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Discovery and Characterization of ACT-451840: an Antimalarial Drug with a Novel Mechanism of Action

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More than 40% of the world's population is at risk of being infected with malaria. Most malaria cases occur in the countries of sub-Saharan Africa, Central and South America, and Asia. Resistance to standard therapy, including artemisinin combinations, is increasing. There is an urgent need for novel antimalarials with new mechanisms of action. In a phenotypic screen, we identified a series of phenylalanine-based compounds that exhibit antimalarial activity via a new and yet unknown mechanism of action. Our optimization efforts culminated in the selection of ACT-451840 [(*S*,*E*)-*N*-(4-(4-acetylpiperazin-1-yl)benzyl)-3-(4-(*tert*-butyl)phenyl)-*N*-(1-(4-(4-cyanobenzyl)piperazin-1-yl)-1-oxo-3-phenylpropan-2-yl)acrylamide] for clinical development. Herein we describe our optimization efforts from the screening hit to the potential drug candidate with respect to antiparasitic activity, drug metabolism and pharmacokinetics (DMPK) properties, and in vivo pharmacological efficacy.

Introduction

Malaria remains a major health problem in large areas of the world, despite significant efforts toward the identification of novel treatments over the last decade.[1] Approximately half of the world's population live in malaria-endemic areas, and the World Health Organization (WHO) estimates that 1.2 billion people are at elevated risk of being infected with a malaria parasite. In 2015 there were an estimated 214 million malaria cases and 438000 deaths, which reflects a decrease from 2004, when death cases peaked at 1.8 million.[2] The recently reported emergence of resistance, or decreased efficacy, of the most modern endoperoxide-containing drug combinations to treat malaria[3] emphasize the importance of identifying new antimalarial drugs with novel mechanisms of action.[4] Novel antimalarial drugs require high safety and tolerability standards, because the vast majority of fatal cases are children under the age of 5 (one child dies from malaria every 40 seconds) and pregnant women.[5] This and the scarcity of novel drug targets in the malaria parasites resulted in approaches to optimize the activity and pharmacokinetic (PK) properties of existing drugs. This, in turn, resulted in moderate advances to prevent the development of resistance against these compounds.[6] The difficulty in developing a safe vaccine[7] leaves pharmacological intervention by drugs as the only current option to treat, cure, or prevent malaria. Therefore, the need for new, inexpensive, and safe antimalarial agents with novel mechanisms of action remains very high.

In humans there are five *Plasmodium* species with distinct and differentiated disease patterns.[8] The most significant, deadly, and virulent is *Plasmodium falciparum* (malaria tropica). The others are *P. vivax*, *P. ovale* (both resulting in malaria tertiana), *P. malariae* (malaria quartana), and, very recently discovered, *P. knowlesi*. *P. falciparum* and *P. vivax* are responsible for 95% of worldwide malaria infections, and nearly all fatal cases are associated with *P. falciparum* infections. Malaria parasites are transmitted by female *Anopheles* mosquitos when biting humans for a blood meal. The mosquito injects sporozoites

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from its salivary glands into the human bloodstream. The sporozoites enter the liver and develop into schizonts, which contain large numbers of merozoites. It takes about 10 days until a schizont ruptures and releases the merozoites into the bloodstream, where these invade red blood cells (RBCs; erythrocytes). In the erythrocyte, the parasite develops from a ring stage into a trophozoite stage and finally into a blood schizont. After 24 (*P. knowlesi*) or 48 (*P. falciparum*, *P. vivax*, *P. ovale*) or 72 h (*P. malariae*), the erythrocyte ruptures and again releases merozoites into the bloodstream, where they invade more erythrocytes. After a few asexual lifecycles, some merozoites develop into gametocytes, a sexual form. Upon another mosquito bite, gametocytes are transferred back into the vector, where they undergo sexual reproduction to finally produce large quantities of sporozoites in the salivary gland, ready to infect the next subject upon the mosquito's next blood meal. When erythrocytes rupture within the infected patient, the parasites' waste products and cell debris are released into the bloodstream, causing some of the clinical symptoms (fever, chill, headache, abdominal and back pain, nausea, diarrhea, vomiting) of malaria.[4d] Additional information about the complex life cycle of the malaria parasite can be found in the literature or the internet.[9]

Results and Discussion

Our approach toward designing inhibitors of food-vacuolar plasmepsins was complicated a few years ago,[10] when knockout experiments with *P. falciparum* demonstrated that these presumed new drug targets were not essential for parasite survival.[11] We therefore performed an erythrocyte-based phenotypic screen[12] of ~5000 compounds at the Swiss Tropical and Public Health Institute against the chloroquine-resistant K1 strain of *Plasmodium falciparum*. In this effort several hits were identified, among them compound **1**, a phenylalanine-based derivative offering several possibilities for structure–activity relationship (SAR) investigations (Figure 1).[13]

Scrutinizing the screening hit **1** immediately reveals seven aspects to be investigated. The cinnamic acid moiety raises the question about the importance of the C=C double bond (**A**) for activity and its potential reactivity as a Michael acceptor. It also needs to be clarified if the phenyl ring can be replaced by

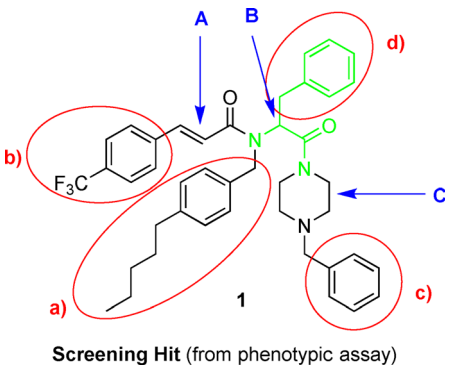


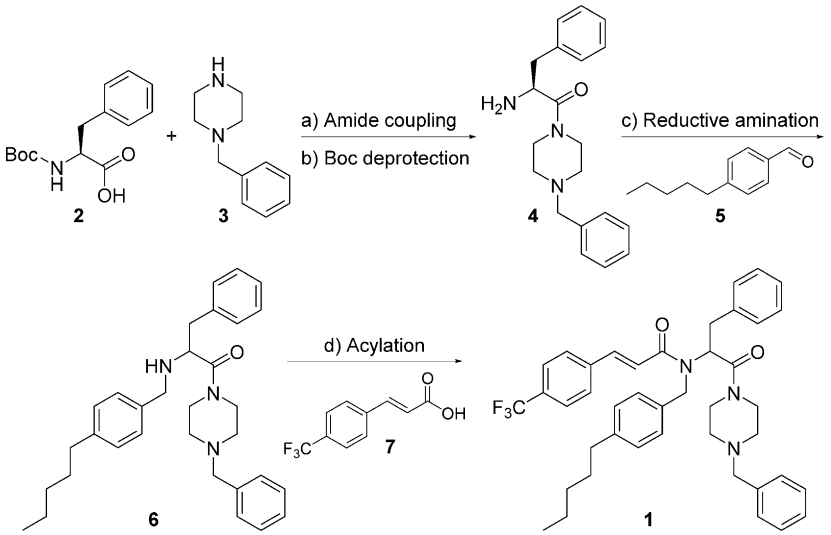
Figure 1. Exemplary screening hit from the phenotypic RBC assay.

heteroaromatic ring systems, and the substitution pattern needs to be investigated (**b**). The *n*-pentylbenzyl unit (**a**) triggers many ideas and should certainly be replaced by more drug-like groups. Another important aspect is the amino acid in the core of the compounds. The influence of chirality (**B**) on activity as well as determining whether the phenylalanine can be replaced by other amino acids (**d**) needs to be assessed. The piperazine moiety (**C**) also inspires ideas for variations (e.g., ring size), and the benzylamine group (**c**) invites investigations of phenyl group replacements and phenyl substitution patterns, as well as clarification as to whether the basic amine is crucial or if acylation could be an option in this region of the compounds. Screening hit **1** was considered a very interesting and valuable starting point, as it offers diverse optimization options to the medicinal chemist. In addition, chemical accessibility of **1** and potential derivatives is straightforward (Scheme 1).[14] This is important in the antimalarial field, as cost of goods is a crucial parameter.

