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Novel in vivo active anti-malarials based on a hydroxy-ethyl-amine scaffold

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ABSTRACT

A novel series of anti-malarials, based on a hydroxy-ethyl-amine scaffold, initially identified as peptidomimetic protease inhibitors is described. Combination of the hydroxy-ethyl-amine anti-malarial phramacophore with the known *Mannich* base pharmacophore of amodiaquine (**57**) resulted in promising in vivo active novel derivatives.

Malaria is an infectious disease caused by *Plasmodium* parasites and is transmitted to humans via the bite of infected female anopheles mosquitoes. According to the World Health Organization (WHO), 3.3 billion people were at risk of being infected with malaria.[1] Every year, about 250 million people are affected by malaria resulting in 1 million deaths. Most vulnerable are children under the age of five and pregnant women. Five *Plasmodium* parasite species can cause malaria in humans, with *Plasmodium falciparum* being responsible for most of the fatal cases. Due to the development of resistance towards existing treatment regimens, there is an urgent need for new anti-malarial drugs.[2]

Hydroxy-ethyl-amine containing compounds (Fig. 1) have previously been studied as potential anti-malarials. Since the hydroxy-ethyl-amine moiety represents a stabilized peptidomimetic inhibitor of aspartic proteases, it has been suggested that these compounds act by blocking the parasite specific aspartic proteases, the plasmepsins.[3]

Food-vacuolar plasmepsins were considered viable drug targets in plasmodium parasites, based on their role in the parasite specific degradation of hemoglobin in the food-vacuole.[4] However, it has been recently shown that the parasite has redundant mechanisms for hemoglobin digestion and that targeting of food-vacuolar plasmepsins is not sufficient to kill the malaria parasites.[5] In our search for a new anti-malarial drug, compounds of type **3** (Fig. 2) were screened with the [^{3}H]hypoxanthine incorporation assay[6]

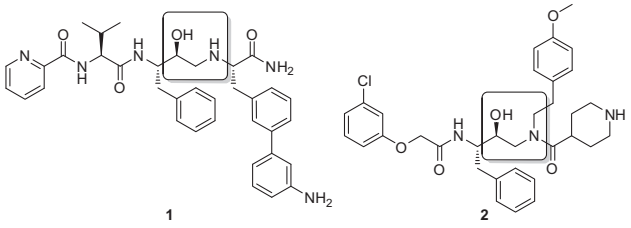

Figure 1. Examples of hydroxy-ethyl-amine compounds targeting plasmepsin I and II.

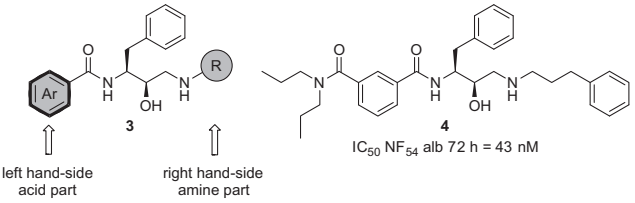

Figure 2. Hydroxy-ethyl-amine scaffold based anti-malarials.

for the inhibition of parasite growth in red blood cells (RBC) infected with the *P. falciparum* NF54 strain (chloroquine-sensitive strain). The initial hit compound **4** (Fig. 2) showed promising activity against the malaria parasite with an IC_{50} value of 43 nM and possesses a lower molecular weight than the previously described

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hydroxy-ethyl-amine plasmepsin inhibitors. This chemotype was also described in the TCAMS dataset released by GSK during the course of this work.[7]

As a starting point **4** displayed a good in vitro activity against *P. falciparum* in the RBC assay, therefore we set out to optimize its activity against the parasite as well as its physico-chemical properties.

Two synthetic routes were developed to access the compounds of class **3** Figure 3. The first one allowed easy modification of the right hand-side of the molecule while the second route permitted variation of the left hand-side. Route 1 started with the homologation of Boc-Phe with nitromethane. After reduction of the ketone, recrystallization afforded the desired diastereomer **6**.[8] Subsequent Boc-deprotection resulted in amine **7**. At this point different acids were introduced by standard amide coupling reactions. The nitro-group was subsequently hydrogenated and the right hand-side substituent introduced by reductive amination affording the desired hydroxy-ethyl-amine compounds **3**.[9] In route 2, epoxide opening of **10**[10] afforded Boc-protected amine **11**. The target compounds of structure **3** were obtained by Boc-deprotection followed by amide-coupling with carboxylic acid derivatives.

