Standard compounds were theophylline, 5-Phenyltetrazole,  
829 benzimidazole, acetophenone, indole, propiophenone, butyrophenone, valerophenone, with  
830  $R^2 > 0.999$ . The Chromatographic Hydrophobicity data expressed as CHI LogD was calculated as  
831 previously described)[28].

#### 832 4.3.3. Permeability Determination by PAMPA-GIT Assay

833 The Parallel Artificial Membrane Permeability (PAMPA)-GIT experiments were conducted  
834 using the Pampa Explorer Kit (Pion Inc) according to manufacturer's protocol. Usually, the  
835 studied compound is dissolved in DMSO at 20 mM. Considering that the studied molecule was  
836 poorly soluble in DMSO, a stock solution at 10 mM was realized, and was then diluted to 50  
837  $\mu\text{M}$  in Prisma HT buffer pH 7.4, 6.0 or 5.0 (Pion Inc). 200  $\mu\text{L}$  of these solutions ( $n = 4$ ) were  
838 added to the donor plate (P/N 110243). GIT-0 Lipid (5  $\mu\text{L}$ , P/N 110669) was used to coat the  
839 membrane filter of the acceptor plate (P/N 110243). The acceptor Sink Buffer (200  $\mu\text{L}$ , P/N  
840 110139) was added to each well of the acceptor plate. The sandwich was incubated at room  
841 temperature for 4h, without stirring. After the incubation the UV-visible spectra were  
842 measured with the microplate reader (Tecan infinite M200), and the permeability value ( $P_e$ )  
843 was calculated by the PAMPA Explorer software v.3.7 (pION) for each compound. Antipyrine  
844 and ketoprofen were used as references.

#### 845 4.3.4. hERG Inhibition Assay

846 hERG channel binding of the compound is determined with the Predictor<sup>TM</sup> hERG  
847 Fluorescence Polarization Assay, that permits hERG channel biochemical binding studies in the  
848 absence of radioligands. The assay is based on the principle of fluorescence polarization, where  
849 a red-shifted fluorescent tracer displays a high polarization when bound to the hERG channel  
850 and a low polarization when displaced by compounds that bind to the channel.

851 Materials and Methods: hERG channel binding was determined with the Predictor<sup>TM</sup> hERG  
852 Fluorescence Polarization Assay (PV5665 ThermoFischer). Briefly, reagents were thawed, and  
853 were mixed by pipetting 20x with Predictor<sup>TM</sup> hERG membranes. The fluorescent tracer was  
854 diluted at 4 nM in the Predictor<sup>TM</sup> hERG buffer. The tested compound was dissolved in DMSO  
855 at 10 mM and was then diluted from 0.4  $\mu\text{M}$  to 0.04 nM in the Predictor<sup>TM</sup> hERG buffer. The  
856 assay was performed in a 96-well Greiner microplate (675093 Dutscher). 25  $\mu\text{L}$  of each  
857 concentration of M1 were dispensed into the plate. Then, 50  $\mu\text{L}$  of the Predictor<sup>TM</sup> hERG  
858 membranes and 25  $\mu\text{L}$  of the fluorescent tracer 4 nM were added. The plate was incubated at  
859 room temperature for 4 hours. The fluorescence polarization measurements were made using

860 a Synergy 2 microplate reader (Biotek Instrument, Colmar, France). Both parallel and  
861 perpendicular fluorescence were measured using 530/25 nm excitation and 590/35 nm  
862 emission filters with a 570 nm dichroic mirror. The gain was fixed at 110. A known hERG  
863 channel blocker (E-4031), provided by the kit, was assayed as positive reference. E-4031,  
864 LP0373 and LP0374 were tested at final concentrations ranging from 0.01 nM to 100  $\mu$ M. IC50  
865 values were determined graphically by plotting the % inhibition versus the logarithm of 9  
866 concentrations of each compound in the assay solution using the GraphPad Prism software  
867 (version 6.01, GraphPad Software, La Jolla, CA, USA).

