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Prisca Lagardère, Romain Mustière, Nadia Amanzougaghene, Sébastien Hutter, Marion Casanova, et al.. New antiplasmodial 4-amino-thieno[3,2-d]pyrimidines with improved intestinal permeability and microsomal stability. *European Journal of Medicinal Chemistry*, 2023, 249, pp.115115. 10.1016/j.ejmech.2023.115115 . hal-04503268

HAL Id: hal-04503268

<https://hal.science/hal-04503268v1>

Submitted on 13 Mar 2024

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New antiplasmodial 4-amino-thieno[3,2-*d*]pyrimidines with improved intestinal permeability and microsomal stability.

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Abstract

The increasing number of *Plasmodium falciparum* strains resistant to current treatments justifies the urgent need to discover new compounds active on several stages of the parasite development. Based on the structure of Gamhepathiopine, a 2-*tert*-butylaminothieno[3,2-*d*]pyrimidin-4(3*H*)-one previously identified for its dual activity against the sexual and asexual stages of *P. falciparum*, 25 new 4-amino-substituted analogues were synthesized and evaluated on the erythrocytic and hepatic stages of *Plasmodium*. A promising compound, *N*²-(*tert*-butyl)-*N*⁴-(3-(dimethylamino)propyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine, showed improved physicochemical properties, intestinal permeability (PAMPA model) and microsomal stability compared to Gamhepathiopine, while maintaining a good antiplasmodial activity on the erythrocytic stage of *P. falciparum* and on the hepatic stage of *P. berghei*.

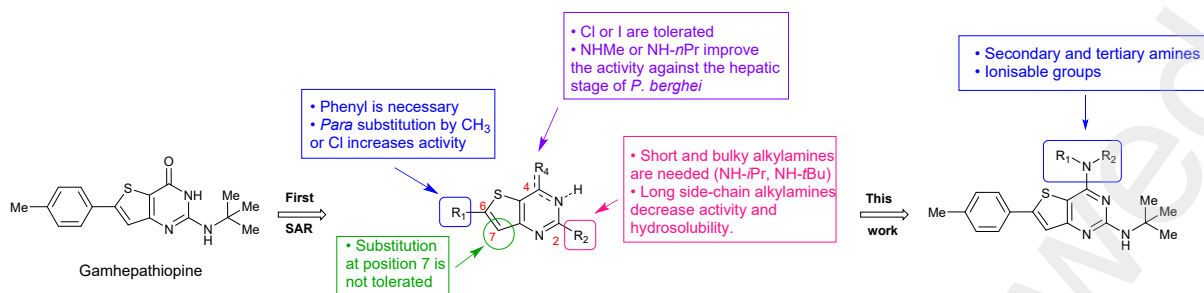
1. Introduction

Malaria is an infectious disease that occurs mainly in Africa¹. According to the 2021 WHO world malaria report, the number of malaria cases was estimated at 241 million people in 2020 and the disease caused the death of 627,000 people^{2,3}. These numbers are significantly increasing since 2019, when 227 million people were infected and 558,000 died⁴. The population in endemic areas remains

38 vulnerable to malaria, despite ongoing government efforts to strengthen vector control, prescription
39 of preventive antimalarial treatments and vaccination of young children.⁵⁻⁷

40 Artemisinin and its derivatives are currently the drugs of choice to treat severe *P. falciparum* malaria.
41 They are particularly effective in combination with slow-acting antimalarial drugs (combination called
42 artemisinin-based combination therapy or ACT). However, partial artemisinin resistance was firstly
43 reported in the 2000s in the Greater Mekong Subregion⁸ and, indeed, resistant to artemisinin-based
44 therapies is now widespread in Southeast Asia and emerging in Africa^{9,10}. In 2015, the WHO established
45 a malaria control program, with the goal of reducing the mortality rate by 90% by 2030, through the
46 strengthening of vector control and the development of new treatments. To achieve this goal, the
47 development of innovative treatments such as those involving multistage activity on *Plasmodium* has
48 been strongly encouraged¹¹⁻¹³.

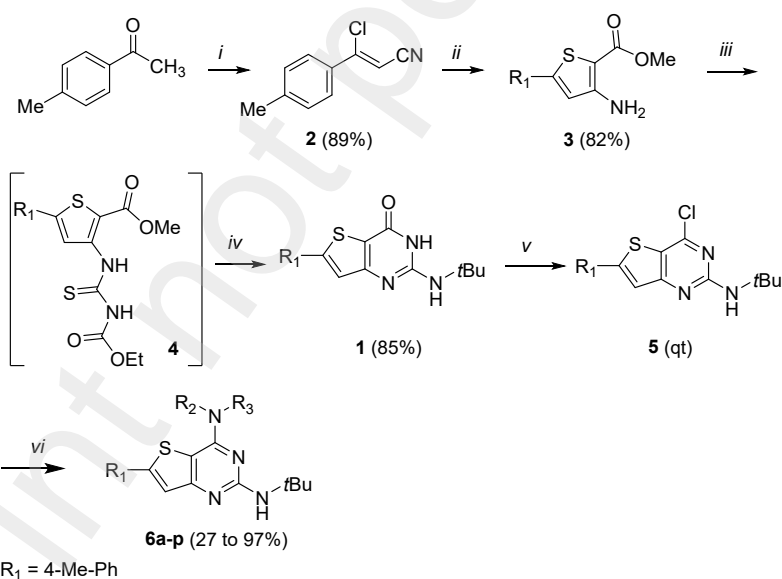
49 In this context, Gamhepathiopine (Figure 1) was identified as a hit compound, with potent
50 antiplasmodial activity against the sexual and asexual stages of *Plasmodium falciparum*.¹⁴ It was
51 subsequently shown that the *in vivo* activity of this compound was limited due to its low aqueous
52 solubility and its poor metabolic stability. Indeed, the *in vivo* activity of this compound could only be
53 demonstrated by co-administration of 1-aminobenzotriazole, a pan-CYP450 inhibitor.¹⁵ Additional
54 structure-activity relationship (SAR) studies highlighted the importance of the thieno[3,2-*d*]pyrimidine
55 scaffold, as well as the presence of a *tert*-butylamine substituent at position 2 and a *p*-tolyl group at
56 position 6, in maintaining antiplasmodial activity.^{14,16-19} Recently, we reported that the introduction of
57 a chlorine atom or a small amino-alkyl group at position 4, such as an NH-Me or an NH-Pr, is tolerated
58 and may even improve the activity of the compound against the hepatic stage of *P. berghei*.¹⁷ In the
59 continuation of this study, we report here the synthesis and biological activity of new 4-amino-
60 substituted thieno[3,2-*d*]pyrimidine derivatives. The introduction of various secondary or tertiary
61 amines was studied at position 4 to determine the influence of the amino substituent and an exocyclic
62 NH toward the antiplasmodial activity (Figure 1). The introduction of diamino moieties as ionizable
63 functions was also considered to improve the aqueous solubility and the microsomal stability of the
64 compounds.



67 **2. Results and Discussion**

68 **2.1. Chemistry**

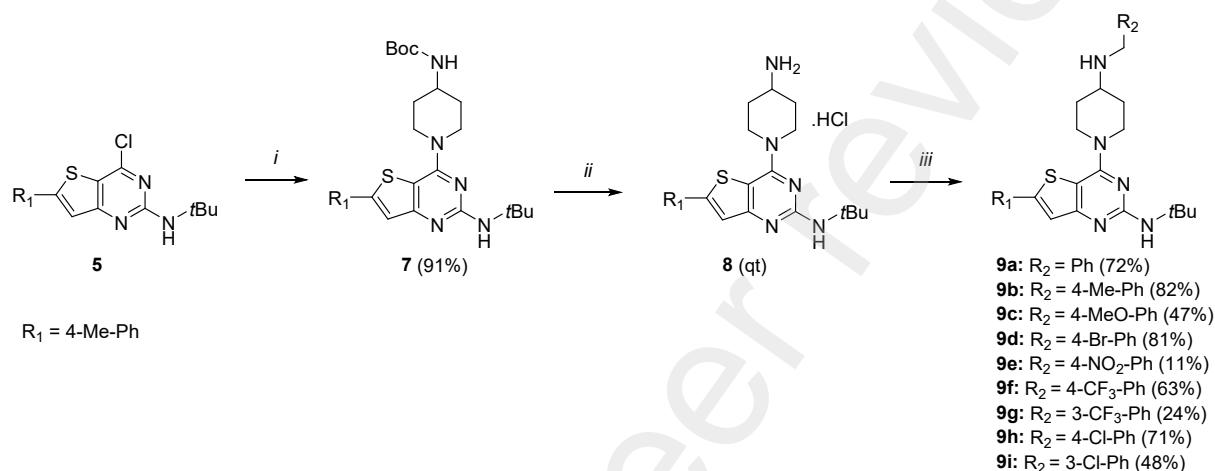
69 All 4-aminothieno[3,2-*d*]pyrimidine derivatives were synthesized from the 4-chloro precursor **5**, using
 70 aromatic nucleophilic substitution, according to our previously published methodology (Scheme 1).¹⁷
 71 Briefly, the reaction of 4-methylacetophenone with phosphorus (V) oxychloride in *N,N*-
 72 dimethylformamide (DMF), followed by treatment with hydroxylamine hydrochloride at room
 73 temperature, afforded compound **2** in good yield (89%). Then, methyl thioglycolate was treated with
 74 sodium methylate and reacted with **2** to obtain methyl 3-amino-5-(*p*-tolyl)thiophene-2-carboxylate **3**
 75 in an 82% isolated yield. Condensation of **3** with ethoxycarbonyl isothiocyanate in DMF gave a thiourea
 76 intermediate.²⁰ This latter reacted with triethylamine, *tert*-butylamine and EDCI.HCl used as a coupling
 77 agent to provide a guanidine intermediate, which cyclized at 170 °C to afford **1** in an overall three-step
 78 yield of 85%. The chlorination of **1** occurred in the presence of phosphorus (V) oxychloride to give **5** in
 79 quantitative yield. Next, targeted compounds were synthesized by aromatic nucleophilic substitution,
 80 under basic conditions (sodium carbonate) and in a polar protic solvent (ethanol). Compounds **6a-p**
 81 were isolated after chromatography in 27-97% yields.



83 **Scheme 1.** Synthesis of targeted compounds **6**. Reagents and conditions: (i) 1. POCl₃, DMF, RT; 2. NH₂OH.HCl, RT,
 84 and 1 h 30; (ii) MeONa, HSCH₂CO₂Me, MeOH, 80 °C, and 40 min; (iii) 1. SCNCO₂Et, DMF, 30 °C, and 2 h; (iv) 1. *t*BuNH₂,
 85 NEt₃, EDCI.HCl, DMF, RT, and 18 h; 2. 170 °C and 2 h; (v) POCl₃, *N,N*-dimethylaniline, ACN, 85 °C, 16 h; 2. H₂O, 40
 86 °C, and 2 h; (vi) Na₂CO₃, Amine, EtOH, reflux.