All compounds were tested for inhibition of parasite growth in red blood cells (RBC) infected with the *P. falciparum* NF54 parasite strain which was cultured in vitro according to Trager and Jensen.[6] IC_{50} values were determined by measuring incorporation of the nucleic acid precursor [^{3}H]hypoxanthine after 72 h of incubation.[11a,11b] IC_{50} values were also determined in the presence of 50% serum (no albumax), since a large activity shift between the assay with 0.5% albumax and the assay with 50% serum has previously led to inactive compounds in vivo, most likely due to high protein binding.

We first optimized the right hand-side of the molecule by replacing the phenyl-propylamine substituent with a range of amines (Fig. 4 and Table 1). Both alkyl and aromatic substituents were tolerated. The best alkyl substituent was a cyclopentyl (**18**) with an IC_{50} in the NF54 albumax assay of 7.1 nM. Reducing the chain length of the original phenylpropylamine in **4** resulted in a loss of activity (**22**–**24**). However, introducing one (**26** and **27**) or two methyl groups (**25**) at the benzylic position of **24** led to very active compounds with IC_{50} values below 10 nM. The chirality of the methyl group was important, with the *R*-epimer **26** being more active. Substituents were then introduced to the aromatic ring of **25** and it was found that *meta*-substitution resulted in the most active compounds, for example derivative **29** which has an IC_{50} below 1.5 nM.

The phenyl-alanine core was studied, too (Fig. 5). The OH group was found to be crucial for the activity of the compounds against the parasite. Indeed, the *S,S*-diastereomer **42** was totally inactive.

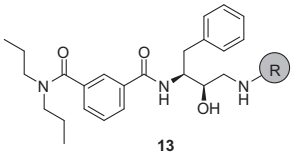


Figure 4. Structure of compounds in Table 1.

Removal of the OH group (**44**) or replacing it with two fluorine atoms (**46**) resulted in inactive compounds as did exchanging the amino-acid core to valine (**47**).

Having identified active substituents for the right hand-side of the molecule, we turned our attention to the modification of the left hand-side of the molecule (Table 2 and Fig. 6). Compound **26** was taken as starting point. First, the position of the amide substituent was varied. The *para*-isomer **49a** was also highly active with an IC_{50} value of 1.6 nM. The *ortho*-isomer **49b** was inactive against the parasite with an IC_{50} >500 nM. Changing the N-substituent of the amide slightly improved the activity (**49c** and **49f**) with IC_{50} values below 1 nM. Compound **49i** suggests that both alkyl chains are necessary for high activity. To further investigate the influence of the left-hand side of the molecule on the anti-malarial activity, the amide group of **48** was replaced by heterocycles and alkyl substitutents (Table 1, Supplementary data). Unfortunately this resulted in a loss of activity against the malaria parasite. Introducing an *N*-methyl-piperazine unit as the R group led to compound **49j** with an IC_{50} below 10 nM. Further modifications allowed us to identify 4-amino-piperidine as in **49m, n** and 4-amino-pyridine as in **49o–r** as active substituents.

In order to prioritize candidates for further investigations, the in vitro metabolic stability of the compounds was evaluated using a mouse liver microsomal preparation.[12] Unfortunately, highly active compounds where R is an amide group (compounds **26**, **49a** and **49f**) mostly showed only moderate in vitro metabolic stability, which may be a potential indicator for high in vivo clearance and for a non-optimal pharmacokinetic profile. However, **49c** together with **49j**, **49m, n** and **49p** had better in vitro metabolic stability (below 100 µl/(min mg)).

A fast onset of action is highly desirable for an anti-malarial drug as it suggests that it is active against all three asexual erythrocytic stages of the parasite. The widely used artemisinins for example exhibit a fast onset of action.[13] In view of selecting compounds for in vivo testing, this property was assessed by measuring the IC_{50} values of the compounds against the parasite after an incubation time of 24, 48 and 72 h. Together with the evaluation of the onset of action, the potency of the compounds against the rodent parasite *Plasmodium berghei* was as well measured, being a prerequisite to test the compounds in vivo in the *P. berghei*