#### 868 4.4. *Microsomal Stability*

869 The tested products and propranolol, used as a reference, were incubated in duplicate  
870 (reaction mixture volume of 0.5 mL) with female mouse microsomes (CD-1, 20 mg mL<sup>-1</sup>; BD  
871 Gentest) at 37 °C in a 50 mM phosphate buffer, pH 7.4, in the presence of MgCl<sub>2</sub> (5 mM), NADP  
872 (1 mM), glucose-6-phosphate dehydrogenase (G6PD) (0.4U per mL), and glucose-6-phosphate  
873 (5 mM). For the estimation of the intrinsic clearance, 50  $\mu$ L aliquots were collected at 0, 5, 10,  
874 20, 30, and 40 min and the reaction was stopped with 4 volumes of acetonitrile (ACN)  
875 containing the internal standard. After centrifugation at 12000 rpm for 10 min at 4 °C, the  
876 supernatants are kept at 4°C for immediate analysis. Controls (time zero and final time point)  
877 in triplicate were prepared by the incubation of the internal standard with microsomes  
878 denatured by acetonitrile. The calculation of the half-life ( $t_{1/2}$ ) of each compound in the  
879 presence of microsomes was carried out according to the equation  $t_{1/2} = (\ln 2)/k$ , where k is the  
880 first-order degradation constant (the slope of the logarithm of the compound concentration  
881 versus incubation time). The intrinsic clearance in vitro ( $Cl_{int}$ , expressed in mL.min<sup>-1</sup>.mg) was  
882 calculated according to the equation  $Cl_{int} = (\text{dose}/AUC_{\infty})/[\text{microsomes}]$ , where dose is the  
883 initial concentration of the product in the sample,  $AUC_{\infty}$  is the area under the concentration–  
884 time curve extrapolated to infinity, and [microsomes] is the microsome concentration  
885 expressed in mg.mL<sup>-1</sup>.

#### 886 5. **Acknowledgements**

887 This work was financially supported by the Agence Nationale de la Recherche (ANR Plasmodrug  
888 18-CE18-0009-01) and the Fondation pour la Recherche Médicale (FRM Palukill  
889 DMC20181039565). We want to thank M Pierre Sanchez (Institut des Biomolécules Max  
890 Mousseron, Montpellier) for his help with MS analyses and Ms Catherine Piveteau (Institut  
891 Pasteur de Lille) for performing microsomal stability studies.