87 Finally, a set of 4-benzylaminopiperidinyl-thienopyrimidine derivatives **9** was prepared from
 88 compound **5** (Scheme 2). The reaction of **5** with 4-*N*-*boc*-aminopiperidine in basic conditions under
 89 refluxing ethanol gave **7** in a 91% yield. After *Boc* removal using a mixture of hydrochloric acid in

90 dioxane (50/50 v/v) at room temperature, the resulting 4-aminopiperidiny derivative **8** was isolated
 91 as a hydrochloride salt in a quantitative yield. Derivatives **9a-i** were obtained by reductive amination
 92 in methanol, using the appropriate commercial aldehyde and sodium borohydride, in the presence of
 93 triethylamine. Compounds **9a-9i** were isolated after chromatography in moderate to good yields (47-
 94 82%), except when electron-deficient benzaldehydes were used (compounds **9e** and **9g**). The proposed
 95 structures of all newly synthesized compounds were in full agreement with their spectral analyses,
 96 such as ¹H NMR, ¹³C NMR and high-resolution mass spectrometry.



97

98 **Scheme 2.** Reagents and conditions: (i) Na_2CO_3 , 4-(*tert*-butoxycarbonylamino)piperidine, EtOH, reflux; (ii)
 99 Dioxane/HCl, 4:1, 45 min; (iii) NEt_3 , aldehyde, NaBH_4 , MeOH, RT.

100

101 2.2. Biological evaluation

102 2.2.1. Blood-stage antiplasmodial activity

103 All newly synthesized 4-aminosubstituted thieno[3,2-*d*]pyrimidines were screened for their activity
 104 against asexual intraerythrocytic stage parasites of a multidrug-resistant *P. falciparum* K1 strain (

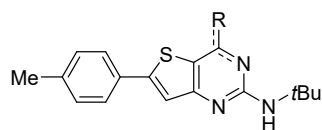
105 Table 1), resistant to chloroquine, pyrimethamine and sulfadoxine. All derivatives were compared to
106 Gamhepathiopine **1**. Chloroquine and doxycycline were included as reference drugs.

107 Efforts to explore SAR requirements to improve potency focused on optimizing amino substituents at
108 position 4. First, the introduction of cyclopropyl- to cyclohexylamines was investigated. Compounds
109 with a 3- to a 5-membered ring showed antiparasitic activity on *Pf.* K1 broadly similar to chloroquine,
110 but less active than Gamhepathiopine **1** (EC_{50} between 0.9 and 1.3 μM), while introduction of a bulkier
111 cyclohexyl group (compound **6d**) decreased the activity (EC_{50} of 2.5 μM). In contrast, the introduction
112 of an *N,N*-ethyl or *N,N*-methylethanediamine or propanediamine (**6e-h**) at position 4 led to compounds
113 with good efficiency against *P. falciparum*, equivalent to the reference compound **1**, with chain size
114 having only a minor influence on activity. Next, a series of tertiary amines was examined. Compounds
115 with an azetidiny (**6i**), pyrrolidinyl (**6j**), piperidinyl (**6k**), 4-aminopiperidinyl (**8**) or 4-methylpiperidinyl
116 (**6l**) group at position 4 showed micromolar activity (EC_{50} between 0.7 and 1.2 μM). This result suggests
117 that the presence of a NH group at position 4 is not essential for the antiparasitic activity. The
118 introduction of cyclic amines incorporating two heteroelements was also studied. However, the
119 resulting compounds **6m** to **6p** were less active than their piperidinyl analogues **6k** and **6l** ($EC_{50} \geq 1.2$
120 μM). Notably, a thiomorpholinyl substituent (**6p**) was not tolerated at position 4 ($EC_{50} = 4.90 \mu\text{M}$).

121 Considering the interesting EC_{50} of the 4-substituted piperidinyl derivatives **6l** and **8**, we decided to
122 synthesize other compounds in this series. Thus, a series of (un)substituted benzylaminopiperidine
123 derivatives was prepared and evaluated. Globally, the activities of these compounds **9a-e** were quite
124 similar, with EC_{50} between 0.7 and 1.7 μM . The efficacy of these compounds was not impacted by the
125 electronic effect and the position (*meta* or *para*) of the benzyl substituent group, except when a
126 trifluoromethyl group (**9f** and **9g**) or a chlorine atom (**9h-9i**) was introduced. In both cases, the
127 antiparasitic activity decreased compared to reference **1** ($EC_{50} > 1 \mu\text{M}$).

128

129 **Table 1.** *In vitro* antiplasmodial activity (EC₅₀) of thienopyrimidines on the erythrocytic stage of a K1 *P. falciparum*
 130 strain.



131

Compound	R	<i>P. falciparum</i> K1 EC ₅₀ (μM) ^a	Compound	R	<i>P. falciparum</i> K1 EC ₅₀ (μM) ^a
1 (Gamhepathiopine)		0.20 ± 0.02 ²⁰	6o		1.60 ± 0.40
6a		1.10 ± 0.30	6p		4.90 ± 1.00
6b		0.90 ± 0.20	7		2.10 ± 0.20
6c		1.30 ± 0.60	8		1.00 ± 0.03
6d		2.50 ± 0.80	9a		0.70 ± 0.10
6e		0.30 ± 0.08	9b		0.70 ± 0.10
6f		0.20 ± 0.05	9c		0.50 ± 0.10
6g		0.30 ± 0.06	9d		0.70 ± 0.06
6h		0.30 ± 0.05	9e ^b		0.70 ± 0.06
6i		0.70 ± 0.15	9f		1.70 ± 0.20
6j		0.90 ± 0.20	9g ^b		1.30 ± 0.20
6k		1.20 ± 0.30	9h		1.00 ± 0.10
6l		0.70 ± 0.10	9i ^b		1.20 ± 0.10
6m		1.60 ± 0.20	Chloroquine	-	0.80 ± 0.10 ²¹
6n		1.20 ± 0.10	Doxycycline	-	5 ²⁰

132 ^a EC₅₀: half-maximal effective concentration. The values are mean ± SD of three independent experiments.

133 ^b isolated as a trifluoroacetate salt

134

135 2.2.2. Hepatic-stage antiplasmodial activity and cytotoxicity

136 Then, all compounds with EC₅₀ less than or equal to 1 μM on the erythrocytic stage of *P. falciparum*

137 were selected for further studies. Thus, thienopyrimidines **6b**, **6e-j**, **6l**, **8**, **9a-e** and **9h** were evaluated

138 for their ability to inhibit hepatic infection caused by the rodent malaria parasite *P. berghei*. Their
 139 cytotoxicity was then determined on primary simian hepatocytes. The results are presented in Table
 140 2.

141 All selected compounds (**6e-h**), except for compounds **6i** and **8**, showed higher activity against the
 142 hepatic stage of *P. berghei* parasites than reference **1**, with an $EC_{50} \leq 16.1 \mu\text{M}$. Thus, as previously
 143 observed with the introduction of a methylamine or a *n*-propylamine at position 4,¹⁷ the presence of a
 144 secondary amine at this position increased the activity against the hepatic stage. Compounds **6b** and
 145 **9e** showed the best potency against the hepatic stage, with EC_{50} of 6.5 and 7.3 μM , respectively.
 146 However, some toxicity was observed against primary simian hepatocytes. Compound **6b** was
 147 cytotoxic with a selectivity index of 1. All other compounds except **6e** showed moderate cytotoxicity
 148 (CC_{50} between 14 and 43.5 μM), whereas compound **6e** was only weakly cytotoxic (45% of cell death
 149 was observed at 100 μM) and showed the best selectivity index of the series (SI of 6.5).

150 Finally, considering both the erythrocytic and hepatic activities of these new 4-aminosubstituted
 151 thienopyrimidine, compounds **6e** to **6h** displayed the lowest EC_{50} and moderate cytotoxicity against
 152 primary simian hepatocytes. These 4 compounds were then selected for evaluation of their drug-like
 153 properties.

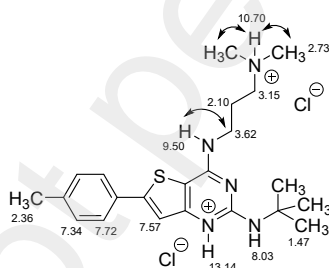
154 **Table 2.** *In vitro* activity (EC_{50}) against the hepatic stage of *P. berghei* parasites and cytotoxicity (CC_{50}) against
 155 primary simian hepatocytes of selected thieno[3,2-*d*]pyrimidines.

Compound	<i>P. berghei</i> EC_{50} (μM) ^a	Primary simian hepatocytes CC_{50} (μM) ^b	Selectivity index (SI) ^c
1 (Gamhepathiopine)	24.3 ± 1.6 ¹⁷	>200 ¹⁷	8.2
6b	6.5 ± 0.3	8.8 ± 0.7	1.3
6e	15.3 ± 1.5	>100	>6.5
6f	11.5 ± 1.4	25.8 ± 2.40	2.2
6g	13.8 ± 5.1	14.0 ± 3.1	1
6h	14.6 ± 1.7	32.6 ± 5.5	2.2
6i	15.1 ± 3.2	25.8 ± 4.4	1.7
6j	14.3 ± 0.1	17.5 ± 1.4	1.2
6l	35.3 ± 4.1	52.5 ± 11.6	1.5
8	31.4 ± 9.1	31.6 ± 6.3	1
9a	16.1 ± 1.6	43.6 ± 6.7	2.7
9b	15.4 ± 1.5	35.5 ± 2.0	2.3
9c	14.0 ± 1.0	28.6 ± 2.3	2.0
9d	13.1 ± 1.3	20.7 ± 5.8	1.6
9e	7.3 ± 0.6	15.8 ± 4.6	2.2
9h	14.2 ± 1.3	36.1 ± 2.2	2.5

156 ^a EC₅₀: half-maximal effective concentration. The values are mean ± SD of three independent experiments.
 157 ^b CC₅₀: half-maximal cytotoxic concentration. The values are mean ± SD of three independent experiments.
 158 ^c Selectivity index (SI) is the ratio of the CC₅₀ value to the EC₅₀ value.

160 2.2.3. Characterization of drug-like properties of compounds 6e-h

161 As compounds 6e to 6h have an additional amine function in their structure, the formation of their
 162 corresponding hydrochloride salts was then studied, in order to improve the aqueous solubility of
 163 these compounds. Indeed, gamhepathiopine 1 showed a very low solubility in phosphate buffer (8 μM,
 164 Table 5), which compromises its further pre-clinic development. The hydrochloride salts of compounds
 165 6e-h were readily obtained after their treatment with a hydrochloric acid solution in dioxane. ¹H NMR
 166 analysis of compound 6e showed the presence of two supplementary broad singlets at 10.70 and 13.14
 167 ppm, attributed to the protonation of the tertiary amine of the amino-alkyl chain and the N1 of the
 168 thienopyrimidine ring, respectively. Indeed, a ¹H-¹H COSY correlation was obtained between the two
 169 methyl groups of the tertiary amine (δ 2.73 ppm) and the new signal at 10.70 ppm. In addition, the
 170 triplet at 9.50 ppm attributed to the secondary amine correlates with the multiplet at 3.62 ppm,
 171 confirming that this position was not charged (Figure 2). The same results were observed for
 172 compounds 6f-g.



173
 174 **Figure 2:** Structural assignment of compound 6e. The main ¹H-¹H COSY correlations are indicated by arrows.

175 First, the activity of the hydrochloride salts in the blood- and hepatic-stage, as well as the cytotoxicity
 176 against primary simian hepatocytes were tested. The results are summarized in Table 4. As expected,
 177 the formation of their corresponding salts did not alter the activity of the compounds.

