Accepting the Invitation to Open Innovation in Malaria Drug Discovery: Synthesis, Biological Evaluation, and Investigation on the Structure-Activity Relationships of Benzo[b]thiophene-2-carboxamides as Antimalarial Agents

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Marco **Pieroni** ,[*,†] ■ ■ Elisa **Azzali** ,[†] ■ Nicoletta **Basilico** ,[2‡] ■ Silvia **Parapini** ,[3§] ■ Michal **Zolkiewski** ,[4∥] ■ Claudia **Beato** , ■ Giannamaria **Annunziato** , ■ Agostino **Bruno** , ■ Federica **Vacondio** , ■ **and** Gabriele **Costantino** ,[6¶] ■

[†] **P4T group**, and [†] **Department of Pharmacy**, **University of Parma** , Parco Area delle Scienze 27/A, Parma, 43124 , Italy

[2‡] **Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche**, **Università di Milano** , via Pascal 36, Milano 20122 , Italy

[3§] **Dipartimento di Scienze Farmacologiche e Biomolecolari**, **Università di Milano** , Milano 20122 , Italy

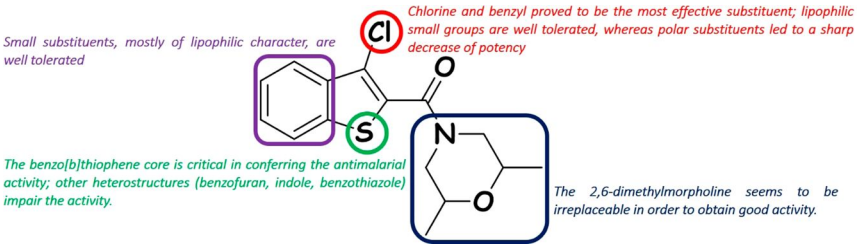
[4∥] **Department of Biochemistry and Molecular Biophysics**, **Kansas State University** , Manhattan, KSKansas 66506 , United States

[6¶] **Centro Interdipartimentale Misure (CIM) 'G. Casnati'**, **University of Parma** , Parco Area delle Scienze 23/A, 43124 Parma, Italy

Author: Postal codes are required for all affiliations. Please verify the postal codes we have added.

[*] **Corresponding Author** Marco Pieroni +39 0521 905054 ; E-mail marco.pieroni@unipr.it . Phone: +39 0521 905054 .

Malaria eradication is a global health priority, but current therapies are not always suitable for providing a radical cure. Artemisinin has paved the way for the current malaria treatment, the so-called Artemisinin-based Combination Therapy (ACT). However, with the detection of resistance to ACT, innovative compounds active against multiple parasite species and at multiple life stages are needed. GlaxoSmithKline has recently disclosed the results of a phenotypic screening of an internal library, publishing a collection of 400 antimalarial chemotypes, termed the "Malaria Box". After analysis of the datasetdata set, we have carried out a medicinal chemistry campaign in order to define the Structure-Activity Relationshipsstructure−activity relationships for one of the released compounds, which embodies a benzothiophene-2-carboxamide core. 35Thirty-five compounds were prepared, and a description of the structural features responsible for the in vitro activity against different strains of *P. falciparum* P. falciparum, the toxicity, and the metabolic stability is herein reported.



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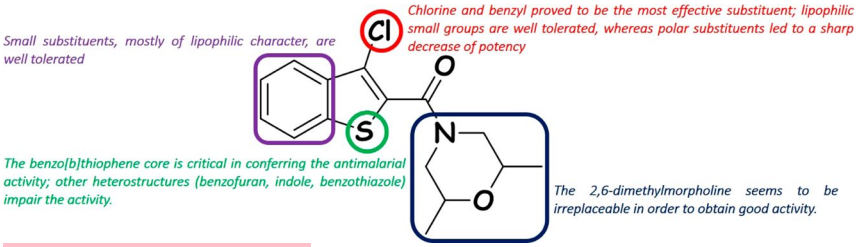

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~~Author Contributions~~ ~~‡These authors contributed equally to this work~~

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~~Keywords: Malaria; Open Innovation; Drug Discovery; Benzo[b]thiophenes; Target Fishing~~

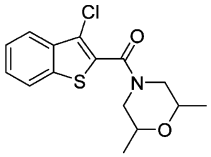
Introduction

Malaria is an infectious disease caused by the unicellular parasite *Plasmodium* ~~Plasmodium~~, that is transmitted by the females of certain species of mosquitoes of the *Anopheles* ~~Anopheles~~ genus.[1] Among the species of plasmodia that may infect humans, *P. vivax* ~~P. vivax~~ and *P. falciparum* ~~P. falciparum~~ are responsible for the majority of malaria infections that are often fatal.[2] Once in the human body, the parasites rapidly multiply in the liver and then in the red blood cells, leading, after ~~7–~~10 days, to the onset of the typical malaria symptoms such as fever, headache, chills, and vomiting. [3,4] If not treated, the hyperparasitemia may cause massive intravascular hemolysis, ~~multi-organ~~multiorgan failure, and eventually death. Over the years, malaria has become a major healthcare challenge, especially in developing countries.[5] Even more disturbing is the fact that among the hundreds of millions people infected with malaria, children under the age of 5 years and expectant mothers account for the majority of cases.[6]

Despite the recent encouraging developments, no effective vaccines are available yet~~,~~; therefore, chemotherapy still remains the only means to fight the infection.[7] Artemisinin-~~Combination Therapies~~combination therapies (ACTs) represent the current pillar of ~~the~~ malaria treatment.[8] After isolation of artemisinin from *Artemisia annua* ~~Artemisia annua~~ by Chinese researchers, [9,10] a discovery awarded ~~with~~ the 2015 Nobel Prize for Medicine,[11] ACTs have been used to cure millions of patients in the last decades. However, as for other tropical neglected infections, for which the first-line therapeutic options are relatively limited, drug resistance represents a persistent threat and patients with resistance to artemisinin and its ~~co-administered~~coadministered drugs are regularly isolated, especially in Asia.[12] In light of these facts, the search for innovative ways to target the parasite is a crucial aim at pursuing the policies of complete malaria eradication in the coming years. [13,14] A major obstacle for drug discovery in the field of many infectious diseases is the lack of adequate resources because of the limited commercial revenues. In spite of this fact, at least in the case of malaria and tuberculosis, some pharmaceutical companies, in collaboration with international agencies such as Medicine for Malaria Venture (MMV), have explored new models of collaboration with academia, encouraging open innovation platforms and making their chemical and biological tools available to academic institutions.[15] In 2010 GlaxoSmithKline released the structures of ~~1353~~13533 chemical probes that were confirmed to inhibit *P. falciparum* ~~P.falciparum~~ growth by at least 80% at 2 ~~μM~~μM concentration.[16] This work was further refined by clustering the molecules according to their structural similarities and cherry-picking 47 hit compounds based on their antimalarial activity, physicochemical properties, and dissimilarity to known antimalarial structures.[17] The final "Open Access Malaria Box" was thus assembled and delivered by MMV, with the overarching aim of catalyzing research ~~towards~~toward the discovery of new efficacious small molecules suitable for clinical development.[18] Among the released hits, five were

subsequently explored in-house by GSK, whereas the malaria community was openly invited to optimize the other chemical series. Due to our interest in novel antimalarial chemotypes,[19–21] we decided to pursue a medicinal chemistry campaign on one of the compounds of the malaria box, namely, (3-chlorobenzo[*b*]thiophen-2-yl)(2,6-dimethylmorpholino)methanone (**77**, Figure 1).