Scheme 1 summarizes the chemistry used to prepare screening hit **1** and derivatives thereof. We started by coupling of *tert*-butyloxycarbonyl (Boc)-protected phenylalanine **2** with benzylpiperazine **3** under standard peptide-bond-forming conditions followed by Boc deprotection by trifluoroacetic acid in dichloromethane to obtain **4**. If hydrochloric acid in dioxane was used to deprotect the amine functionality, we often observed decomposition of the piperazinyl amide. Compound **4** was then treated with aldehyde **5** in a reductive amination reaction in dichloromethane or acetonitrile with sodium triacetoxyborohydride as the reducing agent to give the precursor **6**, which was readily transformed into the final compound **1** by a simple acylation reaction with the cinnamic acid derivative **7**. Details about the synthetic procedures are given in the Supporting Information and in the patent literature.[14,15] Following this strategy, we prepared the compounds depicted in Table 1 by introducing various cinnamic acid moieties in the final step of the sequence.

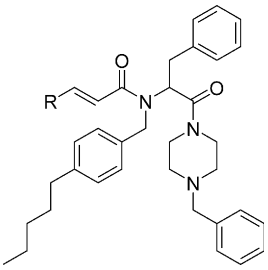
For SAR, all compounds were tested for inhibition of parasite growth in RBCs infected with the *P. falciparum* parasite strain K1 (chloroquine resistant) and/or with the chloroquine-sensitive strain NF54. Both assays produced similar results, indicating that our compounds are active against both chloroquine-sensitive and -resistant strains. Parasites were cultured in vitro according to Trager and Jensen.[16] IC_{50} values were determined by measuring incorporation of the nucleic acid precursor [^{3}H]hypoxanthine after 72 h of incubation.[17] IC_{50} values were usually determined in two ways: in the presence of 0.5% Albumax (a serum substitute corresponding to a final assay concentration of 10% bovine serum albumin), or 50% human serum. The reason for this parallel approach was to identify compounds that lose in vitro potency in the presence of 50% human serum, as this could be an indication of high protein binding.

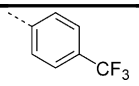
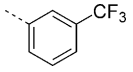
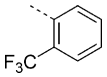
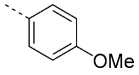
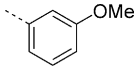
The small cluster of structurally closely related compounds depicted in Table 1 was initially prepared in a completely different project, and they were all identified as hits in the phenotypic antimalarial screen, adding confidence to the results and already giving initial insight into the SAR. Variations in the



Scheme 1. Preparation of compound **1** as an illustration of the general synthetic pathway, showing readily accessible and efficient chemistry. (Further details are given in the Supporting Information).

Table 1. Activities of the initial hit series.

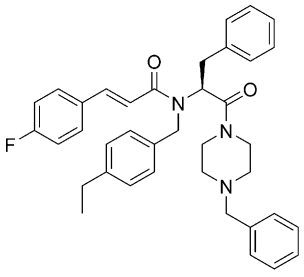


Compd	R	IC_{50} K1 alb 72 h [nM][a]
1		3.8
8		70
9		375
10		7.3
11		19

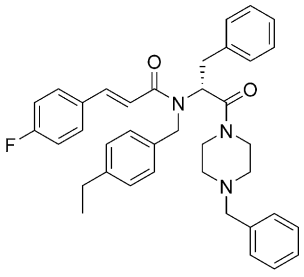
[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined with a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details).

substitution of the phenyl ring in the cinnamoyl unit showed that a *para* substituent as present in **1** and **10** results in more active compounds than the *meta*-substituted (**8** and **11**) or the *ortho*-substituted (**9**) analogues. The next aspect to investigate was the importance of the C=C double bond, the linker length between the aryl moiety, and the N terminus of the core amino acid as well as whether the aryl moiety could be removed or replaced with saturated ring systems or heteroaryl groups. Representative results are summarized in Table 2. In the selected examples we also replaced the initially present *n*-pentyl substituent with a pyridine moiety as a first step toward improved molecular properties. Significant activity can be retained by reducing the C=C double bond to a single bond (**12**), but adding one more methylene unit to the linker resulted in the less active derivative **13**. The indane system present in **14** led to a further decrease in antimalarial activity, and replacement of the phenyl ring by cyclohexyl (**15**), cyclopentyl (**16**), or complete removal of the ring system (**17**) also had negative effects on potency, whereas the introduction of an indole moiety, as shown in **18**, resulted in very potent antimalarial activity. Small substituents such as cyclopropyl (**19**) or cyclopropylacetyl (**20**) were not tolerated.

The two compounds **21** and **22** (Figure 2), which differ only by the chirality of the phenylalanine core, indicate that the *S* configuration present in the naturally occurring amino acid (AA) and in compound **21**, result in significantly higher antimalarial activity than compounds with the unnatural *R* configuration in the core amino acid such as **22**, which is virtually inactive.



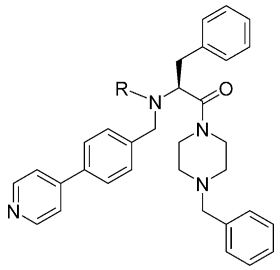
21: *S*-chirality, natural AA
IC_{50} K1 alb 72 h: 13 nM

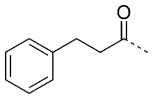
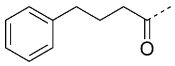
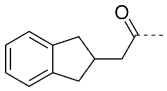
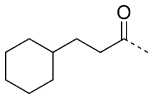
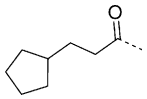
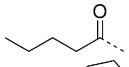
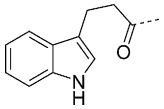
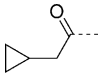


22: *R*-chirality, non-natural AA
IC_{50} K1 alb 72 h: >100 nM

Figure 2. Crucial influence of the chirality of the amino acid core.

Table 2. First results with the cinnamic acid double bond removed.



Compd	R	IC_{50} K1 alb 72 h [nM][a]
12		37
13		80
14		159
15		207
16		458
17		281
18		2.2
19		>500
20		>500

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details).

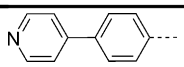
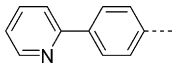
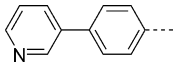
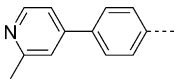
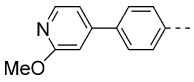
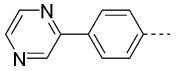
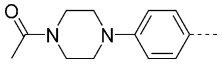
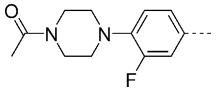
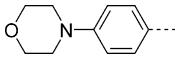
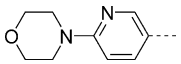
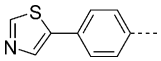
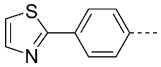
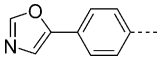
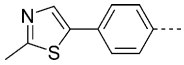
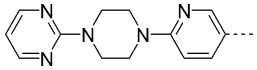
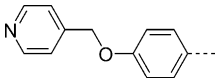
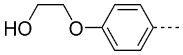
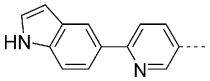
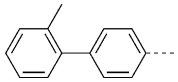
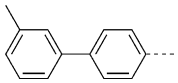
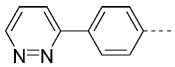
Based on the results described above, we decided to investigate *n*-pentylbenzyl replacements, keeping the compounds otherwise constant as found in compound **1**, with the exception of working with the *S* configuration and avoiding racemates; results are summarized in Table 3. During these investigations we detected limitations of our screening assay, which used Albumax as culture medium supplement. Binding of compounds to serum components decreased the available free compound concentration in the assay. Therefore, the absence of serum components in our assay often led to overestimation of the compounds' potency. We therefore optimized the assay environment by adding 50% human serum (final assay concentration) to the media. Under these conditions, physicochemical properties of the compounds became more relevant for antimalarial activity. In addition, the results became more meaningful for subsequent planning of in vivo experiments based on in vitro results.

Historically, in vivo experiments in antimalarial drug discovery are performed in mice, which are not infected by *P. falciparum*, but with the rodent malaria parasite *P. berghei*. We therefore used an in vitro assay to determine the activity of our antimalarials toward the rodent malaria parasite *P. berghei*. This assay was performed in Albumax containing media and had an incubation time of 24 h. By that we found that our antimalarials were less effective against the murine *P. berghei* parasites than they were against the human parasite strains. This finally forced us to work with a humanized *P. falciparum* SCID mouse model to obtain relevant in vivo results with our best compound (see below).