178 **Table 4:** *In vitro* activity (EC₅₀) against the blood stage of *P. falciparum*, against the hepatic stage of *P. berghei*
 179 parasites and cytotoxicity (CC₅₀) of the hydrochloride salts of compounds 6e-h.

Compound	<i>P. falciparum</i> K1 EC ₅₀ (μM) ^a	<i>P. berghei</i> EC ₅₀ (μM) ^a	Primary simian hepatocytes CC ₅₀ (μM) ^b
6e.2HCl	0.20 ± 0.10	14.4 ± 1.3	35.5 ± 3.5
6f.2HCl	0.20 ± 0.06	12.3 ± 1.1	27.6 ± 11.1
6g.2HCl	0.40 ± 0.10	14.2 ± 1.3	32.5 ± 3.3
6h.2HCl	0.30 ± 0.08	14.4 ± 1.4	59.1 ± 5.1

180 ^a EC₅₀: half-maximal effective concentration. Values are the mean ± SD of three independent experiments.

181 ^b CC₅₀: half-maximal cytotoxic concentration. Values are the mean ± SD of three independent experiments.

182

183 Then, their solubility in Phosphate-Buffered Saline (PBS), lipophilicity (cLog P and Log D) and
 184 topological polar surface area (TPSA) were determined and compared with those of Gamhepathiopine
 185 **1**. In addition, the transmembrane permeability of these compounds was assessed using the parallel
 186 artificial membrane permeation of the gastrointestinal tract (PAMPA-GIT) assay. Finally, microsomal
 187 stability and potential cardiotoxicity due to affinity for the hERG channels were assessed for these
 188 selected compounds **6e-h** (Table and 6).

189 **Table 5.** Physicochemical profile and permeability in the PAMPA gastrointestinal assay of selected thieno[3,2-
 190 *d*]pyrimidines.

Cpd	Solubility ^a (μM)	cLog P ^b	CHI Log D ^c	TPSA (Å ²) ^b	Pe (nm/s) ^d		
					pH		
					5.0	6.0	7.4
1 .HCl	7.92 ± 0.83 ¹⁹	3.83	4.10	45.87	0 ¹⁹	0 ¹⁹	4.6 ± 7.2 ¹⁹
6e .2HCl	1990 ± 82	4.59	4.29	44.81	361.6 ± 45.5	477.9 ± 10.5	672.9 ± 64.8
6f .2HCl	1395 ± 93	5.11	4.23	44.35	489.1 ± 74.4	465.7 ± 52.5	631.4 ± 124.5
6g .2HCl	1636 ± 38	4.56	4.50	44.81	430.1 ± 101.5	411.1 ± 77.4	649.3 ± 102.9
6h .2HCl	1140 ± 40	4.94	4.81	44.35	511.9 ± 69.1	520.9 ± 26.1	739.2 ± 112.2

191 ^a Solubility was determined in Phosphate-Buffered Saline. Values are the mean ± SD of three independent experiments.

192 ^b Calculated using the Molsoft LLC. chemoinformatic tool (2020).

193 ^c CHI: Chromatographic hydrophobicity index at pH = 7.4.

194 ^d Determined using a parallel artificial membrane permeability of gastrointestinal tract (PAMPA-GIT) assay.

195
 196 Insertion of a diamine moiety at position 4 improved the aqueous solubility of the thienopyrimidines
 197 at pH 7.4 by more than a factor of 100, compared to Gamhepathiopine **1**. These results can be
 198 explained by the presence of a tertiary amine on compounds **6e-h** that provides an additional charge
 199 to the molecule. The length of the alkyl chains carried by the tertiary amine slightly affected the
 200 solubility, with the *N,N*-dimethyl derivatives **6e** and **6g** being more soluble than their *N,N*-diethyl
 201 analogues **6f** et **6h**. Despite the introduction of an additional charge, all compounds showed good
 202 permeability in the PAMPA-GIT assay at all three pH levels tested, unlike reference **1**. The observed
 203 results can be explained by the high lipophilicity (cLog P > 4.5 and Log D_{7.4} > 4.1), reduced TPSA and
 204 improved aqueous solubility of these new thienopyrimidines.

205 Moreover, regarding microsomal stability (female mouse microsomes), 4-diamino-thienopyrimidines
 206 were significantly more stable than reference **1** (Table, *t*_{1/2} ≥ 37 min vs. 11 min). Therefore, the intrinsic
 207 clearance of these compounds decreased (Cl_{int} > 64 μL/min/mg vs. Cl_{int} > 240 μL/min/mg). **6e** was the
 208 most stable of these new analogues, with an estimated half-life of 73 min, more than 6-fold improved
 209 compared with Gamhepathiopine **1**. Finally, compounds **6f-h** showed some affinity for hERG channels
 210 (IC₅₀ ≥ 1 μM). Only **6e** did not inhibit hERG channels.

211

212

213

214

215 **Table 6.** Microsomal stability in presence of female mouse liver microsomes and hERG affinity of selected
 216 compounds.

Compound	Microsomal stability		hERG IC ₅₀ (μM)
	t _{1/2} (min)	Cl _{int} (μL/min/mg)	
1.HCl	11 ²²	240	ND ^a
6e.2HCl	> 40 (73 min ^b)	33	>100
6f.2HCl	37	61	1.41
6g.2HCl	37	64	3.40
6h.2HCl	37	63	1.07

^a ND: not determined

^b Extrapolated value.

217

218

219

220

3. Conclusions

221 A library of 25 compounds was synthesized from the scaffold of Gamhepathiopine (**1**), a potent
 222 antiplasmodial agent, active against all stages of *P. falciparum*. Various secondary and tertiary amines
 223 were introduced at position 4 of the thieno[3,2-*d*]pyrimidine scaffold, through a nucleophilic aromatic
 224 substitution reaction and reductive amination. Among the 25 analogues evaluated, 15 of them showed
 225 an EC₅₀ lower than 1 μM against the blood stage of *P. falciparum* K1 and were subsequently evaluated
 226 against the hepatic stage of *P. berghei*. Among them, compounds **6b** and **9** were the most potent and
 227 selective. Moreover, **6e-h** displayed good activity against the erythrocytic stage (EC₅₀ of 0.2-0.3 μM) of
 228 *P. falciparum*, better efficiency against the hepatic stage of *P. berghei* than Gamhepathiopine **1** and
 229 moderate toxicity against primary simian hepatocytes.

230 The physicochemical properties such as aqueous solubility, permeability and microsomal stability of 4-
 231 diaminothienopyrimidines were then evaluated. These new compounds displayed higher aqueous
 232 solubility (more than 1 mM), and better permeability than compound **1**. Compound **6e** appears to be
 233 the best candidate in this series, with a 251-fold higher solubility in PBS, 16-fold improved permeability
 234 and 6-fold improved microsomal stability than Gamhepathiopine **1**, while retaining good activity on
 235 the erythrocytic stage of *P. falciparum* (EC₅₀ of 0.3 μM) and on the liver stage of *P. berghei* (EC₅₀ of 15.3
 236 μM), without affinity for hERG channels. Further modulations are underway based on the
 237 thienopyrimidinone scaffold to improve their antiplasmodial activity and reduce the cytotoxicity of
 238 these analogues.

239

4. Experimental part

240

4.1. Chemistry

241

4.1.1. Material and methods

242 Commercial reagents and solvents were used without further purification. The synthesized products
243 were purified by chromatography on silica gel (40–63 μm) or by preparative HPLC on an apparatus
244 equipped with a Delta Pack C18 radial compression column (100 mm x 40 mm, 15 μm , 100 \AA) at a
245 wavelength of 214 nm, at a flow rate of 28 mL/min with a variable elution gradient from X % A (H_2O +
246 0.1% TFA) to X + 30% B (ACN + 0.1% TFA) in 30 min. The purest fractions are pooled, freeze-dried, and
247 lyophilized to give the final compounds. LC/MS analyses were recorded on a Quattro microTMESI triple
248 quadrupole mass spectrometer (ESI+ electrospray ionization mode) or on a Micromass ZQ
249 spectrometer (ESI+ electrospray ionization mode), coupled to an Alliance HPLC system (Waters,
250 Milford, CT, USA) equipped with a Chromolith High Resolution RP-18e column (25 x 4.6mm), with the
251 samples being previously separated using a gradient from 100% (H_2O + 0.1% HCO_2H) to 100% (ACN +
252 0.1% HCO_2H) in 3 min at a flow rate of 3 mL/min and with UV detection at 214 nm. UPLC/MS analyses
253 were recorded with an Acquity H-Class UPLC system, coupled to a Waters SQ Detector-2 mass
254 spectrometer (Waters, Milford, CT, USA). Chromatographic separation was carried out under the same
255 conditions as above using a Waters Acquity UPLC BEC C18 column (100 x 2.1 mm, 1.5 μm). High-
256 resolution mass spectrometry (HRMS) analyses were performed with a time-of-flight (TOF)
257 spectrometer coupled to a positive electrospray ionization (ESI) source. The elemental analysis was
258 realized with a Elementar Vario Micro Cube. The NMR spectra were recorded on a Brüker 400, 500, or
259 600 spectrometers. Chemical shifts are expressed in parts per million (ppm) relative to the residual
260 signal of the deuterated solvent used (CDCl_3 , DMSO-d_6 , MeOH-d_3 , ACN-d_3), and the coupling constants
261 J are expressed in Hertz. The multiplicities are designated as singlet (s), broad singlet (bs) doublet (d),
262 doublet of doublet (dd), triplet (t), quadruplet (q), quintuplet (qt), sextuplet (st), or multiplet (m).
263 Compounds **1** and **2** were synthesized according to the previous reported procedure and its physical
264 characteristics agreed with the published data^{20,21}.

265 4.1.2. General Procedure for the Synthesis of 4-Amino-thieno[3,2-*d*]pyrimidines **6a-p** and **7**.

266 Compound **5** (1 eq.) was dissolved in EtOH (22 mL/mmol). The appropriate amine (3 eq.) and Na_2CO_3
267 (2 eq.) were added to the solution and the reaction mixture was heated under reflux. If the reaction
268 was not completed after 96 h, another portion of the appropriate amine was added, and the heating
269 was continued until completion of the reaction (1 eq. was added for compound **6a**). Then, the mixture
270 returned to room temperature and was concentrated under reduced pressure. The mixture was
271 dissolved in water. The aqueous layer was extracted three times with EtOAc. Organic layers were
272 washed with brine, dried with MgSO_4 , and filtered and concentrated under vacuum. The crude was
273 purified by the appropriate method.

274 N^2 -(*Tert*-butyl)- N^4 -cyclopropyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6a**)

275 Following the general procedure, using **5** (350 mg, 1.055 mmol) and cyclopropylamine. The crude was
276 purified by chromatography on silica gel (eluent: 9/1 Hexane/AcOEt). The desired product was
277 obtained as a white powder (193 mg, 52%). ¹H NMR (500 MHz, DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.0
278 Hz), 7.46 (bs, 1H, NHCH), 7.35 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 5.82 (bs, 1H, NH), 2.91-
279 2.86 (m, 1H, CH), 2.34 (s, 3H, CH₃-tolyl), 1.42 (s, 9H, (CH₃)₃), 0.76-0.73 (m, 2H, CH₂), 0.61-0.58 (m, 2H,
280 CH₂); ¹³C NMR (125 MHz, DMSO-d₆): δ 162.1, 160.6, 157.8, 147.8, 138.7, 130.6, 129.8, 125.8, 118.7,
281 103.9, 49.9, 29.2, 23.9, 20.9, 6.90; HR-MS (ESI) C₂₀H₂₅N₄S calculated: 353.1794 [M+H]⁺, found:
282 353.1803.