Figure 1. Structure of compound **77** .



Structure of compound **77**

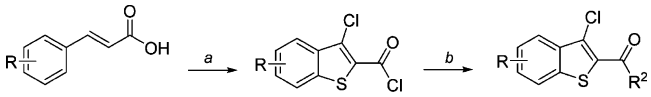
We focused our attention on this compound for several reasons. First, its baseline anti-~~Plasmodium~~*Plasmodium* activity was significantly higher than the average (*Pf*3d7 XC_{50} = 88 nM). Second, the molecule was amenable for chemical manipulation at various positions. The compound modifications could lead to a modulation and, perhaps, an improvement of potency and other pharmacokinetic characteristics. Moreover, the benzothiophene scaffold is found in many marketed drugs (raloxifene, zileuton, sertaconazole)[22] which suggests its usefulness for developing novel pharmaceuticals. Finally, when this work was about to be finalized, additional biological information about compound **77** ~~were~~was disclosed by a comprehensive ~~work~~report coordinated by van Voorhis et al.[23] In this paper, compounds belonging to the Malaria Box were ~~furtherly~~further explored with regard to their biological activity, PK characteristics, and metabolomic and chemogenomic profile. **77** was found to have a similar drug-drug chemogenomic profile. It has been found that the drug-drug chemogenomic profile of compound **77** was similar to that of the artemisinin sensitivity cluster. This result provided an additional rationale for further investigation of compound 77, although the molecular mechanism of its action could not be revealed.[23] In this publication, we report the rational design, synthesis, and biological antimalarial evaluation of a series of derivatives structurally related to compound **77**, leading to the description of SAR and to the characterization of the pharmacophore for this molecule. For the most active compounds, we also investigated their predicted metabolic properties and their putative molecular mechanism of action through a target fishing approach.

Results and ~~discussion~~Discussion

Chemistry

The 3-chlorobenzo[*b*]thiophene-2-carbonyl chloride core was easily prepared through the synthetic protocol reported by Krubsack and Higa,[24] and Brabander[25] starting from commercially available cinnamic acid that was refluxed with thionyl chloride and pyridine in chlorobenzene to give intermediates **6**–**10** (Scheme 1). Subsequent synthesis of amide derivatives was carried out refluxing the acyl chloride and the suitable amine in dioxane, to give the final compounds **11**–**25** (see Table 1).

Scheme 1. ~~*a*, *b*~~ *a*, *b*



1, R = H
2, R = 6-Cl
3, R = 6-F
4, R = 6-Me
5, R = 6,7-Cl

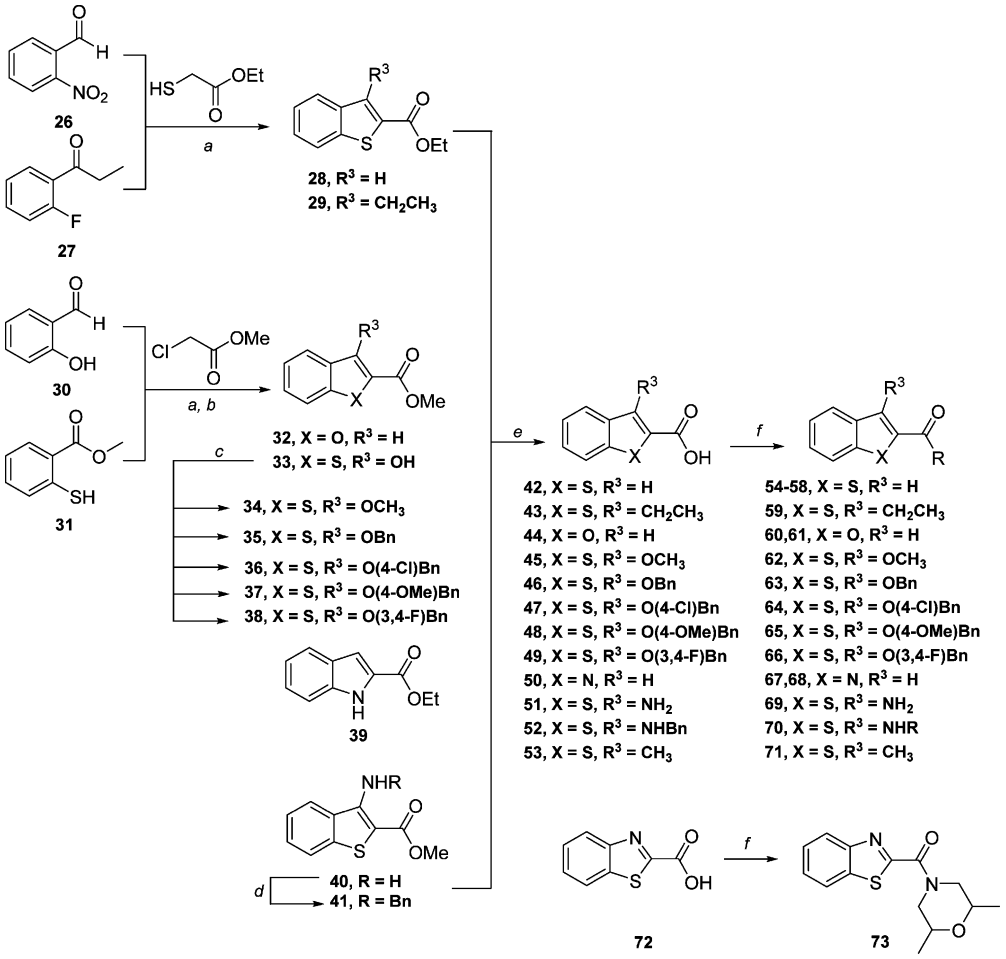
6, R = H
7, R = 6-Cl
8, R = 6-F
9, R = 6-Me
10, R = 6,7-Cl

11-21, R = H
22, R = 6-Cl
23, R = 6-F
24, R = 6-Me
25, R = 6,7-Cl

[a]Reagents and conditions: (a) $SOCl_2$, Pyr, DMF, or Chlorobenzene, 130 °C (16–62%); (b) RNH, dioxane, reflux (23–71%).

[b]For complete structures, see Table 1 .

Scheme 2. ~~*a*, *b*~~ *a*, *b*



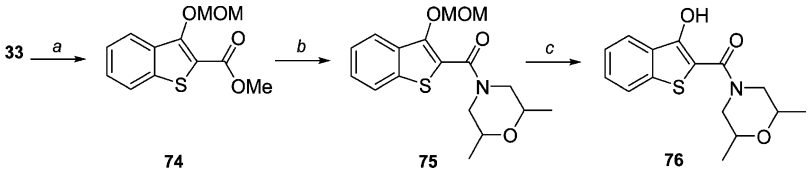
[a]Reagents and conditions: (a) anhydrous DMF, K_2CO_3 , 0 °C to rt (68–80%); (b) for comp **33**; *t*-BuONa anhydrous THF (91%); (c) NaH, R^3-X, anhydrous DMF, rt to 60 °C 1–3 h (35–58%); (d) benzyl bromide, K_2CO_3 , KI, CH_3CN, MW, 120 °C, 10 minutesmin × 3 times (43%); (e) LiOH, MeOH/H_2O/THF, 18 h, rt (91–97%); (f) TBTU, EDC·HCl, Et_3N, DMF, rt, 1–3 h (13–73%).