As a general aspect, all compounds listed in Table 3 revealed significant antimalarial activity under the K1 Albumax assay conditions, but lower activity was observed in the K1 serum assay with shifts for the best compounds in the range of 5- (**29**, **30**) to 70-fold (**27**). A similar pattern was found when analyzing the assay data from the NF54 Albumax and NF54 serum assays, which confirmed the validity of the thinking behind the approach. Analysis of the data obtained with the rodent parasite showed that the potency to kill *P. berghei* was lower for all investigated compounds than their potency to kill the human parasite. This finding was surprising to us, as most of the newer antimalarials work with similar potency against the human and rodent parasites.

The data summarized in Table 3 were analyzed by taking into account the combined assumptions explained above, in order to guide us toward compounds that show low shift factors between the Albumax versus human serum conditions. This meant that we were looking at the absolute activity values with second priority only. Therefore, compounds **29**, **30**, and **32** were among the most interesting derivatives identified so far, as their activity shifts were <10-fold for both parasite strains. In addition to these compounds, derivatives **28**, **38**, and **43** also presented attractive datasets with respect to antiparasitic activity combined with activity shifts between Albumax and human serum conditions. Unfortunately, all three compounds showed potent inhibition of cytochrome P450 (CYP) 3A4 in an assay using testosterone as marker substrate (**28**: 0.31 μM, **38**: 0.2 μM, **43**: 2.5 μM) and were therefore not considered further. It seemed that the needle-like shape of the biphenyl or related moieties, possessing a nitrogen atom in an exposed position toward the "needle-tip", confirmed reported findings of being a perfect binder of the heme unit present in cytochromes. Therefore, compounds with this structural subunit were often found to suffer from cytochrome inhibition liabilities. For further investigations the 4-*N*-acetylpiperazine-substituted benzyl group, as present in **29**, resulting in an unproblematic CYP3A4 inhibition of 8.4 μM with testosterone as marker substrate, was used as substituent of choice whenever possible. Another strategy to overcome strong cytochrome inhibition was applied in **26** and **27**, by introducing a substituent at the *ortho* position to the ring nitrogen atom, resulting in values of 5.3 μM for **26** and >50 μM for **27**.

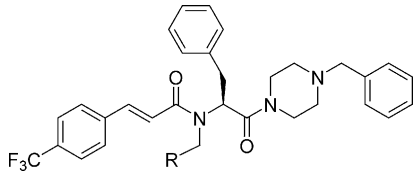
Table 3. Further replacements of the *n*-pentylbenzyl substituent.

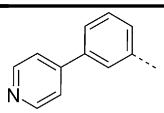
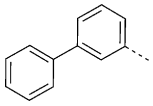
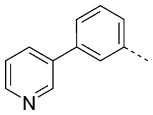
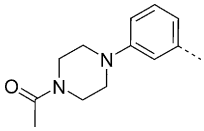
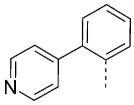
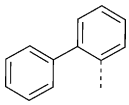
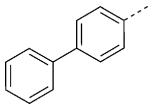
Compd	R	IC_{50} [nM][a] K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
23		0.8	8.7	2.0	>20	27
24		1.1	6.3	3.7	32	>200
25		0.9	18	3.8	>20	ND
26		0.4	10	2.2	26	29
27		1.0	73	2.6	11	91
28		1.3	24	7.9	88	77
29		0.9	5.2	5.2	28	112
30		8.8	35	41	91	ND
31		4.8	34	36	>100	ND
32		10	66	14	106	ND
33		0.4	11	<7.8	34	104
34		<7.8	73	14	369	ND
35		<7.8	12	<7.8	27	107
36		<7.8	17	3	>20	ND
37		3.3	74	4.7	311	ND
38		1.5	14	<7.8	27	73
39		3.4	70	26	243	ND
40		<7.8	29	<7.8	66	115
41		11	>500	ND	ND	ND
42		<7.8	>500	ND	ND	ND
43		1.0	6.5	5.4	41	82

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

Another aspect requiring clarification was the question of optimal substitution position on the benzyl ring. The results listed in Table 4 clearly confirm that the best orientation of the biaryl and related systems is a *para* arrangement (e.g., **50**). Both the *meta* (**44**–**47**) and *ortho* arrangements (**48**, **49**) result in decreased antimalarial potency and were not further pursued.

Table 4. *meta*-Biaryls and *ortho*-biaryls.



Compd	R	IC_{50} [nM][a] K1 alb 72 h	K1 ser 72 h
44		19	451
45		22	>500
46		<7.8	344
47		18	166
48		211	>500
49		375	>500
50		1.6	ND

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

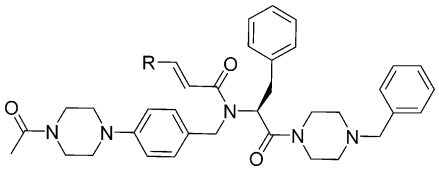
Table 5 shows a summary of the investigations performed in the optimization of the cinnamoyl moiety. Compounds **51**–**53** contain a 2-substituted pyridine ring as phenyl replacement. Although the activity shifts between the Albumax and human serum conditions were low, the absolute antiparasitic activities were rather disappointing. Compound **54** contains a 4-methylsulfone-substituted phenyl unit, a substituent often used in medicinal chemistry to improve PK behavior and absorption. The activity shift for this compound was also low. Unfortunately, **54** showed only low potency against the rodent malaria parasite. The 4-alkyl-substituted-phenyl-containing derivatives (**55**–**58**) are the most promising compounds with respect to activity shifts in the presence and absence of serum, as well as absolute activity levels and activity against the rodent parasite. Derivative **55** can be considered the most promising compound in this subgroup. Results obtained for **59** proved the beneficial effects of a *para* substituent at the phenyl moiety of the cinnamoyl group. Compounds **60**–**63** summarize the subgroup of compounds bearing a *para*-alkoxy substituent at the phenyl ring. Generally, the compounds exhibited good antimalarial potency with low activity differences between the human and rodent parasites, and a tendency to be slightly less active against the rodent parasite than the alkyl-substituted derivatives. Fluoroalkoxy substituents, as present in **64** and **65**, did not result in any advantage, as was also the case for the examples of substitutions given in **66**–**69**.

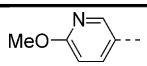
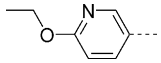
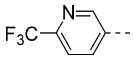
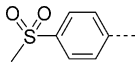
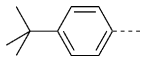
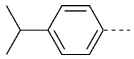
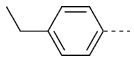
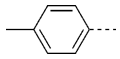
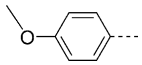
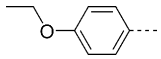
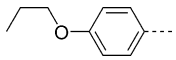
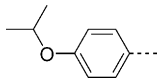
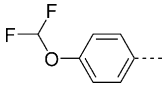
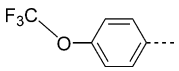
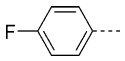
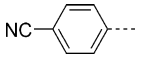
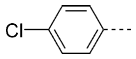
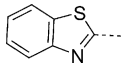
Table 6 summarizes the results with respect to replacement of the phenylalanine core with unnatural amino acids, mainly containing heterocycles or heteroaryl groups (5- and 6-membered) as phenyl replacements.[15a] Analysis of the data revealed that combinations of a morpholinyl moiety as R^1 and a 4-pyridyl unit as R, or a piperidinyl moiety as R^1 and a 4-pyridyl unit as R resulted in compounds **73** and **75**, which exhibited potent inhibitory activity. Unfortunately, these were coupled with very potent CYP3A4 inhibition in an assay using midazolam as marker substrate (**73**: 0.2 μM, **75**: 0.3 μM). Replacement of the 4-pyridyl by a 2-pyridyl group at the R position, a standard strategy to avoid strong cytochrome inhibition, resulted in significant losses in activity, as shown for **72** and **74**. Further substituents, such as *N*-methylpiperazinyl, pyrazinyl, 2-pyrimidinyl, pyrolyl, pyrazolyl, imidazolyl, or isoxazolyl, tested at the R^1 position, all resulted in less active derivatives. A general trend was that the 4-pyridyl-containing compounds always resulted in better antimalarial activity than the 2-pyridyl-containing analogues. Based on these results, it was decided to maintain a phenylalanine as the core of the antimalarials.