283

284 *N*²-(*Tert*-butyl)-*N*⁴-cyclobutyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6b**)

285 Following the general procedure, using **5** (350 mg, 1.055 mmol) and cyclobutylamine. The crude was
286 purified by chromatography on silica gel (eluent: 9.5/0.5 to 9/1 Hexane/EtOAc) to afford the desired
287 product as a yellow solid (105 mg, 27%).

288 ¹H NMR (500 MHz, DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.53 (d, 1H, NHCH, *J* = 7.1 Hz), 7.34 (s, 1H,
289 H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 5.83 (bs, 1H, NH), 4.60 (sx, 1H, CH, *J* = 7.9 Hz), 2.34 (s, 3H,
290 CH₃-tolyl), 2.31-2.25 (m, 2H, CH₂), 2.12-2.04 (m, 2H, CH₂), 1.74-1.61 (m, 2H, CH₂), 1.40 (s, 9H, (CH₃)₃);
291 ¹³C NMR (125 MHz, DMSO-d₆): δ 162.0, 160.6, 155.5, 147.5, 138.7, 130.7, 129.8, 125.7, 118.9, 103.9,
292 49.8, 45.5, 30.5, 29.2, 20.9, 14.8; HR-MS (ESI) C₂₁H₂₇N₄S calculated: 367.1951 [M+H]⁺, found: 367.1967.

293

294 *N*²-(*Tert*-butyl)-*N*⁴-cyclopentyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6c**)

295 Following the general procedure, using **5** (350 mg, 1.055 mmol) and cyclopentylamine. The crude was
296 purified by chromatography on silica gel (eluent: 95/05 Hexane/EtOAc to 90/10) to afford the desired
297 product as a yellow solid (298 mg, 74%). ¹H NMR (500 MHz, DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.0 Hz),
298 7.33 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.22 (d, 1H, NHCH, *J* = 6.9 Hz), 5.81 (bs, 1H, NH),
299 4.44 (sx, 1H, CH, *J* = 6.9 Hz), 2.34 (s, 3H, CH₃-tolyl), 1.99-1.91 (m, 2H, CH₂), 1.76-1.66 (m, 2H, CH₂), 1.60-
300 1.50 (m, 4H, CH₂), 1.40 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): δ 161.8, 160.7, 156.2, 147.3,
301 138.7, 130.7, 129.8, 125.7, 118.8, 104.2, 51.8, 49.8, 32.4, 29.3, 23.7, 20.9; HR-MS (ESI) C₂₂H₂₉N₄S
302 calculated: 381.2107 [M+H]⁺, found: 381.2122.

303

304 *N*²-(*Tert*-butyl)-*N*⁴-cyclohexyl-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6d**)

305 Following the general procedure, using **5** (350 mg, 1.055 mmol) and cyclohexylamine. The crude was
306 purified by chromatography on silica gel (eluent: 99/01 Hexane/EtOAc to 90/10) to afford the desired
307 product as a yellow solid (274 mg, 66%). ¹H NMR (500 MHz, DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.0 Hz),
308 7.32 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.13 (d, 1H, NHCH, *J* = 7.5 Hz), 5.81 (bs, 1H, NH),

309 4.01-3.99 (m, 1H, CH), 2.34 (s, 3H, CH₃-tolyl), 1.94-1.91 (m, 2H, CH₂), 1.78-1.75 (m, 2H, CH₂), 1.64-1.61
310 (m, 1H, CH₂), 1.40 (s, 9H, (CH₃)₃), 1.36-1.25 (m, 4H, CH₂), 1.18-1.11 (m, 1H, CH₂); ¹³C NMR (125 MHz,
311 DMSO-d₆): δ 161.9, 160.7, 155.7, 147.3, 138.7, 130.7, 129.8, 125.7, 118.8, 104.1, 49.8, 49.1, 32.6, 29.2,
312 25.4, 25.3, 20.9; HR-MS (ESI) C₂₃H₃₁N₄S calculated: 395.2264 [M+H]⁺, found: 395.2278.

313

314 *N*²-(*Tert*-butyl)-*N*⁴-(3-(dimethylamino)propyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6e**)

315 Following the general procedure, using **5** (400 mg, 1.205 mmol) and 3-dimethylaminopropylamine. The
316 desired product was purified by recrystallization with acetonitrile (ACN) and the desired product was
317 obtained as a yellow solid (416 mg, 87%). ¹H NMR (600 MHz, DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.1 Hz),
318 7.36 (t, 1H, NHCH₂, *J* = 4.9 Hz), 7.33 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 5.76 (s, 1H, NH),
319 3.44 (q, 2H, CH₂NH, *J* = 4.9 Hz), 2.34 (s, 3H, CH₃-tolyl), 2.29 (t, 2H, CH₂N, *J* = 7.1 Hz), 2.13 (s, 6H, (CH₃)₂,
320 1.72 (qt, 2H, CH₂, *J* = 7.1 Hz), 1.41 (s, 9H, (CH₃)₃); ¹³C NMR (150 MHz, DMSO-d₆): δ 161.7, 160.7, 156.4,
321 147.2, 138.6, 130.6, 129.7, 125.7, 118.9, 104.2, 57.1, 49.8, 45.2, 38.8, 29.2, 27.0, 20.8; HR-MS (ESI)
322 C₂₂H₃₂N₅S calculated: 398.2378 [M+H]⁺, found: 398.2381.

323

324 *N*²-(*Tert*-butyl)-*N*⁴-(3-(diethylamino)propyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6f**)

325 Following the general procedure, using **5** (200 mg, 0.603 mmol) and 3-diethylaminopropylamine. The
326 crude was purified by recrystallization with ACN to afford the desired product as yellow crystals (249
327 mg, 97%). ¹H NMR (500 MHz, ACN-d₃): δ 7.62 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.30 (bs, 1H, NHCH₂), 7.26 (d, 2H,
328 CH_{ar}, *J* = 8.0 Hz), 7.22 (s, 1H, H-thiophene), 5.02 (s, 1H, NH), 3.59-3.55 (m, 2H, CH₂NH), 2.59-2.52 (m,
329 6H, CH₂N), 2.36 (s, 3H, CH₃-tolyl), 1.79-1.74 (m, 2H, CH₂), 1.45 (s, 9H, (CH₃)₃), 1.07 (t, 6H, CH₃, *J* = 7.1
330 Hz); ¹³C NMR (125 MHz, ACN-d₃): δ 162.8, 162.1, 157.7, 148.7, 140.2, 131.8, 130.6, 126.8, 120.0, 105.9,
331 53.3, 51.0, 47.6, 42.3, 29.5, 26.0, 21.2, 12.3; HR-MS (ESI) C₂₄H₃₆N₅S calculated: 426.2686 [M+H]⁺, found:
332 426.2689.

333

334 *N*²-(*Tert*-butyl)-*N*⁴-(2-(dimethylamino)ethyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6g**)

335 Following the general procedure, using **5** (150 mg, 0.452 mmol) and 2-dimethylaminoethylamine. The
336 desired product was obtained without purification as a yellow solid (158 mg, 91%). ¹H NMR (500 MHz,
337 DMSO-d₆): δ 7.65 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.34 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.22
338 (bs, 1H, NHCH₂), 5.86 (s, 1H, NH), 3.53 (q, 2H, CH₂NH, *J* = 6.4 Hz), 2.46 (t, 2H, CH₂N, *J* = 7.0 Hz), 2.34 (s,
339 3H, CH₃-tolyl), 2.18 (s, 6H, (CH₃)₂), 1.40 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): δ 161.9, 160.8,
340 156.5, 147.4, 138.7, 130.7, 129.8, 125.8, 118.9, 104.1, 58.3, 49.9, 45.4, 38.3, 29.3, 20.9; HR-MS (ESI)
341 C₂₁H₃₀N₅S calculated: 384.2222 [M+H]⁺, found: 384.2221.

342

343 *N*²-(*Tert*-butyl)-*N*⁴-(2-(diethylamino)ethyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine (**6h**)

344 Following the general procedure, using **5** (250 mg, 0.753 mmol) and *N,N*-diethylethylenediamine. The
345 desired product was obtained after purification by recrystallisation in ACN as a yellow solid (217 mg,
346 70%). ¹H NMR (500 MHz, ACN-d₃): δ 7.63 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.26 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.24 (s,
347 1H, H-thiophene), 5.72 (bs, 1H, NHCH₂), 5.04 (bs, 1H, NH), 3.57-3.52 (m, 2H, CH₂NH), 2.66 (t, 2H, CH₂N,
348 *J* = 6.6 Hz), 2.56 (q, 2H, CH₂N, *J* = 7.1 Hz) 2.36 (s, 3H, CH₃-tolyl), 1.45 (s, 9H, (CH₃)₃), 1.00 (t, 6H, CH₃, *J* =
349 7.1 Hz); ¹³C NMR (125 MHz, ACN-d₃): δ 163.2, 162.2, 157.9, 149.0, 140.3, 131.8, 130.7, 126.9, 120.1,
350 105.9, 52.6, 51.1, 47.8, 39.4, 29.6, 21.3, 12.5; HR-MS (ESI) C₂₃H₃₄N₅S calculated: 412.2529 [M+H]⁺,
351 found: 412.2526.

352
353 4-(Azetidin-1-yl)-*N*-(*tert*-butyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6i**)

354 Following the general procedure, using **5** (200 mg, 0.603 mmol) and azetidine. The desired product
355 was obtained without purification as a white powder (200 mg, 94%). ¹H NMR (500 MHz, DMSO-d₆): δ
356 7.64 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.32 (s, 1H, H-thiophene), 7.27 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 5.49 (s, 1H, NH),
357 4.28 (t, 4H, CH₂N, *J* = 7.5 Hz), 2.45-2.39 (m, 4H, CH₂), 2.36 (s, 3H, CH₃-tolyl), 1.43 (s, 9H, (CH₃)₃); ¹³C NMR
358 (125 MHz, DMSO-d₆): δ 162.2, 160.2, 157.6, 148.5, 138.4, 130.1, 129.2, 125.4, 118.2, 102.7, 50.1, 49.6,
359 28.8, 20.2, 16.2; HR-MS (ESI) C₂₀H₂₅N₄S calculated: 353.1800 [M+H]⁺, found: 353.1808.

360
361 *N*-(*Tert*-butyl)-4-(pyrrolidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6j**)

362 Following the general procedure, using **5** (200 mg, 0.603 mmol) and pyrrolidine. The desired product
363 was obtained after a recrystallization with ACN as yellow crystals (199 mg, 90%). ¹H NMR (500 MHz,
364 DMSO-d₆): δ 7.67 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.36 (s, 1H, H-thiophene), 7.27 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 5.83
365 (s, 1H, NH), 3.76-3.74 (m, 4H, CH₂N), 2.34 (s, 3H, CH₃-tolyl), 1.99-1.95 (m, 4H, CH₂), 1.40 (s, 9H, (CH₃)₃);
366 ¹³C NMR (125 MHz, DMSO-d₆): δ 162.7, 160.2, 155.7, 148.0, 138.8, 130.3, 129.8, 125.7, 118.7, 104.0,
367 49.8, 47.4, 29.3, 24.9, 20.9; HR-MS (ESI) C₂₁H₂₇N₄S calculated: 367.1956 [M+H]⁺, found : 367.1963.