[b]forFor complete structures, see Table 1 .

The final compounds **54**–**71**, **73** , and intermediate **75** were obtained employing a conventional amide coupling protocol (SchemeSchemes 2 and 3), reacting the proper carboxylic acids with the suitable amines in the presence of 1-ethyl-3-(3-dimethylaminopropyl(dimethylamino)propyl)carbodiimide (EDC) and 2-(1H1*H* -Benzotriazolebenzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as coupling agents and triethylamine as the base. In the case of compounds **50**, **51**, and **73**, either the carboxylate ester **39**, **40** or the carboxylic acid **72** precursors were commercially available, whereas for the majority of the derivatives reported the synthesis of the carboxylic acid core was carried out from commercially available precursors. The benzofuran-2-carboxylic acid intermediate **32** was obtained according to a previously reported protocol,[26] reacting salicylaldehyde with methylchloroacetate in DMF and K_2CO_3, to obtain the corresponding methyl ester, subsequently cleaved through basic hydrolysis. Following a similar procedure, but using methyl thiosalicylate in the place of salicylaldehyde, the ethyl 3-hydroxybenzo[*b*]thiophene-2-carboxylate precursor **33** was obtained. However, it was not possible to hydrolyze **33**, also in harsh conditions, likely because of the formation of a pseudo-cyclepseudocycle between the 2-carboxylic moiety and the vicinal 3-hydroxyl. Indeed, when the hydroxyl group was masked either with a methyl or with a substituted benzyl group (intermediates **34**–**38**) or protected with a

methoxymethyl group (intermediate **74**), the corresponding carboxylic acid derivatives (**45**−**49**) were easily obtained in the ~~above~~previously reported conditions. After coupling with 2,6-dimethylmorpholine, MOM derivative **75** was deprotected with conc HCl to give the final derivative **76**~~.~~.

Scheme 3. [a]



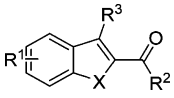
[a] ~~R eagents~~Reagents and conditions: (a) MOMCl, anhydrous DCM, then DIPEA at 0 °C to rt, 2 h (86%); (b) 1. LiOH, MeOH/H_2O/THF, 18 h, rt (91%); 2. TBTU, EDC·HCl, Et_3N, DMF, rt, ~~1~~–3 h (41%); (c) HCl ~~6N~~6 N, MeOH, 1 h rt (82%).

The benzo[*b*]thiophene-2-carboxylic acid was obtained reacting *o*-nitrobenzaldehyde with ethyl-2-mercaptoacetate in DMF and K_2CO_3 as the base to yield the ethyl ester **28**, that was hydrolyzed in basic conditions to afford the corresponding acid **42**. Following a similar procedure, but using 2-fluoropropiophenone in place of *o*-nitrobenzaldehyde, the ethyl ester intermediate **29** was obtained and hydrolyzed to give the precursor **43**. All of the ester hydrolyses were carried out reacting the suitable ester in a mixture of THF/MeOH/H_2O with LiOH at room temperature.

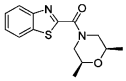
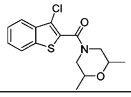
In this work, a total of 35 compounds were prepared and tested for their capability to inhibit the growth of *P. falciparum* ~~P. falciparum~~ using the pLDH assay. The compounds were tested both against the CQ susceptible (D10) and CQ resistant (W2) strains, in order to evaluate their susceptibility to cross resistance. Among the 35 derivatives, 9 showed a remarkable potency in the low ~~nM~~nanomolar range and therefore were further evaluated for their toxicity against a ~~Human Microvascular Endothelial Cell~~human microvascular endothelial cell line, and their metabolic stability in human liver microsomes. Rational modifications of the main structure led to a variable range of activities and allowed us to construct a plausible SAR for this series of benzo[*b*]thiophene-2-carboxamides, that could eventually inspire the design of improved analogues. In addition, an attempt to identify the actual molecular target of these derivatives was made through a “target fishing” approach.

We identified four portions of hit compound **77** that were amenable for chemical modifications: (a) the carboxamide substituent, (b) the substituent a position C-3, (c) the phenyl portion of the benzo[*b*]thiophene core, and (d) the benzo[*b*]thiophene ring.

Table 1. Antiparasitic and ~~cytotoxic activity~~Cytotoxic Activity of ~~compounds~~Compounds **11**−**25**, **54**−**71**, **73** , and **76**~~.~~



Comp	X	R^1	R^2	R^3	ClogP[a]	IC_{50} (nM)[b]		IC_{50} HMEC-1[e] (μM)	$T_{1/2}$ HLM min[f]
						D10[c]	W2[d]		
11	S	H		Cl	3,73	37±9	184±61	195±64	40±3
12	S	H		Cl	3,73	645±64	2685±542	154±6	nd[g]
13	S	H		Cl	3,71	4909±893	14749±203	nd	nd
14	S	H		Cl	2,69	>15000	>15000	nd	nd
15	S	H		Cl	3,62	>15000	>15000	nd	nd
16	S	H		Cl	4,73	>15000	>15000	nd	nd
17	S	H		Cl	3,87	>15000	>15000	nd	nd
18	S	H		Cl	3,03	>15000	>15000	nd	nd
19	S	H		Cl	3,03	>15000	>15000	nd	nd
20	S	H		Cl	3,58	>15000	>15000	nd	nd
21	S	H		Cl	4,76	100±6	461±42	149±26	nd
22	S	6-Cl		Cl	4,45	407±29	2094±532	nd	nd
23	S	6-F		Cl	3,88	74±27	455±52	nd	nd
24	S	6-Me		Cl	4,22	86±22	596±145	201±68	nd
25	S	6,7-Cl		Cl	5,01	2223±388	7436±114	nd	nd
54	S	H		H	2,97	1823±363	8541±259	nd	nd
55	S	H		H	2,96	>15000	>15000	nd	nd
56	S	H		H	1,51	10893±46	7754±175	nd	nd
57	S	H		H	2,49	>15000	>15000	nd	nd
58	S	H		H	1,93	>15000	>15000	nd	nd
59	S	H		Et	5,02	682±82	2310±241	nd	nd
60	O	H		H	2,37	>15000	>15000	nd	nd
61	O	H		H	2,36	>15000	>15000	nd	nd
62	S	H		OMe	4,18	429±20	1726±102	nd	nd
63	S	H			5,94	38±5	142±13	73±3	2,1±0
64	S	H			6,65	186±14	728±75	48±19	nd
65	S	H			5,86	146±5	877±197	83±12	nd

Comp	X	R^1	R^2	R^3	ClogP[a]	IC_{50} (nM)[b] D10[c]	IC_{50} (nM)[b] W2[d]	IC_{50} HMEC-1[e] (μM)	$T_{1/2}$ HLM min[f]
66	S	H			6,16	141±43	747±163	97±34	nd
67	N	H		H	2,21	>15000	>15000	nd	nd
68	N	H		H	2,20	>15000	>15000	nd	nd
69	S	H		NH_2	3,29	8761±113	>15000	nd	nd
70	S	H			5,52	2615±359	9768±235	nd	nd
71	S	H		Me	4,50	226±14	753±214	142±38	44±1
76	S	H		OH	3,89	>15000	>15000	nd	nd
73					2,13	9788±941	>15000	nd	nd
77[h]					3,73	na[h]	260	nd	nd
			CQ			16±4	400±120	>100	

[f]The data reported are the means of 3 different experiments ± SD ~~made~~run in duplicate~~;~~. *indicates the point of attachment.