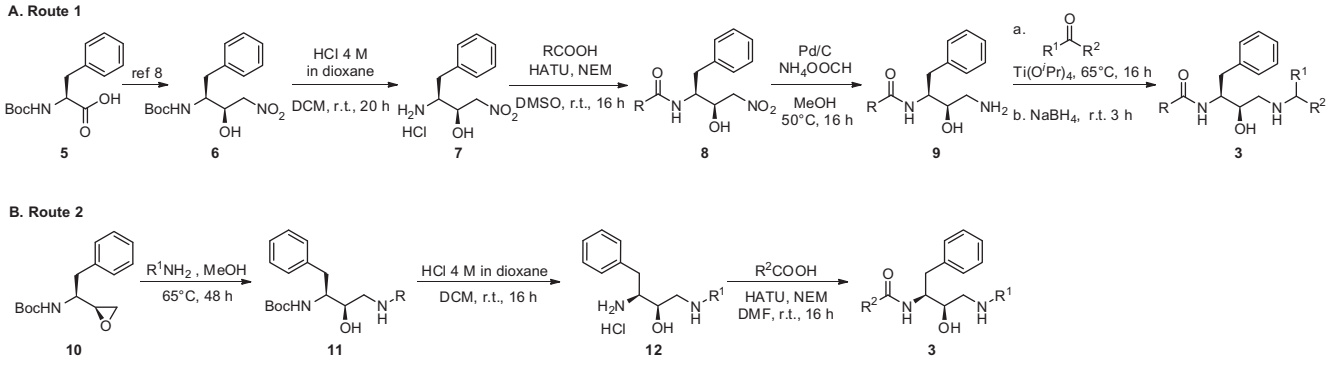
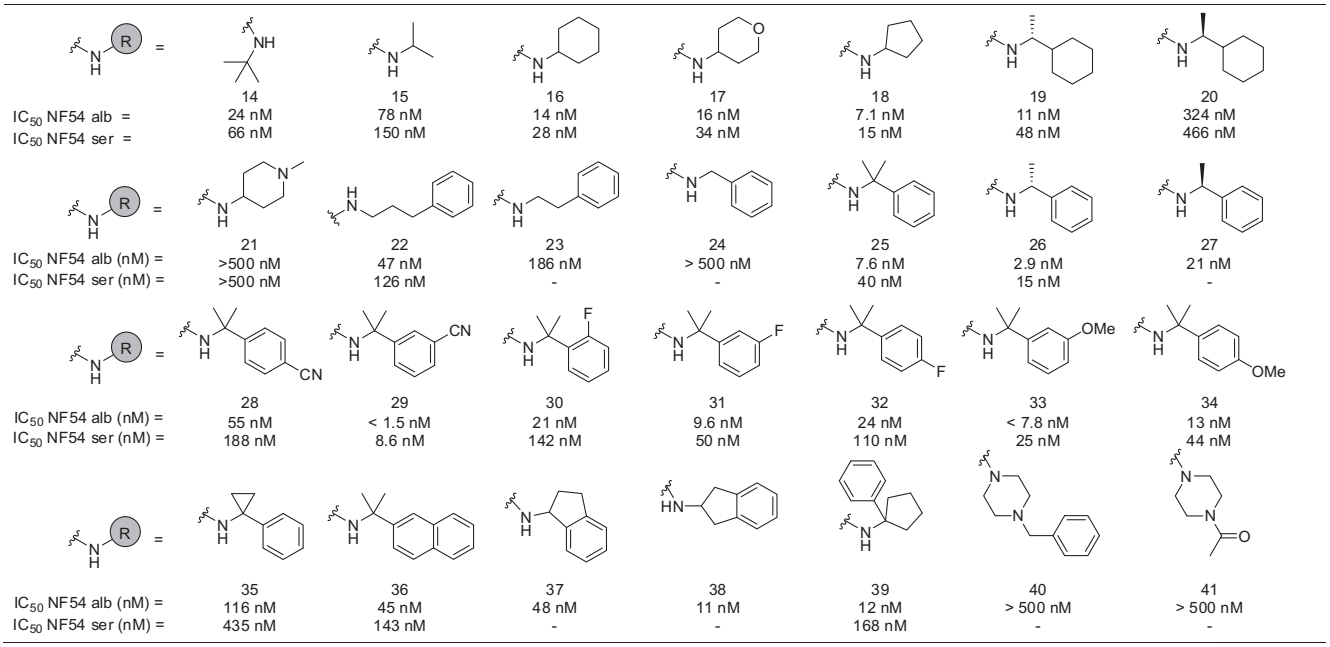


Figure 3. (A) Route 1: synthesis of hydroxy-ethyl-amine compounds with variations on the left hand-side. (B) Route 2: synthesis of hydroxy-ethyl-amine compounds with variations on the right hand-side.