368
369 *N*-(*Tert*-butyl)-4-(piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6k**)

370 Following the general procedure, using **5** (299 mg, 0.901 mmol) and piperidine. The crude was purified
371 by recrystallization with ACN to afford the desired product yellow crystals (313 mg, 91%). ¹H NMR (500
372 MHz, DMSO-d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.41 (s, 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz),
373 5.99 (s, 1H, NH), 3.84-3.82 (m, 4H, CH₂), 2.34 (s, 3H, CH₃-tolyl), 1.68-1.66 (m, 2H, CH₂), 1.63-1.61 (m,
374 2H, CH₂), 1.40 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): δ 163.8, 60.0, 157.1, 147.5, 139.0, 130.0,
375 129.8, 125.9, 119.0, 102.9, 49.8, 46.8, 29.2, 25.7, 24.3, 20.9; HR-MS (ESI) C₂₂H₂₈N₄S calculated:
376 381.2107 [M+H]⁺, found: 381.2123.

377
378 *N*-(*Tert*-butyl)-4-(4-methylpiperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6l**)

379 Following the general procedure, using **5** (150 mg, 0.452 mmol) and 4-methylpiperidine. The crude was
380 purified by recrystallization with ACN to afford the desired product as yellow crystals (243 mg, 71%).
381 ¹H NMR (500 MHz, DMSO-d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.40 (s, 1H, H-thiophene), 7.27 (d, 2H,
382 CH_{ar}, *J* = 8.0 Hz), 6.0 (s, 1H, NH), 4.60-4.58 (m, 2H, CH₂), 3.07 (t, 2H, CH₂, *J* = 12.2 Hz), 2.34 (s, 3H, CH₃-
383 tolyl), 1.74-1.67 (m, 3H, CH₂ + CH), 1.39 (s, 9H, (CH₃)₃), 1.18-1.10 (m, 2H, CH₂), 0.91 (d, 3H, CH₃, *J* = 6.2
384 Hz); ¹³C NMR (125 MHz, DMSO-d₆): δ 164.0, 160.1, 157.1, 147.5, 139.0, 130.0, 129.8, 125.9, 119.0,
385 102.9, 49.8, 46.1, 33.9, 30.7, 29.2, 21.8, 20.9; HR-MS (ESI) C₂₃H₃₁N₄S calculated: 395.2269 [M+H]⁺,
386 found : 395.2278.

387

388 *N*-(*Tert*-butyl)-4-(piperazin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6m**)

389 Following the general procedure, using **5** (200 mg, 0.6025 mmol) and piperazine. The crude was
390 purified by recrystallization in ACN to afford yellow crystals (200 mg, 87%). ¹H NMR (500 MHz, DMSO-
391 d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.41 (s, 1H, H-thiophene), 7.27 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 6.01 (s, 1H,
392 NH), 3.76-3.74 (m, 4H, CH₂N), 2.82-2.80 (m, 4H, CH₂NH), 2.34 (s, 3H, CH₃-tolyl), 1.39 (s, 9H, (CH₃)₃); ¹³C
393 NMR (125 MHz, DMSO-d₆): δ 164.0, 160.0, 157.6, 147.7, 139.0, 130.0, 129.8, 125.9, 119.0, 103.1, 49.8,
394 47.1, 45.7, 29.1, 20.9; HR-MS (ESI) C₂₁H₂₈N₅S calculated: 382.2065 [M+H]⁺, found: 382.2072.

395

396 *N*-(*Tert*-butyl)-4-(4-methylpiperazin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6n**)

397 Following the general procedure, using **5** (150 mg, 0.452 mmol) and 1-methylpiperazine. The crude
398 was purified by recrystallization in ACN to afford the desired product as yellow solid (138 mg, 77%). ¹H
399 NMR (500 MHz, DMSO-d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.43 (s, 1H, H-thiophene), 7.27 (d, 2H, CH_{ar},
400 *J* = 8.0 Hz), 6.05 (s, 1H, NH), 3.83-3.81 (m, 4H, CH₂), 2.44-2.42 (m, 4H, CH₂), 2.34 (s, 3H, CH₃-tolyl), 2.21
401 (s, 3H, CH₃N), 1.39 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): δ 164.1, 159.9, 157.5, 147.8, 139.1,
402 129.9, 129.8, 125.9, 119.0, 103.1, 54.5, 49.8, 45.7, 45.5, 29.1, 20.9; HR-MS (ESI) C₂₂H₃₀N₅S calculated:
403 396.2222 [M+H]⁺, found: 396.2229.

404

405 *N*-(*Tert*-butyl)-4-morpholino-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6o**)

406 Following the general procedure, using **5** (200 mg, 0.603 mmol) and morpholine. The crude was
407 purified by recrystallization with ACN to afford the desired compound as yellow crystals (168 mg, 73%).
408 ¹H NMR (500 MHz, DMSO-d₆): δ 7.67 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.37 (s, 1H, H-thiophene), 7.28 (d, 2H,
409 CH_{ar}, *J* = 8.1 Hz), 5.63 (s, 1H, NH), 3.84-3.81 (m, 4H, CH₂), 3.78-3.76 (m, 4H, CH₂), 2.36 (s, 3H, CH₃-tolyl),
410 1.44 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): δ 163.7, 159.6, 157.5, 147.8, 138.6, 129.7, 129.2,
411 125.5, 118.6, 103.4, 65.6, 49.5, 45.8, 28.8, 20.3; HR-MS (ESI) C₂₁H₂₇N₄OS calculated: 383.1906 [M+H]⁺,
412 found: 383.1911.

413

414 *N*-(*Tert*-butyl)-4-morpholino-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**6p**)

415 Following the general procedure, using **5** (300 mg, 0.904 mmol) and thiomorpholine. The crude was
416 purified by chromatography on silica gel (eluent: 90/10 Hexane/EtOAc to 70/30) to afford as a
417 yellowish solid (290 mg, 80%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.43 (s, 1H,
418 H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 6.09 (s, 1H, NH), 4.16-4.14 (m, 4H, CH₂), 2.73-2.71 (m, 4H,
419 CH₂), 2.34 (s, 3H, CH₃-tolyl), 1.39 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 164.2, 160.0, 156.8,
420 147.9, 139.1, 129.9, 129.8, 125.9, 119.1, 102.9, 49.8, 48.6, 29.1, 26.2, 20.9; HR-MS (ESI) C₂₁H₂₇N₄S₂
421 calculated: 399.1672 [M+H]⁺, found: 399.1679.

422 *Tert*-butyl(1-(2-(*tert*-butylamino)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-4-yl)piperidin-4-yl)carbamate (**7**)

423 Following the general procedure, using **5** (573 mg, 1.728 mmol) and 4-boc-aminopiperidine. The crude
424 product was purified by chromatography on silica gel (eluent: 5/5 Hexane/EtOAc) to afford the desired
425 product as a yellow solid (783 mg, 91%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.72-7.70 (m, 2H, CH_{ar}), 7.44
426 (s, 1H, H-thiophene), 7.29-7.27 (m, 2H, CH_{ar}), 6.93-6.91 (m, 1H, NHCH), 6.24 (bs, 1H, NH), 4.52-4.50 (m,
427 2H, CH₂), 3.62-3.62 (m, 1H, CHNH), 3.24 (m, 2H, CH₂), 2.34 (s, 3H, CH₃-tolyl), 1.87-1.85 (m, 2H, CH₂),
428 1.40-1.38 (m, 20H, (CH₃)₃ + CH₂); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 162.0, 158.8, 156.7, 154.9, 148.3,
429 139.3, 129.8, 129.7, 125.9, 118.1, 102.9, 77.7, 50.1, 47.2, 44.9, 42.3, 31.9, 29.0, 28.3, 20.9; HR-MS (ESI)
430 C₂₇H₃₇N₅O₂S calculated: 496.2746 [M+H]⁺, found: 496.2747.

431 4.1.3. Synthesis of 4-(4-aminopiperidin-1-yl)-*N*-(*tert*-butyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-
432 amine hydrochloride **8**

433 Compound **7** (465 mg, 0.9381 mmol) was dissolved in a 4/1 v/v solution of dioxane and 37% aqueous
434 HCl. The reaction mixture was stirred at room temperature for 1h. The reaction was concentrated
435 under reduce pressure. The solid was dissolved in ACN and water and freeze-dried. An off-white
436 powder was obtained (405 mg, 100%).

437 ¹H NMR (500 MHz, MeOH-*d*₃): δ 7.72 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.51 (s, 1H, H-thiophene), 7.34 (d, 2H,
438 CH_{ar}, *J* = 8.1 Hz), 4.96 (bs, 2H, CH₂), 3.66-3.64 (m, 1H, CH), 3.60-3.53 (m, 2H, CH₂), 2.41 (s, 3H, CH₃-tolyl),
439 1.81-1.73 (m, 2H, CH₂), 1.57-1.55 (m, 11H, CH₂ + (CH₃)₃); ¹³C NMR (125 MHz, DMSO-*d*₆): δ 157.6, 155.8,
440 152.7, 150.4, 142.7, 131.3, 129.9, 127.6, 113.0, 105.8, 53.7, 48.2, 46.7, 31.2, 29.0, 21.4; HR-MS (ESI)
441 C₂₂H₃₀N₅S calculated: 396.2216 [M+H]⁺, found: 396.2213.

442 4.1.4. General Procedure for the Synthesis of 4-(4-(Benzylamino)piperidin-1-yl)-thieno[3,2-
443 *d*]pyrimidines **9a-i**.

444 Compound **8** (1 eq.) was dissolved in methanol. Molecular sieve (4 Å) was added. Triethylamine (1 eq.)
445 and the appropriate aldehyde (5-8.5 eq.) were added. The reaction was stirred for 2h then NaBH₄ (5-

446 8.5 eq.) was added slowly at 0°C. The reaction mixture was stirred at room temperature until the end
447 of the reaction and water is added to destroy the excess of borohydride. The aqueous phase was
448 extracted three times with EtOAc. Then, organic layers were combined and washed with a saturated
449 sodium bicarbonate solution, dried with MgSO₄, filtered and concentrated under vacuum. The residue
450 was purified by the appropriate purification method.

451 4-(4-(Benzylamino)piperidin-1-yl)-*N*-(tert-butyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine (**9a**)

452 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
453 32 μL), benzaldehyde (123 mg, 1.158 mmol, 117 μL) and NaBH₄ (44 mg, 1.158 mmol). The crude
454 product was purified by chromatography on silica gel (eluent: 50/50 Hexane/EtOAc to 100% EtOAc) to
455 afford the desired compound as a white solid (81 mg, 72%). ¹H NMR (600 MHz, DMSO-*d*₆): δ 9.56 (bs,
456 2H, NH), 7.75 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.63-7.61 (m, 2H, CH_{ar}), 7.53 (s, 1H, H-thiophene), 7.46-7.39 (m,
457 3H, CH_{ar}), 7.31 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 4.71-4.69 (m, 2H, CH₂), 4.19 (s, 2H, CH₂NH), 3.48-3.43 (m, 1H,
458 CHNH), 3.29-3.27 (m, 2H, CH₂), 2.36-2.32 (m, 5H, CH₃-tolyl + CH₂), 1.83-1.76 (m, 2H, CH₂), 1.43 (s, 9H,
459 (CH₃)₃); ¹³C NMR (150 MHz, DMSO-*d*₆): 164.0, 160.0, 157.0, 147.5, 140.2, 138.9, 130.9, 130.0, 129.7,
460 128.2, 128.0, 126.1, 115.9, 103.2, 53.6, 50.7, 47.0, 44.3, 28.8, 27.8, 20.8; HR-MS (ESI) C₂₉H₃₅N₅S
461 calculated: 486.2686 [M+H]⁺, found: 486.2687.