[a]Calculated with molinspiration sotware (http://www.molinspiration.com/services/faq.html~~);~~.

[b]Indicates the concentration to inhibit the growth of half of parasitic population~~;~~.

[c]CQ-susceptible strain~~;~~.

[d]CQ -resistant strain~~;~~.

[e]Human ~~Microvascular Endothelial Cells;~~microvascular endothelial cells.

[f]Calculated in ~~Human Liver Microsomes~~human liver microsomes and expressed in minutes~~;~~.

[g]Not determined~~;~~.

[h]for complete set of data on different strains see ~~reference~~ref 23. ~~*indicates the point of attachment.~~

Substituents at the 2-~~carboxamide nitrogen~~Carboxamide Nitrogen

Modifications at this position were carried out in order to answer the following structural questions: (a)~~can~~Can the morpholine be substituted by other heteroaliphatic structures? (b) Are the methyl groups important for the activity? (c) Can an aromatic group be located at this position, in order to make the nitrogen a hydrogen bond donor (HBD)? First, we deemed it relevant to establish which configuration was the most favorable for the two methyl groups of the morpholine. Indeed, in the work that has inspired our research, it was not specified whether the *cis* or *trans* stereoisomer is more active. Compounds **11** and **12** were then prepared and, although both did show ~~the~~ activity, the *cis* stereoisomer was found to be about 20 times more potent than the corresponding *trans* analogue (**11**, IC_{50D10} = 37.1 nM vs **12**, IC_{50D10} = 646 nM). Thus, the *cis* configuration was maintained in all of the other analogues bringing a 2,6-dimethylmorpholine in their structure (see the following paragraphs). Next, the obvious step was to investigate the impact of the dimethyl pattern on the overall activity. Surprisingly, removal of the dimethyl substitution on the morpholine ring (**14**, IC_{50D10} ~~= >15000~~> 15000 nM), as well as on the piperidine (**15**, IC_{50D10} ~~= >15000~~> 15000 nM), led to a complete loss of activity. The same was observed when a linear aliphatic amine such as diethylamine (**20**, IC_{50D10} ~~= >15000~~> 15000 nM) was used in place of a cycloaliphatic one. We then investigated whether the substitution of the oxygen with a nitrogen atom, that could serve as a hydrogen bond donor, could play a role in the interactions with

the target binding site and affect the activity. Substituting the 2,6-dimethylmorpholine core with a 3,5-dimethylpiperazine (**13**, IC_{50D10} = 4909 nM) led to a drastic reduction ~~of~~in the antiparasitic activity, that was restored to some extent when 3,5-dimethylpiperidine was used as the substituent (**21**, IC_{50D10} = 100 nM). Finally, we investigated whether the 2-carboxamide could have a hydrogen bond donor property to better interact with the target binding site. Therefore, compounds **16**–**19**, bearing either cycloaliphatic or aromatic/heteroaromatic rings as substituents, were synthesized and tested. In particular, the 4-aminopyridyl derivative was prepared due to its recurrent presence ~~both~~ in both some marketed antimalarial compounds and preclinical candidates. Unfortunately, all of these modifications only led to the loss of activity (**16**–**19**, IC_{50D10} ~~= >15000~~> 15000 nM). Finally, some representative compounds, namely, **11**, **19** , and **23**, were evaluated with regard to their stability in RPMI 1640, that is, the medium used for the antiplasmodial assays. Indeed, by virtue of the 3-chloro substitution, these compounds might behave as vinylogue acid chlorides, which are very reactive and may decompose or unselectively interact with every protein and generate a false positive. However, the resulting compounds ~~resulted~~were unmodified at the test conditions also after 24 h (Supporting Information, S19).

From this first round of modifications, we can conclude that the 2,6-dimethylmorpholine moiety is crucial for conferring high antimalarial activity. Attempts to substitute the ring with other structures, as well as to remove the dimethyl group, all led to a loss/decrease of activity. The only exception, especially considering the difference in the activity with compound **13**, is represented by the 3,5-dimethylpiperidine derivative **21**, that has an activity similar to that of the hit compound **77**. It can ~~be~~ then be speculated that, in order to maintain the activity, hydrogen bond donor groups cannot be introduced in place of the morpholine oxygen.

Substitutions at the ~~phenyl portion~~Phenyl Portion of the ~~benzo~~Benzo[*b*]thiophene ~~core~~Core

After exploring the best substituent for the amide, and maintaining the chlorine atom at position C-3 as for the hit compound, we investigated whether adding small functional groups at the phenyl portion of the benzo[*b*]thiophene scaffold might affect the activity and the pharmacokinetic parameters. Electron withdrawing groups (EWGs) of different size, and an electron donor group (EDG) such as the methyl, were introduced at position C-6 of the benzo[*b*]thiophene scaffold. In addition, a dichloro substitution pattern was investigated as well. Small ~~size~~sized substituents, regardless of their EDG or EWG nature, were found to properly maintain strong antimalarial activity (**23**, IC_{50D10} = 74 nM; **24**, IC_{50D10} = 86 nM). At the same position, a chlorine is still well tolerated, although decreasing the activity by 5-fold compared to the fluorine atom (**22**, IC_{50D10} = 407 nM), whereas the dichloro substitution led to a considerable drop in the activity (**25**, IC_{50D10} = 2223 nM). Therefore, ~~this~~the structural portion of ~~the~~this molecule is amenable for various modifications, which can affect the activity and other pharmacological properties.

Substituents at ~~position~~Position C-3

The 3-chlorobenzothiophene scaffold has ~~been~~ seldom been used in medicinal chemistry, possibly due to the difficulties in its preparation. The chlorine atom at C-3 might affect the electronic characteristics of the main benzo[*b*]thiophene, and also those of the amide moiety. To explore the effect of various substituents, we decided to introduce at C-3 a wide range of functional groups, differing in size, and electronic and physicochemical properties. Removing the chlorine atom produced a compound that was found to be 50-fold less active than the parent derivative (**54**, IC_{50D10} = 1823 nM). As expected, the same results were obtained when cycloaliphatic moieties other than the 2,6-dimethylmorpholine core were used to modify the carboxamide portion (**56**, IC_{50D10} = ~~10893~~10893 nM; **55**, **57**, **58**, IC_{50D10} ~~= >15000~~> 15000 nM). Substitution with polar groups, having a hydrogen bond donor/acceptor nature, led to a sharp decrease, or even loss of activity (**69**, IC_{50D10} = 8761 nM; **76**, IC_{50D10} ~~= >15000~~> 15000 nM). On the other hand, apolar substituents at the same position, such as the methyl (**71**, IC_{50D10} = 226 nM), methoxy (**62**, IC_{50D10} = 429 nM), or ethyl (**59**, IC_{50D10} = 682 nM) were able to maintain a remarkable activity, only 4-fold lower than that of compound **71**. Therefore, it might be speculated that, at the position ~~being object~~ of the described modifications, the lipophilicity of the substituent, rather than its EWG/EDG properties, plays an important role in conferring good overall activity. Thus, we evaluated next how the size of the substituent at C-3 could modulate the compound activity. A benzyl moiety was used to functionalize the amino and hydroxyl group of compounds **69** and **76**, to yield derivatives **70** and **63**, respectively. Although derivative **69** is more active than **76**, the corresponding benzyl derivative was found to be 70-fold less active than the benzyloxy counterpart (**63**, IC_{50D10} = 38 nM vs **70**, IC_{50D10} = 2615 nM). Indeed, compound **63** can be considered the most active derivative of the series, ~~being~~with the activity being toward both the chloroquine susceptible and resistant strain ~~on~~in the same range of the hit compound **11**. Moreover, with this compound we have moved from the 3-chlorobenzothiophene scaffold that could be unselectively reactive, as mentioned above. Modifications of the benzyl appendage were attempted as well, in particular, by adding a lipophilic EWG such as the chlorine, a more polar EDG such as the methoxy, and a disubstitution such as the 3,4-difluoro