In Table 7 (compounds **90**–**92**), the results of a fluorine scan around the phenyl ring of the phenylalanine core are summarized and indicate no advantage of additional substituents in this area of the compounds. From the important perspective of cost of goods, this was considered a very positive point.

Table 8 summarizes the results obtained in investigating the SAR around the benzyl substituent connected to the piperazine moiety of our antimalarials.[15b] Most of this work was done in the presence of the initially detected 4-trifluoromethylcinnamoyl group. Analyzing the results of compounds **93**–**104** (CF_3-cinnamoyl) showed that all compounds exhibited good potencies in both Albumax as well as serum-based assay conditions. In general, a moderate loss in activity was observed when moving to the human serum conditions, with shifts below a factor of 10-fold. A few compounds were also investigated with regard to their antimalarial potency toward the rodent parasite, *P. berghei*. Compound **97**, with a hydroxyethylmethylamino substituent showed potent activities. Integrating the substituent into a benzoxazine moiety, as shown in **98**, resulted in a significant loss of potency, which was also the case upon exchanging it with an ethylene glycol unit, as present in compound **95**. Comparing **96** with **102**, in which the cyano substituent is moved from the *meta* to the *para* position, showed that both regioisomers have similar activities toward the K1 strain, but the *para*-substituted compound was found to be significantly more potent toward the NF54 strain. Finally, the methylsulfone- and ethylsulfone-substituted compounds **103**, **104**, **106**, and **107** were among the most potent compounds identified so far, especially against the NF54 parasite

Table 5. SAR on the cinnamoyl moiety in combination with the acetylpiperazine unit.



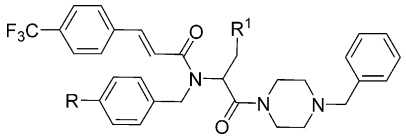
Compd	R	IC_{50} [nM][a] K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
51		5.8	16	47	46	ND
52		2.6	9.0	13	22	ND
53		6.0	>7.8	45	52	ND
54		1.4	6.1	9.4	32	363
55		0.8	5.3	1.9	8.5	47
56		1.4	6.8	2.9	8.3	115
57		0.7	3.7	2.0	9.9	81
58		1.2	7.4	8.0	52	ND
59		<7.8	100	60	>500	ND
60		1.0	5.8	5.4	21	153
61		0.7	3.2	1.9	10	100
62		0.6	3.7	1.8	11	75
63		0.9	5.2	2.4	5.6	88
64		0.7	3.7	3.3	21	117
65		0.7	5.4	3.7	23	90
66		5.1	20	70	>100	ND
67		6.2	13	66	>100	ND
68		<7.8	11	27	46	ND
69		2.2	18	24	>100	ND

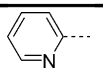
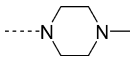
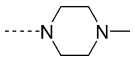
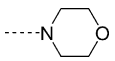
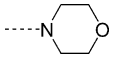
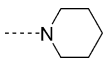
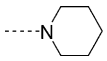
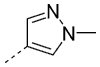
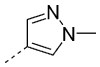
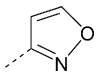
[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

and the rodent *P. berghei* strain. Activity shifts between the Albumax and serum conditions were small, as with the other derivatives. We therefore cannot really expect a better general in vivo behavior as we correlate activity shifts with potential PK properties. Based on the intrinsic activity the compounds might be interesting tools.

In Table 9 we summarize our investigations with respect to variations and potential replacements of the benzylpiperazinyl moiety.[15a,c] Bicyclic systems containing a piperazine ring were investigated as isosteres, as depicted in **108** or **109**. These two compounds showed less promising antimalarial activity than the compounds from Table 8. Further bicyclic piperazine-con-

Table 6. Phenylalanine replacements.

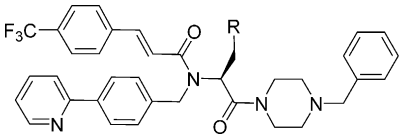


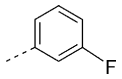
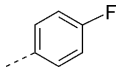
Compd	R	R^1	IC_{50} [nM][a] K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h
70			156	>500	ND
71			23	103	ND
72			<7.8	105	ND
73			2.9	18	36
74			<7.8	76	ND
75			1.2	10	17
76			236	>500	ND
77			86	>500	ND
78			156	>500	ND
79			41	483	ND
80			24	377	ND
81			11	90	ND
82			36	298	ND
83			16	105	ND
84			195	>500	ND
85			167	194	ND
86			67	>500	ND
87			32	270	ND
88			256	>500	ND
89			88	>500	ND

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

taining isosteres are depicted in **116**–**118**, and again resulted in a significant loss in antimalarial activity. Removing the basic character of the second piperazine nitrogen atom by changing to a piperazinone (**110**), by acylating the piperazine (**111**), or by directly attaching the electron-poor pyrimidinyl moiety (**112**) also resulted in low activities, especially against the NF54 strain under the more relevant human serum conditions. Replacing the benzyl substituent with hydroxyethyl (**113**), me-

Table 7. SAR on fluorophenylalanine derivatives.



Compd	R	IC_{50} [nM][a]			
		K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h
90		<7.8	84	8.6	>100
91		<7.8	168	16	>100
92		<7.8	69	4.9	135

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details).

thoxyethyl (**114**), or phenethyl (**115**) at the piperazine nitrogen atom also resulted in a clear loss of activity. Further isosteric replacements such as tetrahydroisoquinolinyl (**119**, **120**), morpholinyl (**121**), or an open-chain analogue of the morpholine group in **122**, were all unsuccessful as well. From these investigations no benzylpiperazine unit replacement could be identified. Therefore, this building block was kept for further explorations, allowing for additional small substituents at the phenyl ring only.

We decided to repeat the optimization on the cinnamoyl portion of compounds by implementing the results obtained thus far into the selection of the remaining parts of the molecules. This work is summarized in Table 10. The 4-cyanobenzylpiperazine was combined with phenylalanine and the 4-(4-acetylpiperazinyl)benzyl substituent to give the template to be combined with a set of diversely *para*-substituted cinnamic acids. The small series of final compounds were assessed for their activity against the NF54 parasite strain under both the Albumax and human serum conditions and against the *P. berghei* rodent parasite strain. All compounds exhibited excellent antiparasitic potency below 4 nM in the most artificial Albumax assay, except **133**, containing the methylsulfone substituent. This substituent provided highly potent compounds when attached to the benzylpiperazinyl unit (**103**, **104**, **106**, and **107**), but was not tolerated here. Most of the compounds also showed excellent potency in the NF54 human serum containing assay, whereas the situation with respect to the activity in the rodent *P. berghei* assay was more complex. With respect to absolute potencies and activity shifts between assays, **132** was the most promising derivative from this series.

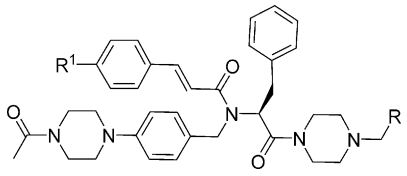
Table 11 summarizes further optimization efforts of the cinnamic acid substituent on a slightly different scaffold. This series of compounds confirmed the findings from Table 10 wherein the 4-*tert*-butylphenyl moiety of **162** resulted in the best overall profile. Compound **162** showed an even more promising overall profile than compound **127**. And again, the 4-methylsulfone substituent (in **156**) resulted in the least active derivative.

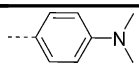
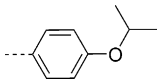
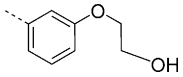
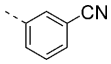
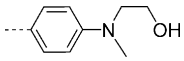
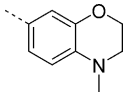
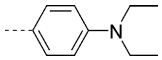
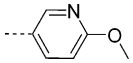
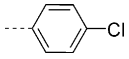
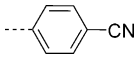
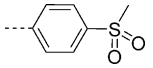
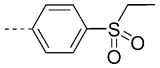
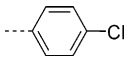
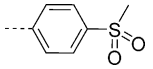
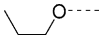
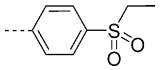
Table 12 depicts the results obtained when trying to replace the cinnamoyl moiety by bicyclic bioisosteres (**144**–**148**). These attempts resulted in significantly less potent derivatives and were stopped at this level.