462 *N*-(Tert-butyl)-4-(4-((4-methylbenzyl)amino)piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine
463 (**9b**)

464 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
465 32 μL), 4-methylbenzaldehyde (167 mg, 1.389 mmol, 164 μL) and NaBH₄ (66 mg, 1.736 mmol). The
466 crude product was purified by chromatography on silica gel (eluent: 70/30 Hexane/EtOAc to 40/60) to
467 afford the desired compound as a transparent solid (95 mg, 82%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 7.70
468 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.41 (s, 1H, H-thiophene), 7.27 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.23 (d, 2H, CH_{ar}, *J* = 7.9
469 Hz), 7.10 (d, 2H, CH_{ar}, *J* = 7.9 Hz), 6.00 (s, 1H, NH), 4.46-4.43 (m, 2H, CH₂), 3.71 (s, 2H, CH₂), 3.23-3.18
470 (m, 2H, CH₂), 2.74-2.69 (m, 1H, CHNH), 2.34 (s, 3H, CH₃-tolyl), 2.27 (s, 3H, CH₃-tolyl), 1.95-1.93 (m, 2H,
471 CH₂), 1.40 (s, 9H, (CH₃)₃), 1.36-1.28 (m, 2H, CH₂); ¹³C NMR (150 MHz, DMSO-*d*₆): 164.0, 160.1, 157.1,
472 147.5, 139.0, 138.1, 135.4, 130.0, 129.8, 128.7, 127.9, 125.9, 119.0, 102.9, 53.2, 49.8, 49.4, 44.5, 32.0,
473 29.2, 20.9, 20.8; HR-MS (ESI) C₃₀H₃₈N₅S calculated: 500.2842 [M+H]⁺, found: 500.2857.

474 *N*-(Tert-butyl)-4-(4-((4-methoxybenzyl)amino)piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-
475 amine (**9c**)

476 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
477 32 μL), 4-methoxybenzaldehyde (268 mg, 1.968 mmol, 239 μL) and NaBH₄ (74 mg, 1.968 mmol). The

478 crude product was purified by chromatography on silica gel (eluent: 70/30 Hexane/EtOAc to 100%
479 EtOAc) to afford the desired compound as a transparent solid (56 mg, 47%). ¹H NMR (500 MHz, DMSO-
480 d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.41 (s, 1H, H-thiophene), 7.28-7.26 (m, 4H, CH_{ar}), 6.87 (d, 2H, CH_{ar},
481 *J* = 8.7 Hz), 6.02 (s, 1H, NH), 4.48-4.45 (m, 2H, CH₂), 3.72-3.72 (m, 5H, CH₂NH + CH₃O), 3.23-3.18 (m,
482 2H, CH₂), 2.78-2.75 (m, 1H, CH), 2.34 (s, 3H, CH₃-tolyl), 1.97-1.95 (m, 2H, CH₂), 1.40 (s, 9H, (CH₃)₃), 1.37-
483 1.33 (m, 2H, CH₂); ¹³C NMR (150 MHz, DMSO-d₆): 164.0, 160.1, 158.1, 157.1, 147.5, 139.0, 132.4, 130.0,
484 129.8, 129.3, 125.9, 119.0, 113.6, 102.9, 55.0, 53.2, 49.8, 48.9, 44.5, 31.7, 29.2, 20.9; HR-MS (ESI)
485 C₃₀H₃₈N₅OS calculated: 516.2792 [M+H]⁺, found: 516.2792.

486 4-(4-((4-Bromobenzyl)amino)piperidin-1-yl)-*N*-(*tert*-butyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine
487 (**9d**)

488 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
489 32 μL), 4-bromobenzaldehyde (297 mg, 1.389 mmol) and NaBH₄ (53 mg, 1.389 mmol). The crude
490 product was purified by chromatography on silica gel (eluent: 70/30 Hexane/EtOAc to 100% EtOAc) to
491 afford the desired compound as a transparent solid (104 mg, 80%). ¹H NMR (500 MHz, DMSO-d₆): δ
492 7.70 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.49 (d, 2H, CH_{ar}, *J* = 8.3 Hz), 7.41 (s, 1H, H-thiophene), 7.32 (d, 2H, CH_{ar}, *J*
493 = 8.3 Hz), 7.27 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 6.01 (s, 1H, NH), 4.46-4.43 (m, 2H, CH₂), 3.72 (s, 2H, CH₂NH),
494 3.23-3.18 (m, 2H, CH₂), 2.73-2.67 (m, 1H, CH), 2.34 (s, 3H, CH₃-tolyl), 1.94-1.92 (m, 2H, CH₂), 1.39 (s,
495 9H, (CH₃)₃), 1.34-1.29 (m, 2H, CH₂); ¹³C NMR (125 MHz, DMSO-d₆): 164.0, 160.1, 157.1, 147.5, 140.9,
496 139.0, 131.0, 130.2, 130.0, 129.8, 125.9, 119.4, 119.0, 102.9, 53.3, 49.8, 48.9, 44.5, 32.1, 29.2, 20.9;
497 HR-MS (ESI) C₂₉H₃₅BrN₅S calculated: 564.1791 [M+H]⁺, found: 564.1791.

498 *N*-(*Tert*-butyl)-4-(4-((4-nitrobenzyl)amino)piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2-amine
499 bis(2,2,2-trifluoroacetate) (**9e**)

500 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
501 32 μL), 4-nitrobenzaldehyde (245 mg, 1.62 mmol) and NaBH₄ (61 mg, 1.62 mmol). The crude was
502 purified by preparative HPLC (ACN/H₂O + 0.1% TFA) to afford the desired compound as a white solid
503 (19 mg, 11%). ¹H NMR (500 MHz, DMSO-d₆): δ 9.32 (bs, 2H, NH), 8.33 (d, 2H, CH_{ar}, *J* = 8.8 Hz), 8.17 (bs,
504 1H, NH), 7.81 (d, 2H, CH_{ar}, *J* = 8.8 Hz), 7.78 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.55 (s, 1H, H-thiophene), 7.36 (d,
505 2H, CH_{ar}, *J* = 8.0 Hz), 4.74 (bs, 2H, NH + CH), 4.42 (s, 2H, CH₂NH), 2.39-2.36 (m, 5H, CH₃-tolyl + CH₂),
506 1.78-1.72 (m, 2H, CH₂), 1.46 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-d₆): 158.9 (q, ²*J* = 32.4 Hz),
507 155.8, 152.6, 151.7, 149.7, 147.8, 140.8, 139.5, 131.3, 130.2, 128.3, 126.4, 123.8, 116.9 (q, ¹*J* = 297.4),
508 112.3, 103.6, 53.5, 52.0, 46.3, 45.0, 29.1, 28.5, 28.0, 21.0; HR-MS (ESI) C₂₉H₃₅N₆O₂S calculated:
509 531.2537 [M+H]⁺, found: 531.2529.

510 *N*-(*Tert*-butyl)-6-(*p*-tolyl)-4-(4-((4-(trifluoromethyl)benzyl)amino)piperidin-1-yl)thieno[3,2-
511 *d*]pyrimidin-2-amine (**9f**)

512 Following the general procedure, using **8** (47 mg, 0.1088 mmol), triethylamine (11 mg, 0.1088 mmol,
513 15 μ L), 4-(trifluoromethyl)benzaldehyde (114 mg, 0.6527 mmol, 89 μ L) and NaBH₄ (24 mg, 0.6527
514 mmol). The crude product was purified by chromatography on silica gel (eluent: 99/01 DCM/MeOH to
515 95/05) to afford the desired compound as a transparent solid (38 mg, 63%). ¹H NMR (600 MHz, DMSO-
516 d₆): δ 7.70 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.66 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.59 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 7.40 (s,
517 1H, H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 8.0 Hz), 5.93 (s, 1H, NH), 4.46-4.44 (m, 2H, CH₂), 3.85 (s, 2H,
518 CH₂NH), 3.28 (s, 1H, NHCH), 3.25-3.21 (m, 2H, CH₂), 2.76-2.71 (m, 1H, CH), 2.34 (s, 3H, CH₃-tolyl), 1.97-
519 1.95 (m, 2H, CH₂), 1.40 (s, 9H, (CH₃)₃), 1.38-1.29 (m, 2H, CH₂); ¹³C NMR (150 MHz, DMSO-d₆): 164.0,
520 160.0, 157.1, 147.5, 146.5, 138.9, 130.0, 129.7, 128.5, 127.0, 125.8, 124.9-124.9 (m), 124.5 (q, ¹*J* =
521 275.5), 119.0, 102.9, 53.3, 49.7, 49.1, 44.4, 32.0, 29.1, 20.8; HR-MS (ESI) C₃₀H₃₅F₃N₅S calculated:
522 554.2560 [M+H]⁺, found : 554.2553.

523 *N*-(*Tert*-butyl)-6-(*p*-tolyl)-4-(4-((3-(trifluoromethyl)benzyl)amino)piperidin-1-yl)thieno[3,2-
524 *d*]pyrimidin-2-amine bis(2,2,2-trifluoroacetate) (**9g**)

525 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
526 32 μ L), 3-(trifluoromethyl)benzaldehyde (201 mg, 1.157 mmol, 156 μ L) and NaBH₄ (53 mg, 1.389
527 mmol). The crude was purified by preparative HPLC (ACN/H₂O + 0.1% TFA) to afford the desired
528 compound as a white solid (34 mg, 24%). ¹H NMR (500 MHz, DMSO-d₆): δ 9.13 (bs, 2H, NH₂CH₂), 7.96
529 (s, 1H, CH_{ar}), 7.84-7.81 (m, 2H, CH_{ar}), 7.78 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.72-7.69 (m, 1H, CH_{ar}), 7.58 (s, 1H,
530 H-thiophene), 7.36 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 4.75-4.72 (m, 2H, CH₂), 4.36 (s, 2H, CH₂NH), 3.61-3.50 (m,
531 3H, NHCH + CH₂), 2.37-2.36 (m, 5H, CH₃-tolyl + CH₂), 1.77-1.71 (m, 2H, CH₂), 1.46 (s, 9H, (CH₃)₃); ¹³C
532 NMR (125 MHz, DMSO-d₆): δ 158.6 (q, ²*J* = 31.8 Hz), 155.9, 152.5, 140.7, 134.4, 133.5, 130.1, 129.9,
533 129.4 (q, ²*J* = 31.8 Hz), 128.4, 126.8-128.8 (m), 126.4, 125.9-125.8 (m), 124.1 (q, ¹*J* = 272.5 Hz), 118.3,
534 115.9, 112.6, 103.6, 53.6, 51.8, 46.7, 44.9, 28.5, 28.0, 21.0; HR-MS (ESI) C₃₀H₃₅F₃N₅S calculated: 554.3
535 [M+H]⁺, found : 554.3.