Table 1
In vitro anti-malarial activity of hydroxy-ethyl-amine compounds of structure **13**: optimization of the amine part (IC_{50} values are the mean of at least three independent experiments)



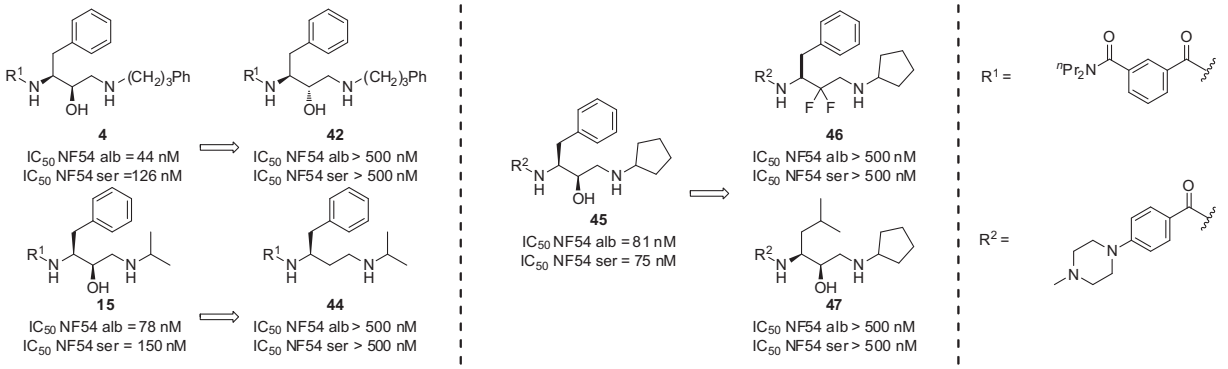


Figure 5. Variation of the core of hydroxy-ethyl-amine compounds.

Table 2
In vitro anti-malarial activity of hydroxy-ethyl-amine compounds: optimization of the acid part

Entry	Compound	R	IC_{50} NF_{54} alb 72 h (nM)	IC_{50} NF_{54} ser 72 h (nM)	IC_{50} NF_{54} alb 24 h (nM)	IC_{50} NF_{54} alb 48 h (nM)	IC_{50} *P. berghei* 24 h (nM)	MLM (µl/(min mg))
1	**26**	3-CON^{n}Pr$_2$	2.0	10	>500	<3.1	>500	>1250
2	**49a**	4-CON^{n}Pr$_2$	1.6	6.5	>500	–	>500	>1250
3	**49b**	2-CON^{n}Pr$_2$	>500	>500	–	–	–	–
4	**49c**	4-CO-Me-piperazine	0.6	<0.6	>500	–	>500	75
5	**49d**	3-CO-Me-piperazine	98	102	–	–	–	–
6	**49e**	3-SO_2-Me-piperazine	98	138	–	–	–	908
7	**49f**	4-CO-piperidine	0.9	1.3	>500	–	>500	860
8	**49g**	3-CO-pyrolidine	4.9	9.3	–	–	–	–
9	**49h**	3-CO-azepane	3.8	13	–	–	–	–
10	**49i**	3-CONH^{n}Pr	12	30	–	–	–	–
11	**49j**	4-Me-piperazine	8.7	8.5	>500	–	>500	80
12	**49k**	3-Me-piperazine	190	300	–	–	–	–
13	**49l**	4-Bn-piperazine	21	39	–	–	–	–
14	**49m**	4-NH-Me-piperidine	4	12	>500	<7.8	–	76
15	**49n**	4-NMe-Me-piperidine	4.4	14	>500	<7.8	–	71
16	**49o**	4-NH-2,6-diMe-pyridine	8.7	23	198	11	329	–
17	**49p**	4-NH-2-Me-pyridine	9.8	29	173	–	228	69
18	**49q**	4-NH-pyridine	20	36	169	26	307	112
19	**49r**	4-NMe-pyridine	21	35	249	27	>500	180

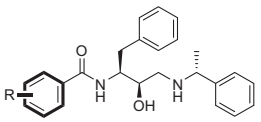


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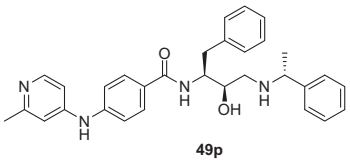
Figure 6. Structure of compounds in Table 2.