A few further isosteric replacements investigated for the cinnamoyl moiety are given in Table 13. Compounds **149** and **150**, containing a 1,4-dimethyl-substituted thiazole moiety to replace the substituted phenyl ring of the cinnamoyl unit, showed lower potencies in the NF54 assays than previously discussed analogues. Compound **151**, bearing a methylsulfone substituent at the *para* position of the phenyl ring, was the most interesting example out of a series of antimalarials containing a phenoxyacetic acid group as an isosteric replacement. All other examples from this series, lacking the methylsulfone substituent, exhibited significant activity losses between the artificial Albumax conditions and the more physiological human serum conditions. Derivative **151** was tested for its in vitro metabolic stability and CYP3A4 inhibition and unfortunately could not be further pursued based on the obtained results (HLM = 974 μL (min mg)$^{-1}$, CYP3A4T = 1.4 μM).

To further cross-check our SAR we prepared the compounds summarized in Table 14. The purpose of this was to confirm that the acetylpiperazine is the best option for the R^1 position. The investigation focused on thiomorpholines (**152**, **153**), variously substituted piperazines (**154**–**157**, **168**), and nitrogen-containing heteroaryls such as pyridines (**158**–**166**) and pyrimidines (**167**) combined with either the trifluoromethyl-substituted cinnamoyl or the isopropoxy-substituted cinnamoyl unit, and in one case the *tert*-butyl-substituted cinnamoyl moiety. None of the compounds listed in Table 14 exhibited an improved activity profile over our most promising compounds (e.g., **132**). Comparison of **156**, **157**, and **168** showed that the cinnamoyl moiety has only a moderate effect on activity in combination with the isopropyl-substituted piperazinyl group

Table 8. SAR on benzylpiperazine substitutions.



Compd	R^1	R	IC_{50} [nM][a] K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
93	F_3C-----		1.8	6.2	4.0	21	ND
94	F_3C-----		1.4	7.5	2.6	14	ND
95	F_3C-----		3.2	29	11	34	34
96	F_3C-----		4.2	15	18	47	ND
97	F_3C-----		0.5	4.7	0.9	5.3	18
98	F_3C-----		3.9	36	17	ND	ND
99	F_3C-----		1.9	15	5.6	35	ND
100	F_3C-----		1.8	16	4.3	>20	ND
101	F_3C-----		2.1	15	3.7	48	ND
102	F_3C-----		1.6	16	1.8	14	54
103	F_3C-----		ND	ND	0.5	3.5	13
104	F_3C-----		ND	ND	0.8	3.9	7.0
105			ND	ND	1.9	11	64
106			ND	ND	0.2	1.8	0.9
107			ND	ND	0.5	1.8	3.7

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

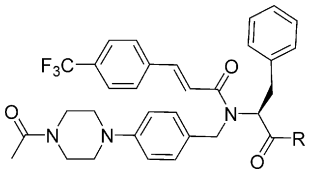
as R^1, confirming that the correct combination of both substituents is key for highly potent compounds.

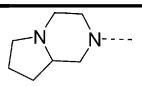
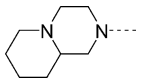
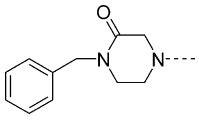
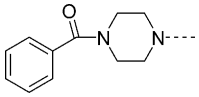
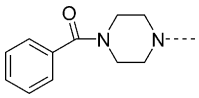
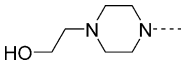
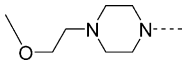
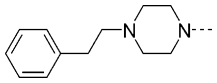
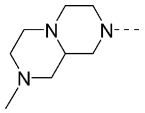
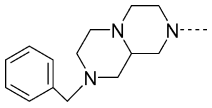
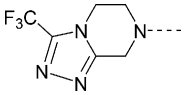
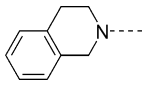
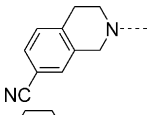
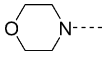
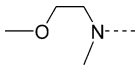
DMPK and pharmacology of a selected set of compounds

In the course of optimizing the in vitro antimalarial potency, the most interesting compounds were investigated with respect to their rat in vitro and in vivo as well as human in vitro drug metabolism and pharmacokinetics (DMPK) properties. A selection of results is summarized in Table 15. Descriptions of the experimental procedures used to generate the data depicted in Tables 15 and 16 are given in the Supporting Information.

As the in vivo rat hepatic clearance was high for the majority of the compounds investigated, the in vitro/in vivo extrapolation (IVIVE) of hepatic clearance in rat using the well-stirred liver model[18] was evaluated in order to establish how the in vitro intrinsic clearance (CL_{int}) data could predict in vivo clearance (CL_H), to allow selection of the most promising compounds to be tested in pharmacology in the murine *P. berghei* malaria model. The following Equation (1) was used:

Table 9. Benzylpiperazine replacements/alternatives.



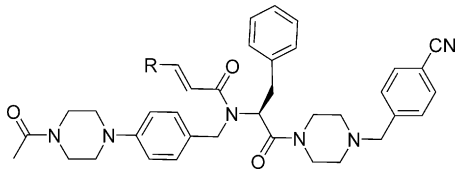
Compd	R	IC_{50} [nM][a]			
		K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h
108		<7.8	29	28	113
109		30	294	ND	ND
110		<7.8	49	36	236
111		ND	ND	59	>100
112		<7.8	176	28	118
113		45	102	ND	ND
114		<7.8	41	43	220
115		39	233	ND	ND
116		213	>500	ND	ND
117		97	>500	ND	ND
118		ND	ND	182	>500
119		ND	ND	8.3	47
120		ND	ND	38	98
121		ND	ND	42	>100
122		<7.8	18	20	135

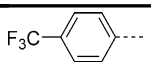
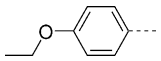
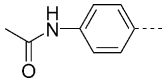
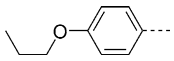
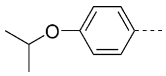
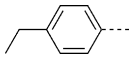
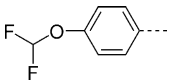
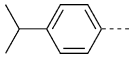
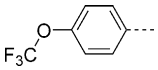
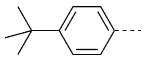
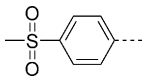
[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

$$CL_H = \frac{Q_H \times \left(\frac{\text{invitro}CL_{\text{int}} \times SF}{f_{\text{uinc}}}\right) \times f_{\text{ub}}}{Q_H + \left(\frac{\text{invitro}CL_{\text{int}} \times SF}{f_{\text{uinc}}}\right) \times f_{\text{ub}}} \quad (1)$$

in which Q_H = liver blood flow; SF = scaling factor (quantity of microsomes/hepatocytes in the body); f_{ub} = free fraction in blood; f_{up} = free fraction in plasma; f_{uinc} = fraction unbound in

Table 10. Cinnamic acid optimization.



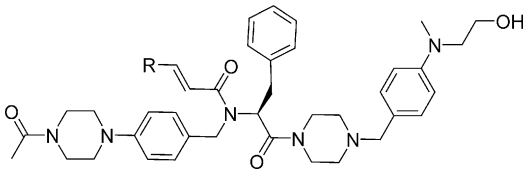
Compd	R	IC_{50} [nM][a] NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
123		3.8	14	54
124		1.7	1.9	36
125		1.9	19	126
126		1.1	3.3	29
127		0.8	3.2	33
128		0.8	5.4	32
129		1.7	4.4	72
130		0.5	2.8	27
131		3.3	8.9	78
132		0.4	6.7	14
133		18	33	ND

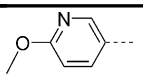
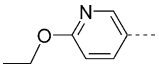
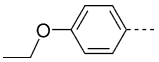
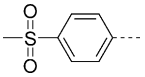
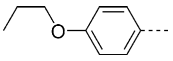
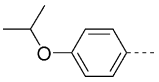
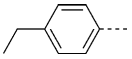
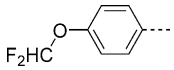
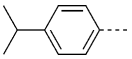
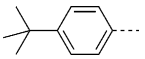
[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

incubation. The blood-to-plasma ratio was not measured, but predicted by the Gastroplus™ software package.