536 *N*-(*Tert*-butyl)-4-(4-((4-chlorobenzyl)amino)piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2- amine
537 (**9h**)

538 Following the general procedure, using **8** (55 mg, 0.1273 mmol), triethylamine (13 mg, 0.1273 mmol,
539 18 μ L), 4-chlorobenzaldehyde (107 mg, 0.7638 mmol, 90 μ L) and NaBH₄ (29 mg, 0.7638 mmol). The
540 crude product was purified by chromatography on silica gel (eluent: 98/02 DCM/MeOH to 90/10) to
541 afford the desired compound as a transparent solid (47 mg, 71%). ¹H NMR (600 MHz, DMSO-d₆): δ 7.70
542 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.40-7.35 (m, 5H, CH_{ar} + H-thiophene), 7.28 (d, 2H, CH_{ar}, *J* = 7.9 Hz), 5.94 (s, 1H,

543 NH), 4.46-4.44 (m, 2H, CH₂), 3.76 (s, 2H, CH₂NH), 3.25-3.20 (m, 2H, CH₂), 2.75-2.72 (m, 1H, CH), 2.35 (s,
544 3H, CH₃-tolyl), 1.96-1.91 (m, 2H, CH₂), 1.40 (s, 9H, (CH₃)₃), 1.37-1.32 (m, 2H, CH₂); ¹³C NMR (150 MHz,
545 DMSO-d₆): 164.0, 160.0, 157.0, 147.5, 140.2, 138.9, 130.9, 130.0, 129.7, 128.2, 128.0, 125.8, 118.9,
546 102.9, 53.2, 49.7, 48.8, 44.4, 31.9, 29.1, 20.8; HR-MS (ESI) C₂₉H₃₅ClN₅S calculated: 520.2296 [M+H]⁺,
547 found : 520.2282.

548
549 *N*-(*Tert*-butyl)-4-(4-((3-chlorobenzyl)amino)piperidin-1-yl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidin-2- amine
550 bis(2,2,2-trifluoroacetate) (**9i**)

551 Following the general procedure, using **8** (100 mg, 0.2315 mmol), triethylamine (23 mg, 0.2315 mmol,
552 32 μL), 3-chlorobenzaldehyde (163 mg, 1.157 mmol, 131 μL) and NaBH₄ (53 mg, 1.389 mmol). The
553 crude was purified by preparative HPLC (ACN/H₂O + 0.1% TFA) to afford the desired compound as a
554 white solid (82 mg, 48%). ¹H NMR (500 MHz, DMSO-d₆): δ 9.24 (bs, 2H, NH₂CH₂), 8.22 (bs, 1H, NH), 7.77
555 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.66 (bs, 1H, CH_{ar}), 7.53 (s, 1H, H-thiophene), 7.52-7.47 (m, 3H, CH_{ar}), 7.35 (d,
556 2H, CH_{ar}, *J* = 8.1 Hz), 4.75-4.73 (m, 2H, CH₂), 4.27 (s, 2H, CH₂NH), 3.59-3.50 (m, 3H, CHNH + CH₂), 2.39-
557 2.36 (m, 5H, CH₃-tolyl + CH₂), 1.80-1.72 (m, 2H, CH₂), 1.47 (s, 9H, (CH₃)₃); ¹³C NMR (125 MHz, DMSO-
558 d₆): δ 158.9 (q, ²*J* = 32.7 Hz), 155.7, 152.7, 151.6, 149.6, 140.8, 134.4, 133.2, 130.6, 130.1, 129.9, 129.0,
559 128.7, 128.2, 126.4, 116.7 (q, ¹*J* = 297.1 Hz), 112.1, 103.6, 53.2, 51.9, 46.5, 44.9, 28.4, 27.9, 20.9; HR-
560 MS (ESI) C₂₉H₃₅ClN₅S calculated: 520.2296 [M+H]⁺, found : 520.2316.

561 4.1.5. General Procedure for the Synthesis of *N*²-(*tert*-butyl)-*N*⁴-(dialkylamino)alkyl)-6-(*p*-
562 tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine dihydrochloride

563 The appropriate substrate **6e-h** was dissolved in a solution of dioxane and HCl 37% in a ratio of 4/1 v/v.
564 The reaction mixture was stirred at room temperature for 1h. The reaction was concentrated under
565 reduce pressure. The solid was dissolved in ACN and water and freeze-dried.

566 *N*²-(*Tert*-butyl)-*N*⁴-(3-(dimethylamino)propyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine
567 dihydrochloride (**6e.2HCl**)

568 Following the general procedure, using **6e** (98 mg, 0.247 mmol). A white powder was obtained (116
569 mg, 100%). ¹H NMR (500 MHz, DMSO-d₆): δ 13.14 (bs, 1H, NH), 10.70 (bs, 1H, NH(CH₃)₂), 9.50 (t, 1H,
570 NHCH₂), 8.03 (bs, 1H, NH), 7.72 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.57 (s, 1H, H-thiophene), 7.34 (d, 2H, CH_{ar}, *J* =
571 8.1 Hz), 3.62 (q, 2H, CH₂NH, *J* = 6.2 Hz), 3.15-3.12 (m, 2H, CH₂N), 2.73 (s, 6H, CH₃N), 2.36 (s, 3H, CH₃-
572 tolyl), 2.10-2.05 (m, 2H, CH₂N), 1.47 (s, 9H, (CH₃)₃); ¹³C NMR (500 MHz, DMSO-d₆): δ 156.1, 152.8, 152.5,
573 146.6, 140.3, 130.0, 129.0, 126.2, 112.3, 105.3, 54.2, 52.0, 42.0, 38.4, 28.6, 23.4, 20.9; HR-MS (ESI)
574 C₂₂H₃₂N₅S calculated: 398.2373 [M+H]⁺, found: 398.2372.

575 *N*²-(*Tert*-butyl)-*N*⁴-(3-(diethylamino)propyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine
576 dihydrochloride (**6f**.2HCl)

577 Following the general procedure, using **6f** (118 mg, 0.277 mmol). A brown solid was obtained (122 mg,
578 88%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 13.15 (bs, 1H, NH), 10.63 (bs, 1H, NH(CH₂CH₃)₂), 9.56 (t, 1H,
579 NHCH₂, *J* = 5.3 Hz), 8.09 (bs, 1H, NH), 7.73 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.59 (s, 1H, H-thiophene), 7.34 (d,
580 2H, CH_{ar}, *J* = 8.2 Hz), 3.62 (q, 2H, CH₂NH, *J* = 6.2 Hz), 3.14-3.06 (m, 6H, CH₂N), 2.36 (s, 3H, CH₃-tolyl),
581 2.10-2.03 (m, 2H, CH₂N), 1.47 (s, 9H, (CH₃)₃), 1.21 (t, 6H, CH₃CH₂, *J* = 7.2 Hz); ¹³C NMR (500 MHz, DMSO-
582 *d*₆): δ 156.2, 152.7, 152.6, 146.5, 140.4, 130.1, 129.0, 126.3, 112.2, 105.3, 52.1, 48.3, 46.0, 38.7, 28.7,
583 22.7, 21.0, 8.4; HR-MS (ESI) C₂₄H₃₆N₅S calculated: 426.2686 [M+H]⁺, found : 426.2702.

584 *N*²-(*Tert*-butyl)-*N*⁴-(2-(dimethylamino)ethyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine
585 dihydrochloride (**6g**.HCl)

586 Following the general procedure, using **6g** (60 mg, 0.156 mmol). A yellow solid was obtained (70 mg,
587 98%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 13.31 (bs, 1H, NH), 10.80 (bs, 1H, NH(CH₃)₂), 9.70 (s, 1H, NHCH₂),
588 8.19 (bs, 1H, NH), 7.73 (d, 2H, CH_{ar}, *J* = 8.2 Hz), 7.59 (s, 1H, H-thiophene), 7.33 (d, 2H, CH_{ar}, *J* = 8.2 Hz),
589 3.92 (q, 2H, CH₂NH, *J* = 5.5 Hz), 3.40 (s, 2H, CH₂N), 2.84 (s, 6H, (CH₃)₂N), 2.36 (s, 3H, CH₃-tolyl), 1.46 (s,
590 9H, (CH₃)₃); ¹³C NMR (500 MHz, DMSO-*d*₆): δ 156.4, 153.0, 152.8, 146.7, 140.5, 130.1, 128.9, 126.3,
591 112.1, 105.7, 55.0, 52.1, 42.3, 36.4, 28.7, 21.0; HR-MS (ESI) C₂₁H₃₀N₅S calculated: 384.2216 [M+H]⁺,
592 found : 384.2231.

593 *N*²-(*Tert*-butyl)-*N*⁴-(2-(diethylamino)ethyl)-6-(*p*-tolyl)thieno[3,2-*d*]pyrimidine-2,4-diamine
594 dihydrochloride (**6h**.HCl)

595 Following the general procedure, using **6h** (128 mg, 0.311 mmol). A brown powder was obtained (150
596 mg, 100%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 13.29 (bs, 1H, NH), 10.81 (bs, 1H, NH(CH₂CH₃)₂), 9.75 (t,
597 1H, NHCH₂, *J* = 4.9 Hz), 8.19 (bs, 1H, NH), 7.33 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 7.59 (s, 1H, H-thiophene), 7.33
598 (d, 2H, CH_{ar}, *J* = 8.1 Hz), 3.96 (q, 2H, CH₂NH, *J* = 5.8 Hz), 3.39-3.36 (m, 2H, CH₂NH), 3.22-3.19 (m, 4H,
599 CH₂N,) 2.36 (s, 3H, CH₃-tolyl), 1.46 (s, 9H, (CH₃)₃), 1.24 (t, 6H, CH₃, *J* = 7.2 Hz); ¹³C NMR (500 MHz, DMSO-
600 *d*₆): δ 156.3, 153.1, 152.8, 146.7, 140.5, 130.1, 128.9, 126.3, 112.1, 105.6, 52.1, 49.5, 46.5, 36.0, 28.7,
601 21.0, 8.4; HR-MS (ESI) C₂₃H₃₄N₅S calculated: 412.2529 [M+H]⁺, found: 412.2537.

602 4.2. Biology

603 4.2.1. Blood-Stage Antiplasmodial Evaluation

604 A K1 culture-adapted *P. falciparum* strain resistant to chloroquine, pyrimethamine, and proguanil was
605 used in an in vitro culture. It was maintained in a continuous culture, as described previously by Trager
606 and Jensen²³. Cultures were maintained in fresh A+ human erythrocytes at 2.5% hematocrit in a

607 complete medium (RPMI 1640 with 25 mM HEPES, 25 mM NaHCO₃, and 10% of A+ human serum) at
608 37°C under a reduced O₂ atmosphere (gas mixture: 10% O₂, 5% CO₂, and 85% N₂). Parasitemia was
609 maintained daily between 1 and 3%. The *P. falciparum* drug susceptibility test was carried out by
610 comparing quantities of DNA in treated and control cultures of parasites in human erythrocytes,
611 according to a SYBR Green I fluorescence-based method²⁴ using a 96-well fluorescence plate reader.
612 Compounds, previously dissolved in DMSO (final concentration less than 0.5% v/v), were incubated in
613 a total assay volume of 200 µL (RPMI, 2% hematocrit and 0.4% parasitemia) for 72 h in a humidified
614 atmosphere (10% O₂ and 5% CO₂) at 37°C, in 96-well flat bottom plates. Duplicate assays were
615 performed for each sample. After incubation, plates were frozen at 20°C for 24 h. Then, the frozen
616 plates were thawed for 1 h at 37°C. A total of 15 µL of each sample was transferred to 96-well flat-
617 bottom non-sterile black plates (Greiner Bio-One, Kremsmünster, Austria) already containing 15 µL of
618 the SYBR Green I lysis buffer (2X SYBR Green I, 20 mM Tris base pH 7.5, 20 mM EDTA, 0.008% w/v
619 saponin, 0.08% w/v Triton X-100). Negative control treated by solvents (DMSO or H₂O) and positive
620 controls (chloroquine) were added to each set of experiments. Plates were incubated for 15 min at
621 37°C and then read on a TECAN Infinite F-200 spectrophotometer with excitation and emission
622 wavelengths at 485 and 535 nm, respectively. The concentrations of compounds required to induce a
623 50% decrease in parasite growth (EC₅₀ K1) were calculated from three independent experiments.