moiety (**64**, IC_{50D10} = 186 nM; **65**, IC_{50D10} = 146 nM; **66**, IC_{50D10} = 141 nM). All of these derivatives maintained activity against both the susceptible and resistant strain of *P. falciparum* ~~P. falciparum~~, although they were 6- to 7-fold less active than the unsubstituted parent compound **63**. In summary, at position C-3, along with the phenyl portion of the benzo[*b*]thiophene ~~above described~~described earlier, we have noticed the highest freedom for substitution. Polar groups were not found to be suitable substituents, whereas lipophilic functional groups, especially the bulky ones, supported the highest activity. In particular, the presence of polar functionalities (**69**, **70** , and **76**) strongly hampers the activity of these derivatives. Thus, it can be speculated that the target binding site is predominantly hydrophobic and lacks hydrogen bonding groups.

Modification of the ~~benzo~~Benzo[*b*]thiophene ~~core~~Core

The final part of our SAR assessment was dedicated to ~~explore~~exploring the possibility of substituting the benzo[*b*]thiophene core with similar heterocycles, maintaining the same pattern of substitution at the C-2. The sulfur atom, although present in several drugs, might be oxidized in the biological systems, and this may represent a limitation for further development of these derivatives. Therefore, the benzo[*b*]thiophene scaffold was substituted by a benzofuran, an indole, and a benzothiazole. As reported in Table 1 , benzofuran and indole analogues (**60**, **61**, **68**, **69**, IC_{50D10} ~~= >15000~~> 15000 nM) failed to show any ~~clue~~hint of activity, when compared to the benzo[*b*]thiophene counterpart; the benzothiazole **73**, that still maintains a sulfur atom at the same position as the hit compound, showed only ~~a~~ negligible activity (IC_{50D10} ~~= >~~> 9788 nM). It can be then hypothesized that the sulfur atom, by virtue of its size and physical characteristics, ~~it~~ is crucial in conferring the desired antiparasitic activity.

The most active derivatives (**11**, **12**, **21**, **24**, **63**−**66**, **71**) were tested against HMEC-1 cells to gain information about their general toxicity. All of them proved to be reasonably safe, the selectivity index spanning from 250 to 5000.

Target ~~fishing experiments~~Fishing Experiments

Next, we attempted to find a putative target for the compound derivatives. Finding the target could help in further optimization of analogues with improved drug-like characteristics. An extended study sponsored by Medicine for Malaria Venture reported a close analogue of the hit compound **11** as inhibitor of *P. falciparum* ~~P. falciparum~~ choline kinase.[27] Although this compound inhibits ~~Choline Kinase~~choline kinase quite efficiently (IC_{50} *Pf*CK = 6.3 ~~μM~~μM), its inhibitory activity toward the parasite is considerably higher (EC_{50} *Pf*3d7 = 0.3 ~~μM~~μM), suggesting that choline kinase is not the main target for this series of derivatives. In another work, Tate and colleagues have reported molecules based on a benzo[*b*]thiophene-2-carboxylic scaffold as potent inhibitors of the *P. falciparum* ~~P. *falciparum*~~*N* ~~N~~-myristoyltransferases (*Pf*NMT).[28,29] However, the SAR for our molecules (see above) does not match that described by Tate et al., making it unlikely that *Pf*NMT would be the target for our compounds. Finally, in the recent paper ~~reported~~ by Van Voorhis et al. ~~above mentioned,~~[23] a mechanism-of-action screening was successfully carried out for a number of compounds of the Malaria Box, but only a generic set of information was retrieved for **77**. In an attempt to ~~find out~~determine the putative target for this series, we have performed an in silico target fishing study.[30] We performed a 2D similarity search (cutoff at 70%) using **11** as query in ChEMBL database[31] to verify if similar compounds have been tested against some specific targets that can be somehow related to *P. falciparum* ~~P. falciparum~~ proteins. From this similarity search, we obtained 116 resulting hits. The results were analyzed in order to cluster compounds according to chemical classes and to rule out those molecules showing results only on whole cell assays, with no specific target annotated. For each surviving hit and its target, we tried to find a corresponding protein in *P. falciparum* ~~P. falciparum~~ proteome. To this end, we queried the proteome of *P. falciparum* ~~P. falciparum~~ 3d7 available in PlasmoDB[32] with the protein sequences belonging to the targets of the ChEMBL hits by using BLASTP[~~33~~ .33] The hit compounds **a** and **b** (Table S1) became the most promising in terms of the putative target identity. Compound **a** was tested as a potential antimicrobial agent targeting ClpB,[34] whereas hit **b** was first developed as an antiproliferative agent and evaluated for DNA binding propensities and ~~topoisomerases~~topoisomerase I/II inhibition as part of its mechanism of action.[35] Analyzing the activity of these derivatives on the annotated target, we found that only compound **a** had some hint of activity, whereas compound **b** was inactive. It must also be noticed that phosphatidylinositol 4-kinase (PI4K) was one of the putative targets obtained from the study. Although the benzo[*b*]thiophenes were known to inhibit choline kinase, we decided against evaluating our derivatives toward this protein, due to a low sequence similarity between PI4K and choline kinase catalytic domains (N1261- -M1559, 21.5% of identity, Figure S5). This preliminary result prompted us to investigate ~~more~~ in ~~details~~more detail the similarity between compounds **11** and **a**, also because the molecular target under consideration plays a crucial role in the microbial life cycle.

ClpB is a member of the Hsp100/ClpB family of AAA+ ATPases that, in bacteria, yeast, and plants, is involved in supporting survival of bacteria, yeast, and plants under stress conditions by mediating ATP-dependent protein dis-