The data used for the prediction and the calculated CL_{int} values are summarized in Table 16. More accurate predictions were obtained for the highly protein-bound compounds relative to those showing a higher free fraction, which is rather uncommon, as a low f_u (fraction unbound) value should propagate in calculation errors. The factor of the underprediction of the rat plasma clearance ranged from <1.4 to 7.8, showing that the investigation of new compounds within this structural class on CL_{int} in rat liver microsomes (RLM) would not help to identify differences in the in vivo clearance data. In the human liver microsomal (HLM) assay, the CL_{int} value determined for the investigated compounds was in the same range as for the values obtained in the RLM assay. IVIVE would therefore predict higher in vivo clearance in humans than in rats. The half lives ($t_{1/2}$) in rat after intravenous (i.v.) administration were all in the same range of 0.76–2.5 h, in accordance with high clearance of the compounds. The terminal half lives might not have been captured properly due to the limit of quantification of the analytical LC–MS/MS systems. The volumes of distribution (V_{ss}) in rats were all above the volume of total body water, indicating a vast tissue distribution, consistent with the high lipophilicity of the compounds. These facts limit the concentration of compound in the systemic compartment. Plasma protein binding values for the compounds were high and also in accordance with the elevated cLog*P* values. The exposures of the compounds measured after i.v. administration were all in the same range. This indicated that the structural modifications of the discussed set of compounds did not have a pronounced influence on the clearance and the distribution of the drug. The bioavailability for the selected derivatives ranged from 1 to 44%, even though exposures after i.v. administration were very similar. The oral bioavailability is usually limited by high clearance. The high bioavailability observed for some compounds (e.g., **130** and **132**) was not completely understood. A potential explanation could be that bioavailability (F) was calculated with lower $AUC_{i.v.}$ values than the $AUC_{p.o.}$ values, which potentially neglects saturation of some hepatic clearance processes. We have the impression that working with compounds

Table 11. Optimization of the cinnamic acid in combination with the 4-(*N*-methyl-*N*-2-hydroxyethyl)benzylpiperazine moiety.



Compd	R	IC_{50} [nM][a] NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
134		10	33	58
135		4	12	69
136		1.5	5.7	24
137		5.8	11	102
138		1.9	6.6	14
139		1.3	4.0	11
140		1.6	3.3	14
141		3	8.2	17
142		1.2	6.2	12
143		0.8	4.5	5.6

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details).

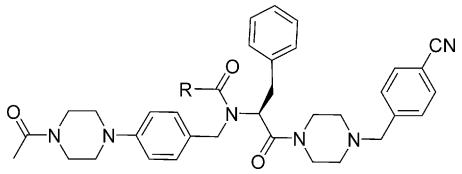
at the edge of the rule of five, for example, exhibiting values for three or more parameters above the defined upper limits, increases the difficulties in predicting in vivo PK behavior based on in vitro assessments of the compounds.[19]

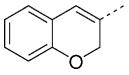
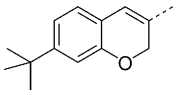
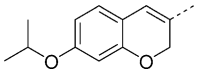
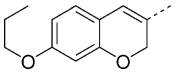
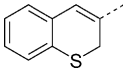
Based on the pharmacology results obtained with several examples, especially **63**, **97**, and **132**, we have shown that large molecules may still have the potential to become drugs, but rational approaches are rather difficult under these circumstances.

With a number of compounds we assessed antimalarial effects in vivo using a mouse malaria model infected with *P. berghei*. Using this test system, both antimalarial activity and survival time can be analyzed (Table 17). Compounds were administered 24 h post-infection (single-dose regimen) or 24, 48, and 72 h post-infection (triple-dose regimen). With the single-dose regimen, blood was collected on day 3 (72 h post-infection). Samples for the triple-dose regimens were collected on day 4 (96 h post-infection). Antimalarial activity was calculated as the difference between the mean percent parasitemia for the control ($n=5$ mice) and treated groups ($n=3$ mice) expressed as a percent relative to the control group. Control mice were euthanized on day 3 (single-dose regimen) or day 4 (triple-dose regimen) to prevent death typically occurring on day 6. Animals were considered cured if there were no detectable parasites on day 30 post-infection. Compound **23** was inactive at the lower dose of 30 mg kg^{-1}, independent of the route of administration, whereas **97** showed signs of antimalarial activity at 30 mg kg^{-1} when administered subcutaneously (s.c.) (77%) as well as per os (p.o.) (66%). At a dose of 100 mg kg^{-1}, **23** showed activity after p.o. administration (82%), but, to our surprise, was inactive after s.c. administration (21%). This behavior was confirmed in a second independent experiment. We assume that this is due to the solid-state properties of compound **23** which are different from those of the other compounds of the series. Compound **97** showed remarkable activity at 100 mg kg^{-1} of 98% (p.o.) and 99.2% (s.c.), which resulted in a minutely prolonged survival of the animals up to 7.7 days in the p.o. experiment. At 100 mg kg^{-1}, compound **100** showed lower activity (75% p.o.; 85% s.c.), but still prolongs survival of the mice to nearly the same extent as **97**.

The antimalarial activities of **26**, **29**, and **43** were compared in two different p.o. dosing regimens of either one dose of 100 mg kg^{-1}, or three dosings of 100 mg kg^{-1} on three consecutive days. Compounds **26** and **43** were inactive in both

Table 12. Cinnamic acid isosteres.



Compd	R	IC_{50} NF54 alb 72 h [nM][a]
144		92
145		178
146		50
147		69
148		253

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details).

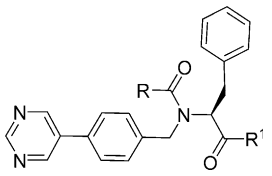
dosing regimens tested. Compound **29** exhibited weak activity (66%) when dosed once at 100 mg kg^{-1} and 85% when dosed three times 100 mg kg^{-1} on three consecutive days, resulting in a minute prolongation of survival time of the *P. berghei* infected mice. Compounds **149** and **150** were found to be inactive in vivo at single and triple dosings of 100 mg kg^{-1}, consistent with the finding that they were poorly active in vitro against *P. berghei*. The other examples summarized in Table 17 were tested under standardized conditions (formulated in Tween-EtOH/water) except example **63**, which was formulated in corn oil due to physicochemical limitations.

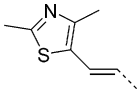
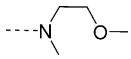
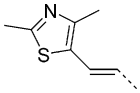
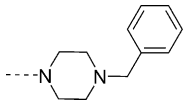
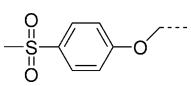
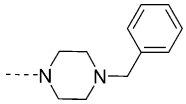
Compounds **63** (activity 98%), **126** (activity 99.3%), **127** (activity 99.2%), **130** (activity 99.0%), **132** (activity 99.2%), and **143** (activity 99.4%) all showed significant effects on mouse survival of at least nine days. For compound **126** and **127**, 10.3 and 13.3 days were reached, respectively. The combination of the mouse in vivo results with the in vitro activity data against *P. berghei* and *P. falciparum* and the results obtained in the PK experiments (Table 15) led us to decide to further profile and investigate **63**, **97**, and **132** in vivo under various dosing regimens summarized below. The standard formulation was changed to corn oil, based on the results obtained for **63**.

In a single-administration dose-escalating experiment with **63**, the survival time improved in a dose-dependent manner, reaching 13.0 days after a single administration of 300 mg kg^{-1} (Table 18). In the experimental setting with repeated administration on three consecutive days, average survival times of 22 (3×100 mg kg^{-1}) and 25 days (3×300 mg kg^{-1}) were obtained with some of the animals reaching cure. For the first time we had confirmation that it was possible to cure *P. berghei* infected mice with compounds of the class described in this report.

Compound **97** was our frontrunner for a certain period of the project and was therefore broadly investigated. The pharmacological characterization of **97** is summarized in Table 19. Antimalarial effects, with respect to survival, were similar at 1× 100 mg kg^{-1} and 3×33 mg kg^{-1} at $t=0$, 8, and 16 h. Administration of 3×100 mg kg^{-1} at the same schedule enhanced survival time to 11.3 days. A further prolongation of survival to 14, 19, and 27 days respectively, was observed when **97** was administered at 100, 350, and 500 mg kg^{-1} at three consecutive days. The same dosing regimen with **97** at 700 mg kg^{-1} resulted in a survival of 30 days, with no detectable parasites, which,

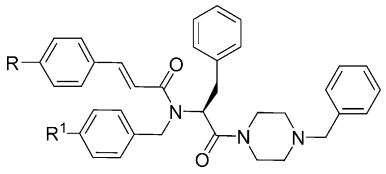
Table 13. Further cinnamic acid isosteres.