49p

In vitro properties

IC_{50} NF54 alb 72 h =	9.8 nM
IC_{50} NF54 ser 72 h =	29 nM
IC_{50} NF54 24 h =	173 nM
IC_{50} P.berghei 24 h =	228 nM
MLM =	69 µl/min.mg

In vivo properties

Dose: 1x100 mg/kg
33% reduction of parasitemia
Survival days : 3 days

Figure 7. In vivo activity of **49p** in the *P. berghei* infected mouse model.

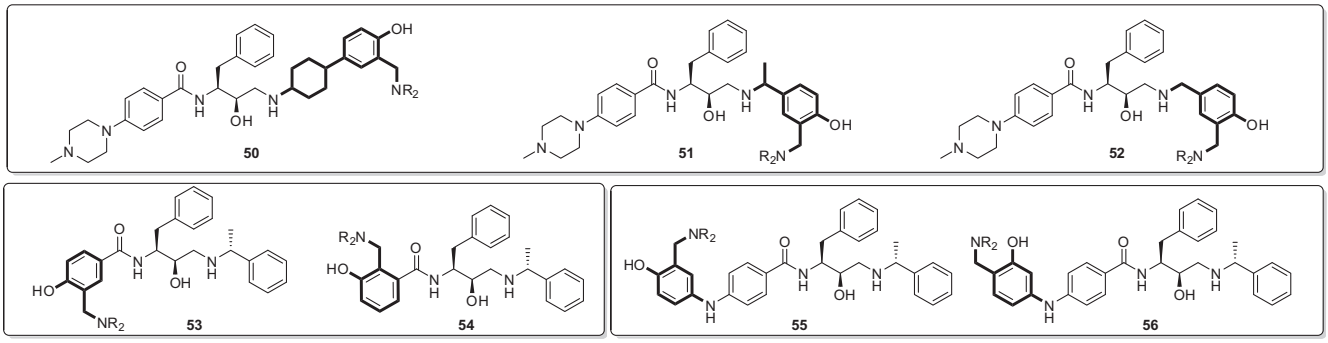
infected mouse model of malaria. The IC_{50} against *P. berghei* was measured after 24 h of incubation (IC_{50} *P. berghei* 24 h).[14] Unfortunately, most compounds were inactive against *P. falciparum* as well as *P. berghei* after 24 h of incubation time. This seems to be a major drawback as it suggests that many of our compounds suffer from a slow onset of action. However, compounds bearing a 4-amino-pyridine **49o–r** showed efficacy against the parasite after 24 h of incubation time albeit with a significant shift between the IC_{50} at 24 h and the IC_{50} at 48 and 72 h.

Compound **49p** (Fig. 7) which exhibited the best combination of activities so far and the best in vitro metabolic stability was tested for efficacy in the *P. berghei* infected mouse model.[15] A single oral dose of 100 mg/kg did not result in a significant reduction of parasitemia (33% of control) and mouse survival time was similar to that of non-treated animals. Despite low nM anti-parasitic activity and reasonable metabolic stability in mice, this series of compounds exhibited poor behavior in in vivo experimental settings, possibly due to the slow onset of action.

We expected that improving the speed of action of this series would permit to obtain an efficient anti-malarial drug. Inspired by the work of Meunier[16a] and O'Neill and co-workers[16b] on hybrid anti-malarial drugs in which two pharmacophores were combined to obtain molecules exhibiting both modes of action, we set out to introduce a *Mannich* base pharmacophore into our compounds. We hoped to improve the speed of action by combining the mechanism of the *Mannich* base pharmacophore with the mechanism of the hydroxy-ethyl-amine pharmacophore. The *Mannich* base motif is part of the amodiaquine **57** pharmacophore, a drug used for the treatment of acute malaria, and was also used by O'Neill and co-workers[16b] in hybrid anti-malarial **58** (Fig. 8).