- 893 [1] World Health Organization, World malaria report 2022, (n.d.).  
 894 <https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2022>  
 895 (accessed June 7, 2022).
- 896 [2] R.O. Makanjuola, A.W. Taylor-Robinson, Improving Accuracy of Malaria Diagnosis in Underserved  
 897 Rural and Remote Endemic Areas of Sub-Saharan Africa: A Call to Develop Multiplexing Rapid  
 898 Diagnostic Tests, *Scientifica* (Cairo). 2020 (2020) 3901409.  
 899 <https://doi.org/10.1155/2020/3901409>.
- 900 [3] T.E. Nkya, U. Fillinger, O.P. Sangoro, R. Marubu, E. Chanda, C.M. Mutero, Six decades of malaria  
 901 vector control in southern Africa: a review of the entomological evidence-base, *Malar. J.* 21  
 902 (2022) 279. <https://doi.org/10.1186/s12936-022-04292-6>.
- 903 [4] A.O. Adeyemo, A.T. Aborode, M.A. Bello, A.F. Obianuju, M.M. Hasan, D.O. Kehinde, M.S. Hossain,  
 904 M. Bardhan, J.O. Imisioluwa, A.A. Akintola, Malaria vaccine: The lasting solution to malaria  
 905 burden in Africa, *Ann. Med. Surg. (Lond)*. 79 (2022) 104031.  
 906 <https://doi.org/10.1016/j.amsu.2022.104031>.
- 907 [5] H. Tripathi, P. Bhalerao, S. Singh, H. Arya, B.S. Alotaibi, S. Rashid, M.R. Hasan, T.K. Bhatt, Malaria  
 908 therapeutics: are we close enough?, *Parasites & Vectors*. 16 (2023) 130.  
 909 <https://doi.org/10.1186/s13071-023-05755-8>.
- 910 [6] B. Hanboonkunupakarn, J. Tarning, S. Pukrittayakamee, K. Chotivanich, Artemisinin resistance  
 911 and malaria elimination: Where are we now?, *Front. Pharmacol.* 13 (2022) 876282.  
 912 <https://doi.org/10.3389/fphar.2022.876282>.
- 913 [7] C. Wongsrichanalai, C.H. Sibley, Fighting drug-resistant *Plasmodium falciparum*: the challenge of  
 914 artemisinin resistance, *Clinical Microbiology and Infection*. 19 (2013) 908–916.  
 915 <https://doi.org/10.1111/1469-0691.12316>.
- 916 [8] H. Bosson-Vanga, N. Primas, J.-F. Franetich, C. Lavazec, L. Gomez, K. Ashraf, M. Tefit, V. Soulard,  
 917 N. Dereuddre-Bosquet, R. Le Grand, M. Donnette, R. Mustière, N. Amanzougaghene, S. Tajeri, P.  
 918 Suzanne, A. Malzert-Fréon, S. Rault, P. Vanelle, S. Hutter, A. Cohen, G. Snounou, P. Roques, N.  
 919 Azas, P. Lagardère, V. Lisowski, N. Masurier, M. Nguyen, L. Paloque, F. Benoit-Vical, P. Verhaeghe,  
 920 D. Mazier, A New Thienopyrimidinone Chemotype Shows Multistage Activity against *Plasmodium*  
 921 *falciparum*, Including Artemisinin-Resistant Parasites, *Microbiol. Spectr.* 9 (2021) e00274-21.  
 922 <https://doi.org/10.1128/Spectrum.00274-21>.
- 923 [9] A. Cohen, P. Suzanne, J.-C. Lancelot, P. Verhaeghe, A. Lesnard, L. Basmaciyan, S. Hutter, M. Laget,  
 924 A. Dumètre, L. Paloque, E. Deharo, M.D. Crozet, P. Rathelot, P. Dallemagne, A. Lorthiois, C.H.  
 925 Sibley, P. Vanelle, A. Valentin, D. Mazier, S. Rault, N. Azas, Discovery of new thienopyrimidinone  
 926 derivatives displaying antimalarial properties toward both erythrocytic and hepatic stages of  
 927 *Plasmodium*, *Eur. J. Med. Chem.* 95 (2015) 16–28.  
 928 <https://doi.org/10.1016/j.ejmech.2015.03.011>.
- 929 [10] P. Lagardère, R. Mustière, N. Amanzougaghene, S. Hutter, J.-F. Franetich, N. Azas, P. Vanelle, P.  
 930 Verhaeghe, N. Primas, D. Mazier, N. Masurier, V. Lisowski, 4-Substituted Thieno[3,2-  
 931 d]pyrimidines as Dual-Stage Antiplasmodial Derivatives, *Pharmaceuticals*. 15 (2022) 820.  
 932 <https://doi.org/10.3390/ph15070820>.
- 933 [11] P. Lagardère, C. Fersing, N. Masurier, V. Lisowski, Thienopyrimidine: A Promising Scaffold to  
 934 Access Anti-Infective Agents, *Pharmaceuticals*. 15 (2022) 35.  
 935 <https://doi.org/10.3390/ph15010035>.
- 936 [12] R. Mustière, P. Lagardère, S. Hutter, C. Deraeve, F. Schwalen, D. Amrane, N. Masurier, N. Azas, V.  
 937 Lisowski, P. Verhaeghe, D. Mazier, P. Vanelle, N. Primas, Pd-catalyzed C–C and C–N cross-coupling  
 938 reactions in 2-aminothieno[3,2-d]pyrimidin-4(3H)-one series for antiplasmodial  
 939 pharmacomodulation, *RSC Adv.* 12 (2022) 20004–20021. <https://doi.org/10.1039/D2RA01687G>.
- 940 [13] R. Mustière, P. Lagardère, S. Hutter, V. Dell’Orco, N. Amanzougaghene, S. Tajeri, J.-F. Franetich,  
 941 S. Corvaisier, M. Since, A. Malzert-Fréon, N. Masurier, V. Lisowski, P. Verhaeghe, D. Mazier, N.