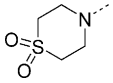
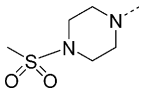
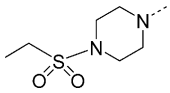
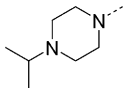
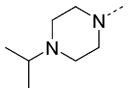
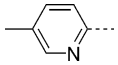
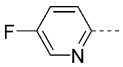
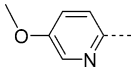
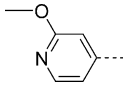
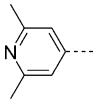
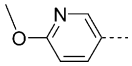
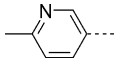
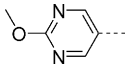
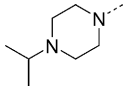


Compd	R	R^1	IC_{50} [nM][a]				
			K1 alb 72 h	K1 ser 72 h	NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
149			23	43	253	418	>500
150			8	44	95	>100	ND
151			<7.8	9.8	5.6	6.7	1281

[a] Data are the geometric mean (±2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

Table 14. Importance of the acetylpiperazine moiety.



Compd	R	R^1	IC_{50} [nM][a] NF54 alb 72 h	NF54 ser 72 h	*P. berghei* 24 h
152			12	18	268
153			10	20	ND
154			2.9	3.9	51
155			3.2	4.4	47
156			56	81	ND
157	F_3C		86	149	ND
158	F_3C		17	49	ND
159	F_3C		8.8	69	ND
160	F_3C		9.4	72	ND
161	F_3C		9.0	76	ND
162	F_3C		<7.8	114	ND
163	F_3C		3.8	27	ND
164	F_3C		2.3	>20	ND
165	F_3C		5.0	70	ND
166	F_3C		4.7	73	ND
167	F_3C		3.3	80	ND
168			39	54	ND

[a] Data are the geometric mean (± 2-fold) of at least three independent experiments; in vitro activity against erythrocytic stages of *P. falciparum* was determined using a [^{3}H]hypoxanthine incorporation assay (see the Supporting Information for assay details). ND: not determined.

Table 15. Selected DMPK parameters and properties of pharmacologically investigated compounds.[a]

Compd	*CL*	V_{ss}	$t_{1/2}$	$AUC_{p.o.}$	$AUC_{i.v.}$	C_{max}	t_{max}	*F*	HLM	RLM	hPPB	rPPB
124	51	3.7	1.8	411	327	151	0.5	13	275	336	99.8	99.8
127	79	7.7	1.6	580	212	223	1	27	306	289	99.6	99.7
130	53	6.1	2.5	996	317	326	1	31	276	181	99.9	99.9
132	57	7.6	2.3	1270	291	288	0.5	44	267	111	99.9	99.9
136	87	4.6	0.76	228	192	93	0.63	12	392	276	99.7	99.8
137	75	3.5	0.87	23.6	223	10.6	0.5	1	314	118	91.1	96.4
139	79	5.3	1.9	274	211	85.2	2	13	304	204	99.8	99.9
143	57	7.5	2	681	291	157	2	23	211	129	99.9	99.9

[a] In vivo data generated in rats; dosing was as follows: 10 mg kg^{-1} p.o. and 1 mg kg^{-1} i.v.

Table 16. Data used for prediction and calculated intrinsic clearance (CL_{int}).

Compd	cLog*P*[a]	B/P ratio	f_u [%] rat plasma	f_u [%] RLM	CL_{int} RLM	Rat pl. *CL* pred.	Rat pl. *CL* obsd.	Fold underprediction	f_u [%] hum. plasma	CL_{int} HLM	Hum. pl. *CL* pred.
124	5.21	0.65	0.2	5.6	336	14	51	3.6	0.2	275	14
127	5.66	0.65	0.3	2.3	289	27	79	2.9	0.4	306	27
130	5.86	0.65	< 0.1	0.3	181	< 32	53	< 1.7	< 0.1	276	< 32
132	6.34	0.65	< 0.1	< 0.1	111	NC[b]	57	NC[b]	< 0.1	267	NC[b]
136	4.92	0.70	0.2	3.7	276	17	87	5.1	0.3	392	17
137	3.84	0.72	3.6	34	116	15	75	5.0	8.9	314	15
139	5.35	0.70	0.1	2.1	204	13	79	6.6	0.2	304	13
143	6.09	0.70	< 0.1	0.1	129	< 41	57	< 1.4	< 0.1	211	< 41

[a] Calculated by Gastroplus™ ver. 0.0 (Simulations Plus Inc.). [b] Plasma clearance could not be calculated, as the f_u values in microsomes and in plasma were measured below the 0.1 % limit of quantification.

Table 17. Summary of in vivo experiments and results.[a]

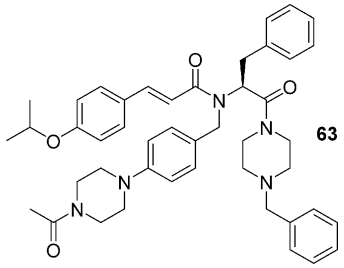
Compd	Dose [mg kg^{-1}]	Administration Repeats	Administration Route	Parasitized RBC/100	% of Ctrl	Activity [%]	Survival days
23	100	1×	s.c.	32.34	79.06	< 40	3.0
	100	1×	p.o.	7.49	18.32	82	6.0
	30	1×	s.c.	40.13	98.10	< 40	3.0
	30	1×	p.o.	30.09	73.57	< 40	3.0
26	100	1×	p.o.	22.88	63.79	< 40	3.0
	100	3×	p.o.	33.20	47.61	< 40	3.0
29	100	1×	p.o.	12.10	33.73	66	7.0
	100	3×	p.o.	10.16	14.56	85	7.3
43	100	1×	p.o.	28.95	80.73	< 40	3.0
	100	3×	p.o.	45.12	64.70	< 40	4.0
63	100	1×	p.o.[b]	0.27	1.55	98	9.0
97	100	1×	s.c.	0.34	0.84	99.2	7.7
	100	1×	p.o.	0.93	2.28	98	6.0
	30	1×	s.c.	9.93	22.96	77	6.0
	30	1×	p.o.	13.95	34.10	66	6.0
100	100	1×	s.c.	5.98	14.63	85	6.7
	100	1×	p.o.	10.13	24.76	75	6.7
	30	1×	s.c.	35.08	85.75	< 40	3.0
	30	1×	p.o.	31.92	78.03	< 40	3.0
123	100	1×	p.o.	7.67	20.77	79	6.7
124	100	1×	p.o.	0.33	0.89	99.1	7.7
125	100	1×	p.o.	38.81	105.14	< 40	3.0
126	100	1×	p.o.	0.25	0.73	99.3	10.3
127	100	1×	p.o.	0.29	0.78	99.2	13.3
128	100	1×	p.o.	0.86	2.33	98	6.7
129	100	1×	p.o.	4.10	11.12	89	7.0
130	100	1×	p.o.	0.54	1.46	99	9.0
131	100	1×	p.o.	0.94	2.56	97	6.7
132	100	1×	p.o.	0.29	0.79	99.2	9.7

Table 17. (Continued)

Compd	Dose [mg kg^{-1}]	Administration		Parasitized RBC/100	% of Ctrl	Activity [%]	Survival days
		Repeats	Route				
134	100	1×	p.o.	32.67	94.15	<40	6.0
135	100	1×	p.o.	16.56	47.72	52	6.0
136	100	1×	p.o.	2.20	6.33	94	6.0
137	100	1×	p.o.	32.28	93.02	<40	5.0
138	100	1×	p.o.	0.61	1.77	98	6.0
139	100	1×	p.o.	0.63	1.81	98	7.0
140	100	1×	p.o.	1.23	3.55	96	7.7
141	100	1×	p.o.	7.53	21.70	78	6.0
142	100	1×	p.o.	0.42	1.22	99.0	6.0
143	100	1×	p.o.	0.21	0.61	99.4	9.7
149	100	1×	p.o.	26.62	83.81	<40	3.0
	100	3×	p.o.	33.77	63.82	<40	4.0
150	100	1×	p.o.	27.58	92.01	<40	3.0
	100	3×	p.o.	45.61	86.20	<40	4.0
151	100	1×	p.o.	33.99	92.08	<40	3.0

[a] Mice were euthanized at the end of the experiment, i.e., after the number of survival days indicated. Standard experiments were performed with three mice per treatment or control group. Compounds were formulated in [Tween-EtOH]/water (10:90). [b] Formulation in corn oil.