aggregation. [36,37] Two isoforms of this ATPase were identified in *P. falciparum* ~~P. falciparum~~, *Pf*ClpB1 and *Pf*ClpB2, the latter usually known as Plasmodium Hsp101.[38] *Pf*ClpB1 is located in the apicoplast and it has been suggested to have a protective role against protein misfolding and aggregation, whereas *Pf*ClpB2 is crucial in *Plasmodium* ~~Plasmodium~~ infectivity as it participates in the export of *Plasmodium* ~~Plasmodium~~ proteins to the erythrocyte cytosol.[39] In addition, the ClpB system is highly conserved among bacteria, yeast, and plants but it is absent in humans~~,~~; therefore, both PfClpB isoforms are regarded as potential new targets for malaria treatment.[40] Although none of the two *Pf*ClpB proteins has been yet crystallized, two bacterial forms of ClpB, one from *E. coli* ~~E. coli~~ (PDB ~~4CIU~~4CIU)[41] and another from *T. thermophilus* ~~T. thermophilus~~ (PDB ~~1QVR~~1QVR),[42] have been solved bound to ADP and an ATP derivative, respectively. The ATP binding sites are strongly conserved among different AAA+ ATPases, and in particular among ClpBs from different species,[37] so the binding site residues are well conserved between bacterial and *Plasmodium* ~~Plasmodium~~ ClpB (Figure S1). In particular, the Walker A motif in the ATP binding site is identical in ~~E~~*E. coli* ~~. coli~~ ClpB and *Pl*ClpB1. Compound **11** appears to dock well in the bacterial ClpB ATP binding site and in the binding site of a homology model of *Pf*ClpB2, that has a lower degree of similarity with *E. coli* ~~E. coli~~ than *Pf*ClpB1. The rates of ATP hydrolysis catalyzed by *E. coli* ~~E. coli~~ ClpB in the absence and presence of compounds **11**, **63**, and **71** were measured (Figure S1). The assay reactions were initiated by adding ClpB to the assay buffer that included ATP and the compound. This procedure mimics the in vivo conditions, where the compounds would compete with ATP for binding to ClpB. We did not detect any inhibition of the ClpB ATPase over a broad range the compound concentrations (Figure S1). Since ClpB uses energy from ATP to produce its biological effects, it is unlikely that the compounds would bind to the ATP site without inhibiting the ATPase, and still have an effect on the biological activity. To further verify the lack of inhibition, ClpB was preincubated with **63** and the reaction was started by adding ATP in order to provide the compound with a competitive advantage over the nucleotide. However, no inhibition of the ClpB ATPase was observed in this case either, effectively ruling out the possibility that ClpB is the molecular target of this series of compounds.

Conclusions

In this work, we have taken advantage of an initiative by GSK to investigate benzo[*b*]thiophene-2-carboxamides as antimalarial agents. The in-depth SAR investigation that we have carried out has revealed the important structural features underlying the pharmacological activity of these derivatives. Also, an attempt to discover the putative molecular target of this series was undertaken through a target-fishing approach.

The backbone structure (see Table 1) allows only for a surprisingly limited number of substitutions that preserve the antimalarial activity. Even small backbone modifications at specific sites lead to a complete loss of antimalarial potency.

Five of the prepared compounds maintained an activity similar to and apparently slightly better than the parent compound **77**, providing useful information to drive the development of next-generation derivatives. The benzo[*b*]thiophene core is crucial for conferring a high potency, while other bicyclic heterostructures such as indole and benzofuran were found to be detrimental for the activity. With regard to the carboxamide at the C-2, the 2,6-dimethylmorpholine appendage not only appears to be the best substituent to provide high potency, but ~~it~~also appears to be the only one that confers ~~a~~ measurable activity. Position C-3 can be adorned with various substituents, to modulate activity and other pharmacological properties~~,~~; however, hydrogen-containing functional groups are detrimental ~~for~~to the potency. Also, small functional groups at the benzyl portion of the benzothiophene scaffold are well tolerated, suggesting a way to modify these derivatives in the future. In spite of our attempts to identify the mechanism of action of these compounds, their molecular target(s) remain unknown. Recent literature has provided information on the importance of further ~~pursuing~~ exploration of the Malaria Box derivatives, and this work sets the stage for further exploration of molecules structurally related to **77**.

Experimental ~~section~~Section

Chemistry

General ~~information~~Information. All the reagents were purchased from Sigma-Aldrich, Alfa-Aesar, and Enamine at reagent purity and, unless otherwise noted, were used without any further purification. Dry solvents used in the reactions were obtained by distillation of technical grade materials over appropriate dehydrating agents. MCRs were performed using CEM Microwave Synthesizer-Discover model. Reactions were monitored by thin layer chromatography on silica gel-coated ~~aluminium~~aluminum foils (silica gel on Al foils, SUPELCO Analytical, Sigma-Aldrich) at both 254 and 365 nm wavelengths. ~~When~~Where indicated, intermediates and final products were purified through silica gel flash chromatography (silica gel, 0.040~~–~~–0.063 mm), using appropriate solvent mixtures.

^{1}H–NMR and ^{13}C–NMR spectra were recorded on a BRUKER AVANCE spectrometer at 300, 400, and 100 MHz, respectively, with TMS as internal standard. ^{1}H–NMR spectra are reported in this order: multiplicity and number of protons. Standard abbreviation indicating the multiplicity was used as follows: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quadruplet, m = multiplet, and br = broad signal. HPLC/MS experiments were performed with (HPLC:) Agilent 1100 series, equipped with a Waters Symmetry C18, 3.5 µmµm, 4.6 mm x× 75 mm column and (MS:) Applied Biosystem/MDS SCIEX, with API 150EX ion source. HRMS experiments were performed with LTQ ORBITRAP XL THERMO. All of the target compounds were tested as 95% purity samples or higher (by HPLC/MS).

Biology: parasite growthParasite Growth and drug susceptibility assayDrug Susceptibility Assay. The CQ sensitive (D10) and the CQ resistant (W2) strains of *P. falciparum* P. falciparum were sustained in vitro as described by Trager and Jensen.[43] Parasites were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMax (Invitrogen, Milan, Italy), 0.01% hypoxanthine, 20 mM HEPES, and 2 mM glutamine. All cultures were maintained at 37 °C in a standard gas mixture consisting of 1% O_2, 5%CO_2, and 94% N_2. Compounds were dissolved in DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is nontoxic to the parasite). Drugs were placed in 96 well flat-bottom microplates (COSTAR) and serial dilutions made. Asynchronous cultures with parasitemia of 1–1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37 °C. Parasite growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler'sMakler's method in control and drug-treated cultures.[44] Antiplasmodial activity is expressed as the 50% inhibitory concentrations (IC_{50}). Each IC_{50} value is the mean ± standard deviation of at least three separate experiments performed in duplicate.

Cytotoxicity assayAssay. The long-term human microvascular endothelial cell line (HMEC-1) immortalized by SV 40 large T antigen38antigen 38 was maintained in MCDB 131 medium (Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum (HyClone, Celbio, Milan, Italy), 10 ng/mlmL of epidermal growth factor (Chemicon), 1 lg/mlmL of hydrocortisone, 2 mM glutamine, 100 U/mlmL of penicillin, 100 lg/mlmL of streptomycin, and 20 mM Hepes buffer (EuroClone). Unless stated otherwise all reagents were from Sigma Italia, Milan, Italy. For the cytotoxicity assays, cells were treated with serial dilutions of test compounds for 72 h and cell proliferation evaluated using the MTT assay already described.3333 The results are expressed as IC50, which is the dose of compound necessary to inhibit cell growth by 50%. Each IC50 value is the mean and standard deviation of at least three separate experiments performed in duplicate.