942 Azas, P. Vanelle, N. Primas, Synthesis of antiplasmodial 2-aminothieno[3,2-d]pyrimidin-4(3H)-  
 943 one analogues using the scaffold hopping strategy, *Eur. J. Med. Chem.* 241 (2022) 114619.  
 944 <https://doi.org/10.1016/j.ejmech.2022.114619>.

945 [14] P. Lagardère, R. Mustière, N. Amanzougaghene, S. Hutter, M. Casanova, J.-F. Franetich, S. Tajeri,  
 946 A. Malzert-Fréon, S. Corvaisier, N. Azas, P. Vanelle, P. Verhaeghe, N. Primas, D. Mazier, N.  
 947 Masurier, V. Lisowski, New antiplasmodial 4-amino-thieno[3,2-d]pyrimidines with improved  
 948 intestinal permeability and microsomal stability, *Eur. J. Med. Chem.* 249 (2023) 115115.  
 949 <https://doi.org/10.1016/j.ejmech.2023.115115>.

950 [15] D. González Cabrera, F. Douelle, C. Le Manach, Z. Han, T. Paquet, D. Taylor, M. Njoroge, N.  
 951 Lawrence, L. Wiesner, D. Waterson, M.J. Witty, S. Wittlin, L.J. Street, K. Chibale, Structure–  
 952 Activity Relationship Studies of Orally Active Antimalarial 2,4-Diamino-thienopyrimidines, *J. Med.*  
 953 *Chem.* 58 (2015) 7572–7579. <https://doi.org/10.1021/acs.jmedchem.5b01156>.

954 [16] D. González Cabrera, C. Le Manach, F. Douelle, Y. Younis, T.-S. Feng, T. Paquet, A.T. Nchinda, L.J.  
 955 Street, D. Taylor, C. de Kock, L. Wiesner, S. Duffy, K.L. White, K.M. Zabiulla, Y. Sambandan, S.  
 956 Bashyam, D. Waterson, M.J. Witty, S.A. Charman, V.M. Avery, S. Wittlin, K. Chibale, 2,4-  
 957 Diaminothienopyrimidines as Orally Active Antimalarial Agents, *J. Med. Chem.* 57 (2014) 1014–  
 958 1022. <https://doi.org/10.1021/jm401760c>.

959 [17] S. Markey, *Pathways of Drugs Metabolism.*, in: *Principles of Clinical Pharmacology - 3rd Edition*,  
 960 2012: pp. 153–172.

961 [18] F.P. Guengerich, Kinetic Deuterium Isotope Effects in Cytochrome P450 Reactions, *Methods*  
 962 *Enzymol.* 596 (2017) 217–238. <https://doi.org/10.1016/bs.mie.2017.06.036>.

963 [19] C. Schmidt, First deuterated drug approved, *Nature Biotechnology.* 35 (2017) 493–494.  
 964 <https://doi.org/10.1038/nbt0617-493>.

965 [20] ClogD values were calculated with ACD/ChemSketch v6.00, Advanced Chemistry Development,  
 966 Inc., Toronto, Canada, 2002. [www.acdlabs.com](http://www.acdlabs.com), (n.d.).

967 [21] A.R. MacKenzie, A.P. Marchington, D.S. Middleton, S.D. Newman, B.C. Jones, Structure–Activity  
 968 Relationships of 1-Alkyl-5-(3,4-dichlorophenyl)-5-{2-[(3-substituted)-1-azetidiny]ethyl}-2-  
 969 piperidones. 1. Selective Antagonists of the Neurokinin-2 Receptor, *J. Med. Chem.* 45 (2002)  
 970 5365–5377. <https://doi.org/10.1021/jm0209331>.

971 [22] W. Trager, J.B. Jensen, Human malaria parasites in continuous culture, *Science.* 193 (1976) 673–  
 972 675. <https://doi.org/10.1126/science.781840>.