624 4.2.2. Hepatic-Stage Antiplasmodial Evaluation

625 4.2.2.1. Parasite Strains and Sporozoite Isolation

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

632 4.2.2.2. Primary Hepatocyte Culture

633 Primary simian hepatocytes were isolated from liver segments collected from healthy *Macaca*
634 *fascicularis* from the facilities of the Centre d'Energie Atomique (CEA) (Fontenay-aux-Roses, France).
635 All hepatocytes were obtained using collagenase perfusion, as previously described²⁶, and were
636 immediately cryopreserved. One day before infection, the cryopreserved hepatocytes were thawed at
637 37°C and were seeded into collagen-coated (5 µg/cm² rat tail collagen I, Invitrogen, Carlsbad, CA, USA)
638 96-well plate, at a density of 80,000 cells per well. They were cultured at 37°C in 5% CO₂ in William's E
639 medium (Gibco, Life Technologies, Saint Aubin, France) supplemented with 10% of Fetal Bovine Serum

640 FCIII, 5×10^{-5} M hydrocortisone hemisuccinate (Serb Laboratories, Paris, France), 5 μ g per ml bovine
641 insulin (Sigma Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, and 0.02 U per ml-0.02 μ g per ml
642 penicillinstreptomycin (Life Technologies) until infection with sporozoites.

643 4.2.2.3. *In Vitro* Infection and Drug Assays

644 Sporozoites of *P. berghei* were re-suspended in the above complete medium used for the hepatocytes
645 culture. Simian hepatocytes were inoculated in 50 μ L of complete media with 20,000 *P. berghei*-
646 sporozoites/well in 96-well plates. The infected culture plates were centrifuged for 6 min at 900x g to
647 allow fast parasite sedimentation onto the target cells and were further incubated with the serial
648 dilution of drugs that were prepared in advance. After 3 h of incubation, cultures were washed and
649 further incubated in a fresh medium containing the appropriate drug concentration, which were
650 changed every 24 h during the study period. Cultured hepatocytes were fixed using 4%
651 paraformaldehyde (PFA) for 15 min at room temperature. Host cell and parasite nuclei were labeled
652 with 4',6-diamidino-2-phenylindole (DAPI). Upon fixation and immunostaining, cell culture plates were
653 analyzed in order to determine the number and size of the parasites using a Cell Insight High Content
654 Screening platform equipped with the Studio HCS software (Thermo Fisher Scientific, Waltham, MA,
655 USA). The parasite size reduction was calculated on the average object area using the total surface
656 area of each selected object (μm^2). To assess the cell toxicity of drugs for hepatic cultures, fixed plates
657 were further scanned for the DAPI signal representing host nuclei. The analysis was based on counting
658 of total cell nuclei. GraphPad Prism 7 statistical software (GraphPad. Software, San Diego, CA, USA)
659 was used for data analysis and graphing. All values were expressed as means and standard deviations
660 (SD).

661 4.3. Physicochemical properties

662 4.3.1. Determination of solubility in PBS

663 1 mg of compound was weighted into an Eppendorf and 1 mL of PBS was added. The mixture was
664 sonicated for 10 min and then stirred at 25°C overnight. The mixture was centrifuged for 10 min at
665 3000 rpm. The supernatant was recovered and filtered. 10 μ L were injected in analytical HPLC. The
666 area under the curve corresponding to the product was measured at 214 nm and compared to the
667 calibration curves obtained after injection of a range of the product previously solubilized in methanol
668 (concentrations 50, 125, 250, 400, 500 μ M). Calibration curves were linear with $R^2 > 0.99$.

669 4.3.2. Chromatographic hydrophobicity index (CHI) Log $D_{7,4}$

670 The determination of Chromatographic Hydrophobicity Index (CHI)²⁷ at neutral pH was realized by
671 retention time measurements using a 1290 Agilent chromatographic system (Agilent Technologies,

672 Santa Clara, California, USA) equipped with a PDA detector 1260 operating at 220, 240, 254, 290 and
673 350 nm for all compounds using a Ultra C18 column (5 μ m, 3 x 50 mm) from Restek (Lisses, France).
674 Before measurements, compounds were dissolved (100 μ M) in methanol. In the used HPLC method,
675 the flow rate was 0.3 mL/min, the temperature was kept constant at 30°C. The mobile phase A was
676 made of 50 mM ammonium acetate aqueous solution, and the mobile phase B was ACN. A HPLC
677 gradient method was applied, with 0 to 0.2 min: 0% B solvent; 0.2 to 3.5 min: 0 to 100% B solvent; 3.5
678 to 4.0 min: 100% B solvent; post run: 2 min. Standard compounds were theophylline, 5-
679 Phenyltetrazole, benzimidazole, acetophenone, indole, propiophenone, butyrophenone,
680 valerophenone, with $R^2 > 0.999$. The Chromatographic Hydrophobicity data expressed as CHI LogD was
681 calculated as previously described)²⁸.

682 4.3.3. Permeability determination by PAMPA-GIT assay

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

694 4.3.4. hERG inhibition assay

695 hERG channel binding of the compound is determined with the PredictorTM hERG Fluorescence
696 Polarization Assay, that permits hERG channel biochemical binding studies in the absence of
697 radioligands. The assay is based on the principle of fluorescence polarization, where a red-shifted
698 fluorescent tracer displays a high polarization when bound to the hERG channel and a low polarization
699 when displaced by compounds that bind to the channel.

700 Materials and Methods: hERG channel binding was determined with the PredictorTM hERG
701 Fluorescence Polarization Assay (PV5665 ThermoFischer). Briefly, reagents were thawed, and were
702 mixed by pipetting 20x with PredictorTM hERG membranes. The fluorescent tracer was diluted at 4
703 nM in the PredictorTM hERG buffer. The tested compound was dissolved in DMSO at 10 mM and was
704 then diluted from 0.4 μ M to 0.04 nM in the PredictorTM hERG buffer. The assay was performed in a

705 96-well Greiner microplate (675093 Dutscher). 25 μ L of each concentration of M1 were dispensed into
706 the plate. Then, 50 μ L of the PredictorTM hERG membranes and 25 μ L of the fluorescent tracer 4 nM
707 were added. The plate was incubated at room temperature for 4 hours. The fluorescence polarization
708 measurements were made using a Synergy 2 microplate reader (Biotek Instrument, Colmar, France).
709 Both parallel and perpendicular fluorescence were measured using 530/25 nm excitation and 590/35
710 nm emission filters with a 570 nm dichroic mirror. The gain was fixed at 110. A known hERG channel
711 blocker (E-4031), provided by the kit, was assayed as positive reference. E-4031, LP0373 and LP0374
712 were tested at final concentrations ranging from 0.01 nM to 100 μ M. IC50 values were determined
713 graphically by plotting the % inhibition versus the logarithm of 9 concentrations of each compound in
714 the assay solution using the GraphPad Prism software (version 6.01, GraphPad Software, La Jolla, CA,
715 USA).

716 4.4. Microsomal stability

717 The tested products and propranolol, used as a reference, were incubated in duplicate (reaction
718 mixture volume of 0.5 mL) with female mouse microsomes (CD-1, 20 mg mL⁻¹; BD Gentest) at 37 °C in a
719 50 mM phosphate buffer, pH 7.4, in the presence of MgCl₂ (5 mM), NADP (1 mM), glucose-6-phosphate
720 dehydrogenase (G6PD) (0.4U per mL), and glucose-6-phosphate (5 mM). For the estimation of the
721 intrinsic clearance, 50 μ L aliquots were collected at 0, 5, 10, 20, 30, and 40 min and the reaction was
722 stopped with 4 volumes of acetonitrile (ACN) containing the internal standard. After centrifugation at
723 12000 rpm for 10 min at 4 °C, the supernatants are kept at 4°C for immediate analysis. Controls (time
724 zero and final time point) in triplicate were prepared by the incubation of the internal standard with
725 microsomes denatured by acetonitrile. The calculation of the half-life ($t_{1/2}$) of each compound in the
726 presence of microsomes was carried out according to the equation $t_{1/2} = (\ln 2)/k$, where k is the first-
727 order degradation constant (the slope of the logarithm of the compound concentration versus
728 incubation time). The intrinsic clearance in vitro (Cl_{int} , expressed in mL.min⁻¹.mg) was calculated
729 according to the equation $Cl_{int} = (\text{dose}/AUC_{\infty})/[\text{microsomes}]$, where dose is the initial concentration of
730 the product in the sample, AUC_{∞} is the area under the concentration–time curve extrapolated to
731 infinity, and [microsomes] is the microsome concentration expressed in mg.mL⁻¹.

732 5. Acknowledgements

733 This work was financially supported by the Agence Nationale de la Recherche (ANR Plasmodrug 18-
734 CE18-0009-01) and the Fondation pour la Recherche M´edicale (FRM Palukill DMC20181039565).

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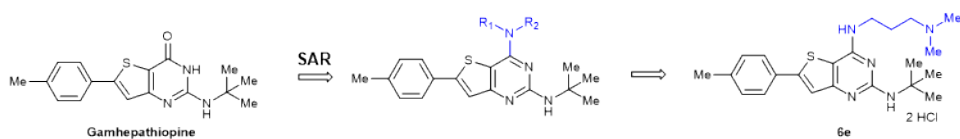
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Graphical abstract



- ✓ Active against *P. falciparum* erythrocytic stage
- ✓ Active against *P. berghei* hepatic stage
- ✗ Poorly soluble in PBS at pH 7.4
- ✗ Not permeable (PAMPA-GIT, pH 7.4)
- ✗ Weak microsomal stability

- ✓ Active against *Pf* erythrocytic stage
- ✓ Active against *Pb* hepatic stage
- ✓ Soluble in PBS at pH 7.4
- ✓ Permeable (PAMPA-GIT, pH 7.4)
- ✓ Improved microsomal stability

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861 Highlights

- 862 • A series of 25 new 4-amino-substituted thienopyrimidines was synthesized.
- 863 • 15 compounds showed an $EC_{50} \leq 1 \mu\text{M}$ against the blood stage of *P. falciparum* K1.
- 864 • 13 compounds showed higher activity against the hepatic stage of *P. berghei* than
- 865 Gamhepathiopine.
- 866 • Compound 6e shows improved aqueous solubility, PAMPA gastro-intestinal permeability and
- 867 microsomal stability compared to Gamhepathiopine.

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