Inhibition of ClpB. *E. coli* E. coli ClpB was produced as previously described.[45] Protein concentration was determined spectrophotometrically and reported in monomer units. To determine the rate of ATP hydrolysis, the samples were incubated in the assay buffer (100 mM Tris/HCl pH 8, 1 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$) at 37 °C. The compounds **11**, **63**, and **71** were dissolved in DMSO and then diluted in the assay buffer to the final concentration. The corresponding amounts of DMSO added to the buffer were used as controls. The reaction was initiated by adding ClpB to the buffer containing the compound and 4 mM ATP or by adding ATP to the buffer containing ClpB and the compound. The concentration of inorganic phosphate generated from ATP by ClpB was measured as described before.[46]

Stability studiesStudies in HLM for compoundsCompounds **11**, **63** , and **71** . Stability of selected compounds in the presence of HLM was assessed by incubation of a 1 µMµM concentration for 60 min in the presence of HLM (1 mg protein mL−1), at 37 °C, in the presence of a NADPH-regenerating system (2 mM NADP+, 10 mM glucose-6-phosphate, 0.4 U mL-1 glucose-6-phosphate dehydrogenase, 5 mM MgCl2$MgCl_2$) in 100 mM PBS buffer solution pH 7.4. The reaction mixtures were preheated (37 °C) for 5 min before adding the parent compound. At fixed time points (t = 0;, 15;, 30;, 60; min), aliquots of samples were withdrawn, deproteinized with two volumes of acetonitrile, centrifuged (9,0009000 *g*, 4 °C, 10 min), and the supernatant analyzed by injection in HPLC-MS/MS system. The chromatographic separation was performed employing a gradient elution starting from 70% water + 0.1% formic acid (solvent A):30% methanol (solvent B) to 90%B:10%A in 10 min; 90%B:10%A was kept for further 5 min; then back to 70%A:30%B and further 5 min of reconditioning time. HPLC-MS analysis employed a Thermo Quantum Access Max TSQ triple quadrupole mass spectrometer (Thermo, USA) equipped with a H-ESI (Heated-ElectroSpray Ionization) interface and coupled to an Accela UHPLC system (Thermo, USA) constituted of a quaternary pump, a degasser, and a thermostated autosampler. Compounds were analyzed in positive ion mode using both total ion monitoring mode, over a mass range from 50 to 500 amu, and single ion monitoring mode. Data were acquired and analyzed employing Thermo Excalibur 1.4 software (Thermo, USA).

Target ~~fishing~~Fishing. Similarity search has been conducted using the similarity search method implemented in the ChEMBL database,[31] with a cutoff for minimum similarity at 70%. In the attempt to find some analogies between the ChEMBL ~~compounds~~compound target and plasmodium proteome, we use the blastp tool available in PlasmoDB, restricting our research to ~~Plasmodium~~*Plasmodium falciparum* ~~Falciparum~~ 3d7 proteome only and applying default parameters. Multiple sequence alignment between PlClpB1, PlClpB2, *E. coli* ~~E. coli~~ ClpB, and *T. th.* ~~.~~ . ClpB were performed with BLASTp. Docking studies were performed using Glide software. [47,48] ~~E~~*E. coli* ~~coli~~ ClpB structure has been prepared using Protein Preparation Wizard and ligand using LigPrep, both from Schrödinger.[49] ClpB2 comparative model has been built using Prime software from Schrödinger. The heteromultimer model methodology was chosen. For the N-terminal domain we took advantage of the available crystal structure of *Pf*ClpB2 N-terminal portion 4IOD (residues 10~~–~~–156). For the remaining part of the protein we used as templates *Tth*1QVR and *E. coli* ~~E.coli~~ 4CIU ClpB. Final alignment is equal to blast alignment reported in Figure S1. The final model was submitted to a long refinement procedure to reduce the steric clashes. The ionization state of the final model has been checked with Protein Preparation Wizard. It should be noted that one of the loop regions presents a quite high degree of uncertainty due to very low similarity between query and template.

General ~~procedure~~Procedure for the ~~synthesis~~Synthesis of ~~compounds~~Compounds **6**–**10** . Freshly distilled thionyl chloride (2 ~~eq~~equiv) and pyridine (0.1 ~~eq~~equiv) were added to a solution of the suitable cinnamic acid (1 ~~eq~~equiv) in anhydrous chlorobenzene (0.5 mL/mmol) and the stirred reaction mixture was heated at 130 °C for 2 ~~hours~~h. Residual thionyl chloride was distilled and the crude was treated with hexane at reflux to afford the respective acyl chloride as pale yellow needles that were collected and used for the following reaction without further purification. Analytical data for compounds **6**–**9** matched the data already published. [24,50,51]

General ~~procedure~~Procedure for the ~~synthesis~~Synthesis of ~~compounds~~Compounds **11**–**25** . A mixture of the suitable 3-chlorobenzothiophene-2-carbonyl chloride (1 ~~eq~~equiv) and the proper amine (1 ~~eq~~equiv) was refluxed in anhydrous dioxane until consumption of the reacting agents according to TLC. The reaction mixture is then poured in ice water and the organic layers were extracted with ethyl acetate (3 × 10 mL), washed with brine, dried over anhydrous Na_2SO_4 , and evaporated under reduced pressure. The oil obtained was purified through flash column chromatography eluting with dichloromethane/methanol, to give the title compounds. Yields, purification methods, and other analytical data are reported in the ~~supporting information~~Supporting Information.

Ethyl ~~benzo~~Benzo[*b*]thiophene-2-carboxylate (**28**). To a solution of 2-nitrobenzaldehyde **26** (2.0 g, 3.0 mmol) in anhydrous DMF (20 mL), K_2CO_3 (2.2 g, 16 mmol) and ethyl thioglycolate (142 ~~µL~~µL, 13 mmol) were added at 0 °C. The reaction mixture was kept at the same temperature for 30 ~~minutes~~min, and then was allowed to react at room temperature until consumption of the limiting reagent according to the TLC. The mixture is then poured in ice water and the precipitate obtained is collected, dried, and used in the next reaction step without further purification. Yield 68%.

Following a similar procedure, but using 1-(2-fluorophenyl)propan-1-one **27** in place of 2-nitrobenzaldehyde, ethyl 3-ethylbenzo[*b*]thiophene-2-carboxylate (**29**) was prepared in 80% yield. Analytical data for compounds **28**, **29** matched the data already published. [52,53]

Ethyl ~~benzofuran~~Benzofuran-2-carboxylate (**32**). To a solution of salicylaldehyde **30** (857 ~~µL~~µL, 8.2 mmol) in anhydrous DMF (15 mL), ethyl bromoacetate (907 ~~µL~~µL, 2.4 mmol) and K_2CO_3 (2.21 g, 160 mmol) were added, and the mixture was stirred at 130 °C until consumption of the limiting reagent according to the TLC. The reaction mixture is then poured in ice water and the organic layers were extracted with ethyl acetate (3 × 10 mL), washed with brine, dried over anhydrous Na_2SO_4 , and evaporated under reduced pressure. The oil obtained was purified through flash column chromatography eluting with dichloromethane/methanol (99:1), to give the title compound as a white solid in 70% yield. Analytical data for compound **32** matched the data already published.[54]

Ethyl 3-~~hydroxybenzo~~Hydroxybenzo[*b*]thiophene-2-carboxylate (**33**). Methyl 2-mercaptobenzoate **31** (1.5 g, 8.92 mmol), ethyl bromoacetate (789 ~~µL~~µL, 8.92 mmol), and sodium *tert*-butoxide (2.68 g, 17.83 mmol) were dissolved in anhydrous THF and reacted at room temperature for 2 ~~hours~~h. After completion, the reaction mixture is poured in ice water and the organic layers were extracted with ethyl acetate (3 × 10 mL), washed with brine, dried over anhydrous Na_2SO_4 , and evaporated under reduced pressure. The crude obtained was purified through flash column chromatography eluting with petroleum ether/ethyl acetate (9:1), to give the title compound as a yellowish solid in 91% yield. Analytical data for compounds **33** matched the data already published.[55]