Table 18. In vivo data for compound **63**.[a]



63

Dose [mg kg^{-1}]	Administration repeats	Parasitized RBC/100	% of Ctrl	Activity [%]	Survival days
300	1×	0.19	1.12	99.0	13
100	1×	0.27	1.55	98	9.0
30	1×	11.66	67.69	<40	3.0
10	1×	18.49	107.32	<40	3.0
3	1×	24.42	141.76	<40	3.0
300	3×	0.16	0.37	99.6	25.0[b]
100	3×	0.14	0.32	99.7	22.0[c]
30	3×	14.59	34.54	65	7.0
10	3×	49.70	117.62	<40	4.0
3	3×	45.44	107.54	<40	4.0

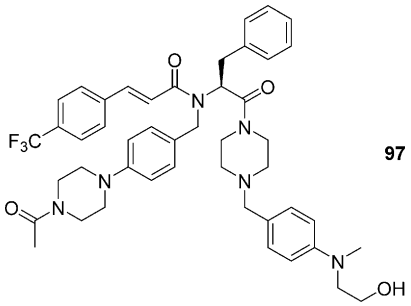
[a] Three mice per experiment; compound was formulated in corn oil for the experiments and administered p.o. Repeated administration was on consecutive days (mice cured: survival was 30 days, and at this time point mice were parasite free). [b] 2 out of 3 mice cured under the experimental conditions. [c] 1 out of 3 mice cured under the experimental conditions.

in the *P. berghei* model, is considered a cure. Single-dose administrations, even at high doses such as 500 or 750 mg kg^{-1}, did not result in cure. These findings were in line with the PK behavior found with compound **97**, with a rather short half-life, which results in fast excretion and allows the parasites to recur.

Our most promising compound at the end of our drug discovery efforts was **132** (ACT-451840). The results of the pharmacological experiments obtained with **132** in the *P. berghei* infected mouse model are summarized in Table 20. The dose-escalating experiments with single doses between 10 and 60 mg kg^{-1} revealed that **132** started to show significant antimalarial effects (parasite reduction) in vivo at 20 mg kg^{-1}. When administered repetitively on three consecutive days, compound **132** exhibited curative activity at 300 mg kg^{-1} and significant antimalarial effects already at 100 mg kg^{-1}. With respect to parasitemia, **132** showed excellent reduction at 30 mg kg^{-1}, resulting in 99.80 % activity.

The in vivo activity of **132** was also assessed in the *P. falciparum* humanized immunodeficient mouse model established and performed at GSK (DDW, Tres Cantos, Spain).[20] After escalating oral dosing on four consecutive days, **132** exhibited

Table 19. In vivo data for compound **97**.[a]

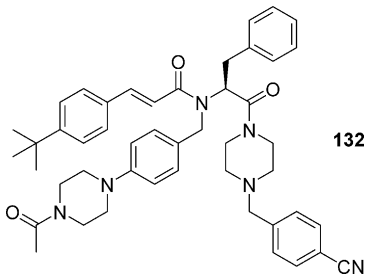


97

Dose [mg kg^{-1}]	Administration repeats	Parasitized RBC/100	% of Ctrl	Activity [%]	Survival days
100	1×	0.98	3.47	97	7.0
33	3×[b]	0.33	1.16	99.0	7.3
100	3×[b]	0.12	0.42	99.6	11.3
100	3×[c]	0.07	0.14	99.9	14.0
350	1×	0.17	0.56	99.4	9.0
350	3×[c]	0.0	0.0	99.9	19.3
500	1×	0.21	0.60	99.4	11.6
500	3×[c]	0.50	0.70	99.3	27.2
750	1×	0.30	0.86	99.1	14.4
750	3×[c]	0.15	0.20	99.8	30.0[d]
100 (−24 h)[e]	1×	26.30	108.31	<40	3.0
100 (−48 h)[f]	1×	27.99	116.44	<40	3.0

[a] Three mice per experiment; compound was formulated in corn oil for the experiments and administered p.o. [b] Repeated administration was at $t=0$ h, $t=8$ h, $t=16$ h. [c] Repeated administration was on consecutive days. [d] Parasite-free mice on day 30 post-infection were considered cured. [e] Administration of **97** 24 h prior to infecting the mice. [f] Administration of **97** 48 h prior to infecting the mice. [e,f] These two experiments indicate that **97** is not useful as preventive treatment under the experimental conditions.

Table 20. In vivo data for compound **132** (ACT-451840).[a]



132

Dose [mg kg^{-1}]	Administration repeats	Parasitized RBC/100	% of Ctrl	Activity [%]	Survival days
100	1×[b]	0.29	0.79	99.2	9.7
60	1×[c]	0.10	0.37	99.6	12.7
30	1×[c]	0.83	3.11	97	8.7
25	1×[c]	2.60	9.71	90	6.7
20	1×[c]	6.37	23.77	76	7.0
15	1×[c]	16.33	60.99	<40	3.0
10	1×[c]	23.80	88.87	<40	3.0
300	3×[c,d]	0.17	0.39	99.6	30.0[e]
100	3×[c,d]	0.15	0.35	99.6	26.7
30	3×[c,d]	0.10	0.23	99.8	13.0
10	3×[c,d]	28.63	67.75	<40	4.0
3	3×[c,d]	50.98	120.65	<40	4.0

[a] Three mice per experiment. [b] Compound was formulated in [Tween-EtOH]/water (10:90) and administered p.o. [c] Compound was formulated in corn oil for the experiments and administered p.o. [d] Repeated administration was on consecutive days. [e] 30 days survival is considered as cure.

a rapid onset of action and an effective dose resulting in 90% antimalarial activity (ED_{90}) of 3.7 mg kg^{-1}, which was similar to that of chloroquine after an oral quadruple-dose regimen in this same model (ED_{90}: 4.9 mg kg^{-1}). After an oral triple-dose regimen in the *P. berghei* mouse model, compound **132** exhibited an ED_{90} value of 13 mg kg^{-1}. This difference in ED_{90} very likely derives from the lower in vitro activity of **132** toward *P. berghei* versus *P. falciparum*. Drug formulation appears to be important for the in vivo activity of **132**. Comparison of the *P. berghei* mouse experiment at 100 mg kg^{-1} formulated in Tween-EtOH/water with the experiment at 60 mg kg^{-1} formulated in corn oil illustrates the additional effect that can be obtained with an optimized formulation.

Taken together, the above results suggest that **132** behaves in a way similar to artemisinin derivatives, with very rapid onset of action and elimination of parasites. However, to result in a cure, repeated high dosing is needed in the case of both artemisinin and **132** to overcome PK limitations. Nevertheless, with optimized drug formulation, compounds such as **132** could be considered as artemisinin replacement options in ACT antimalarial treatment regimens, and could be combined with a long-acting second antimalarial such as lumerantrine or mefloquin, in order to prevent the development of resistance.

Conclusions

We have summarized our efforts in the optimization of phenylalanine-based antimalarials initially detected in a phenotypic screening effort. Our work culminated in compounds that exhibit potent antimalarial activity in vitro and in vivo, including cure in a *P. berghei* mouse model of malaria. The compounds act through a novel and unknown mechanism of action. ACT-451840 (**132**) is a candidate for preclinical and clinical development.[21] Further results will be reported in due course.

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Keywords: antimalarial drugs · malaria · phenotypic drug discovery · phenylalanine derivatives · SCID mouse model

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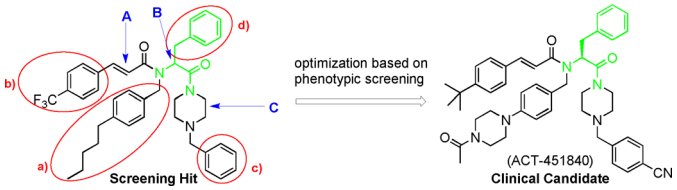

Do it the phenotypic way: After having failed by using a target-based rational approach, we decided to go after malaria parasites by putting phenotypic assays at the center of our efforts.

Herein you will learn the story behind the identification of ACT-451840, which was selected for clinical development as an antimalarial drug candidate.

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Discovery and Characterization of ACT-451840: an Antimalarial Drug with a Novel Mechanism of Action

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