973 [23] W.A. Guiguemde, A.A. Shelat, D. Bouck, S. Duffy, G.J. Crowther, P.H. Davis, D.C. Smithson, M.  
 974 Connelly, J. Clark, F. Zhu, M.B. Jiménez-Díaz, M.S. Martinez, E.B. Wilson, A.K. Tripathi, J. Gut, E.R.  
 975 Sharlow, I. Bathurst, F.E. Mazouni, J.W. Fowble, I. Forquer, P.L. McGinley, S. Castro, I. Angulo-  
 976 Barturen, S. Ferrer, P.J. Rosenthal, J.L. DeRisi, D.J. Sullivan, J.S. Lazo, D.S. Roos, M.K. Riscoe, M.A.  
 977 Phillips, P.K. Rathod, W.C. Van Voorhis, V.M. Avery, R.K. Guy, Chemical genetics of *Plasmodium*  
 978 *falciparum*, *Nature.* 465 (2010) 311–315. <https://doi.org/10.1038/nature09099>.

979 [24] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to  
 980 proliferation and cytotoxicity assays, *Journal of Immunological Methods.* 65 (1983) 55–63.  
 981 [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4).

982 [25] C.J. Janse, B. Franke-Fayard, G.R. Mair, J. Ramesar, C. Thiel, S. Engelmann, K. Matuschewski, G.J.  
 983 van Gemert, R.W. Sauerwein, A.P. Waters, High efficiency transfection of *Plasmodium berghei*  
 984 facilitates novel selection procedures, *Molecular and Biochemical Parasitology.* 145 (2006) 60–  
 985 70. <https://doi.org/10.1016/j.molbiopara.2005.09.007>.

986 [26] O. Silvie, E. Rubinstein, J.-F. Franetich, M. Prenant, E. Belnoue, L. Rénia, L. Hannoun, W. Eling, S.  
 987 Levy, C. Boucheix, D. Mazier, Hepatocyte CD81 is required for *Plasmodium falciparum* and  
 988 *Plasmodium yoelii* sporozoite infectivity, *Nat Med.* 9 (2003) 93–96.  
 989 <https://doi.org/10.1038/nm808>.

990 [27] K. Valkó, C. Bevan, D. Reynolds, Chromatographic Hydrophobicity Index by Fast-Gradient RP-  
 991 HPLC: A High-Throughput Alternative to log P/log D, *Anal Chem.* 69 (1997) 2022–2029.  
 992 <https://doi.org/10.1021/ac961242d>.



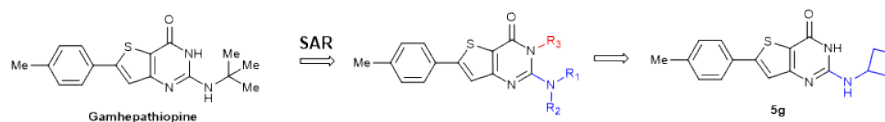
993 [28] K. Valkó, Application of high-performance liquid chromatography based measurements of  
994 lipophilicity to model biological distribution, J Chromatogr A. 1037 (2004) 299–310.  
995 <https://doi.org/10.1016/j.chroma.2003.10.084>.  
996

997

Preprint not peer reviewed

998

## Graphical Abstract



- ✓ Active against *P. falciparum* erythrocytic stage
- ✓ Active against *P. berghei* hepatic stage
- ✗ Not permeable (PAMPA-GIT, pH 5.0 and 7.4)
- ✗ Weak microsomal stability

- ✓ Active against *Pf* erythrocytic stage
- ✓ Active against *Pb* hepatic stage
- ✓ Permeable (PAMPA-GIT, pH 5.0 and 7.4)
- ✓ Improved microsomal stability

999

1000

## Highlights

- A series of 19 new thienopyrimidones was synthesized.
- 6 compounds showed an  $EC_{50} \leq 1.5 \mu\text{M}$  against the blood stage of *P. falciparum* K1.
- 5 compounds showed higher activity against the hepatic stage of *P. berghei* than Gamhepathiopine.
- 3 compounds exhibited improved microsomal stability compared to Gamhepathiopine.
- Compound **5g** showed the better compromise between activity, cytotoxicity and microsomal stability.

1009

1010

1011

1012

1013

1014

1015

1016

1017