General ~~procedure~~Procedure for the ~~alkylation~~Alkylation of ~~compound~~Compound **33** . To a suspension of NaH (2 ~~eq~~equiv) in anhydrous DMF, compound **33** (1 ~~eq~~equiv) is added at room temperature under nitrogen atmosphere. After 10 ~~minutes~~min the suitable alkyl halide (2 ~~eq~~equiv), solubilized in anhydrous DMF, is added dropwise and the

reaction mixture is stirred at 60 °C for a period spanning from 1 to 3 hoursh. After consumption of the limiting reagent monitored through TLC, the mixture is poured in ice water and extracted with ethyl acetate (3 × 5 mL), and the organic layers are washed with brine, dried over anhydrous Na_2SO_4 , and evaporated under reduced pressure. The crude is purified through flash column chromatography eluting with petroleum ether/ethyl acetate (95:5), to give compounds **34**–**38** in good overall yields. Yields, purification methods, and other analytical data are reported in the supporting informationSupporting Information. Analytical data for compound **35** matched the data already published.[56]

Methyl 3-(benzylaminoBenzylamino)benzo[*b*]thiophene-2-carboxylate (**41**). Ethyl 3-aminobenzo[*b*]thiophene-2-carboxylate **40** (30 mg, 0.135 mmol), benzyl bromide (7.7 mg, 0.045 mmol), K_2CO_3 (37 mg, 0.270 mmol), and KI (1 mg, 0.045 mmol) were dissolved in acetonitrile (3mL3 mL), put in a microwave flask, and heated in a microwave oven at 120 °C for 10 minutes for two timesmin twice, and then at 140 °C for 10 minutes formin once. After quenching with brine (5 mL), the mixture was extracted with ethyl acetate (3 × 5 mL), and the combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude material was then purified through flash chromatography eluting with petroleum ether/ethyl acetate 95:5 to give the title compound **41** as a yellow solid in 83% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.88 (s, 3H); 4.95 (s, 2H); 7.21–7.43 (m, 8H); 7.72 (s, 1H); 7.84 (bs, 1H); 8.00 (s, 1H). LRMS (ESI) calcd for $C_{17}H_{15}NO_2S$ ([M-H]$^-$) 296.08; found 296.3.

General procedureProcedure for the ester hydrolysisEster Hydrolysis: synthesisSynthesis of compoundsCompounds **42**–**53** . Esters **28**, **29**, **32**–**41** (1 eqequiv), and LiOH·H_2O (4 eqequiv) were dissolved in a solution of THF/MeOH/H_2O (3/1/1, 1 mlmL/mmol) and stirred overnight at room temperature. The reaction mixture is then evaporated in vacuo, and the crude is taken up with H_2O, acidified with HCl 1N1 N, and extracted with ethyl acetate, that is onin turn washed with brine and dried over anhydrous Na_2SO_4. After the evaporation of the solvent the crude materials were used in the next reaction step without any additional purification. Yields and essential analytical data are reported in the supporting informationSupporting Information. Analytical data for compounds **42**–**46**, **50**–**53** matched the data already published. [56–63]

General procedureProcedure for the synthesisSynthesis of amidesAmides **54**–**71**, **73**, **75** . To a solution of the appropriate carboxylic acid (1 equiv) in anhydrous dichloromethane or dimethylformamide (DMF) (4 mL/mmol) at room temperature were added anhydrous hydroxybenzotriazole (HOBt, 1 equiv) and 1-ethyl-3-(3-dimethylamino-propyl(dimethylamino)propyl)carbodiimide hydrochloride (EDC·HCl, 1 equiv) under nitrogen atmosphere. After stirring for 10 min, the appropriate substituted amine (1 equiv) and triethylamine (3 equiv) were added, and the reaction mixture was stirred at room temperature until disappearance of the starting material (usually 2 to 6 hoursh). After this time water (5 mL) was added, and the mixture was extracted with EtOAc (3 × 10 mL), and the organic layers were separated, washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography eluting petroleum ether/ethyl acetate (8:2) to obtain the title compounds in yields ranging from 13% to 73%.

3-(Methoxymethoxy)benzo[*b*]thiophene-2-carboxylic acidAcid (**74**). To a solution of **33** (0.15 g, 0.63 mmol) in anhydrous dichloromethane (5 mL), chloromethyl methyl ether (72 µLµL, 0.95 mmol) is added dropwise through a dropping funnel at room temperature. After cooling at °C, DIPEA (165 µLµL, 0.95 mmol) is added, and the reaction mixture is allowed to stir at room temperature until consumption of the starting material (2 hoursh). The reaction is quenched with NH_4Cl, and the organic layers washed with water (3 × 5 mL), treated with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The oil obtained, without further purification, was hydrolysedhydrolyzed according to the procedure above reported earlier to give the acid **74** as a white powder in 86% overall yield. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.68 (s, 3H); 5.49 (s, 2H); 7.44–7.56 (m, 2H); 7.81 (d, J = 8 Hz, 1H); 7.98 (d, J = 8 Hz, 1H). LRMS (ESI) calcd for $C_{11}H_{10}O_4S$ ([M–H]$^-$) 237.03; found 237.3.

(2,6-Dimethylmorpholino)(3-hydroxybenzo[*b*]thiophen-2-yl)methanone (**76**). To a solution of compound **75** (0.028 g, 0.083 mmol) in anhydrous methanol, HCl 6N6 N is added over a 20 minutesmin period. After reacting for 1 hourh at room temperature, the solvent is evaporated and the residue taken up with saturated $NaHCO_3$ aq. solution (8 mL), extracted with ethyl acetate (3 × 5 mL), and the organic layers were separated, washed with brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash chromatography eluting petroleum ether/ethyl acetate (8:2) to obtain the title compounds in 82% yield as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.12–1.30 (m, 6H); 2.70–2.82 (m, 2H); 3.60–3.75 (m, 2H); 4.52–4.65 (m, 2H); 7.35–7.50 (m, 2H); 7.82 (d, J = 6 Hz, 1H); 7.99 (d, J = 8 Hz, 1H). δ 17.8, 38.8, 71.6, 122.4, 122.9, 125.7, 128.5, 132.6, 135.9, 138.4, 141.2, 162.0. HRMS (ESI) calcd for $C_{15}H_{17}NO_3S$ ([M + H]$^+$) 292.0929; found 292.1002.

Supporting Information

~~Supporting Information Available~~

~~A Brief description of the biological assays, some of the synthetic procedures, the~~The Supporting Information ~~1~~ ~~H-NMR spectra of the intermediates and title compounds and the methods used for the target fishing experiments are available as supporting information. This material~~ is available free of charge ~~via~~on the ~~Internet~~ACS Publications website at ~~http://pubs.acs.org~~ DOI: 10.1021/acs.jmedchem.6b01685 .

Brief description of the biological assays, some of the synthetic procedures, the ^{1}H NMR spectra of the intermediates and title compounds, and the methods used for the target fishing experiments (PDF)

Smiles structural list (CSV)

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~~Author Contributions~~

~~The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally.~~

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The authors declare no competing financial interest.

Author Contributions

#Marco Pieroni and Elisa Azzali contributed equally to this work.

Abbreviations ~~a~~Used

ACT, Artemisinin-based combination therapy; CK, choline kinase; CQ, chloroquine; DMF, *N,N*-~~dimethyl formamide~~dimethylformamide; EDC, 1-ethyl-3-(3-~~dimethylaminopropyl~~(dimethylamino)propyl)carbodiimide; EDG, electron-donor group; EWG, electron-withdrawing group; MOM, methoxy-methyl; PDB, protein data bank; SAR, ~~Structure-Activity Relationships~~structure−activity relationships; TBTU, 2-(~~1H~~1*H* ~~-Benzotriazole~~benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; THF, tetrahydrofuran

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