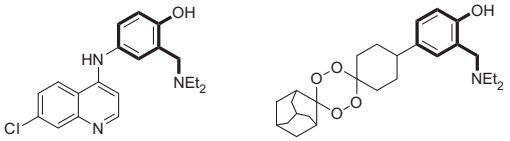
The *Mannich* base pharmacophore was introduced into our compounds as depicted in Table 3. Replacing the phenethyl-amine by a *Mannich* base led to slightly less active compounds (**50a–52d**) however for all compounds no difference in activity was detected for 24–72 h of incubation, confirming the hypothesis that combining the hydroxy-ethyl-amine scaffold and a *Mannich* base pharmacophore accelerates the onset of action. For example, compound **50a** exhibited an IC_{50} below 20 nM against *P. falciparum* NF54 at all time points. It was also active against the rodent parasite with an IC_{50} of 143 nM. Moreover, the introduction of the *Mannich* base pharmacophore did not affect the in vitro metabolic stability. In contrast to previous observations, the presence of a methyl group at the benzylic position did not have a dramatic effect on the activity against NF54 (e.g. **51b** vs **52d**). The nature of the R group on the

Table 3
In vitro anti-malarial activity and in vitro metabolic stability of hybrid compounds



50 51 52 53 54 55 56

Entry	Compound	NR_2	IC_{50} NF_{54} alb 72 h (nM)	IC_{50} NF_{54} ser 72 h (nM)	IC_{50} NF_{54} alb 24 h (nM)	IC_{50} NF_{54} alb 48 h (nM)	IC_{50} *P.berghei* 24 h (nM)	MLM (µl/(min mg))
1	**50a**	N^nPr_2	19	–	20	19	143	74
2	**51a**	Piperidine	203	125	–	–	–	–
3	**51b**	N^nPr_2	89	62	90	75	–	–
4	**52a**	Pyrolidine	>500	295	>500	>500	–	–
5	**52b**	NEt_2	333	167	408	314	–	–
6	**52c**	Piperidine	120	57	158	102	–	–
7	**52d**	N^nPr_2	65	42	69	50	140	90
8	**53a**	NEt_2	357	>500	380	305	–	–
9	**53b**	N^nPr_2	118	334	151	83	>500	–
10	**53c**	Pyrolidine	261	386	–	–	–	–
11	**54a**	Piperidine	4.9	5.9	235	<7.8	>500	–
12	**55a**	NEt_2	8.3	3.6	133	<7.8	–	–
13	**55b**	N^nPr_2	9.4	13	77	11	–	–
14	**55c**	Pyrolidine	17	4.9	174	<7.8	–	–
15	**56a**	NEt_2	<7.8	9	65	15	–	–
16	**56b**	N^nPr_2	22	20	77	–	–	–
17	**56c**	Piperidine	3.9	3.3	25	<7.8	172	415



Amodiaquine **57** Hybrid compound **58**[16b]

Figure 8. Anti-malarial compounds containing a *Mannich* base pharmacophore.

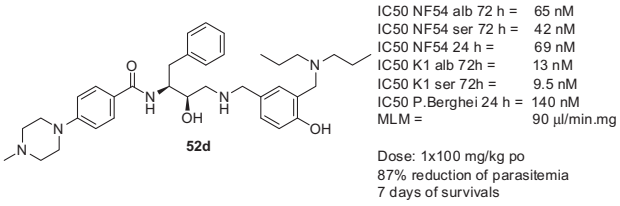


Figure 9. In vivo activity of **52d** in the *P. berghei* infected mouse model.

benzyl amine had however a noticeable effect on the IC_{50}, going from an inactive compound **52a** with a pyrrolidine as a side chain to **52d** with an IC_{50} NF54 alb 72 h = 65 nM bearing a dipropylamine side chain. In addition compound **52d** showed activity against *P. berghei* and an in vitro metabolic stability below 100 µl/(min mg).

The replacement of the *N*,*N*-dipropylbenzamide in **13** by a *Mannich* base resulted in poorly active compounds **53a–c**. Changing the substitution pattern of the *Mannich* base significantly improved the IC_{50} in the 72 h assay. However, despite the presence of the *Mannich* base pharmacophore, compound **54a** showed a considerable shift between the 24 and the 72 h assay.

The toxicity of amodiaquine **57** (Fig. 8) is believed to come from the 4-hydroxyanilino moiety, which can be oxidized by enzymes to the quinoneimine. Nucleophilic addition of proteins to this reactive intermediate can occur, affecting cellular function.[17] Placing the OH group *meta* to the nitrogen would block this oxidation pathway and solve the potential toxicity problem. Therefore, in case the pyridine was replaced by a *Mannich* base, two different substitution patterns were investigated using 4-hydroxy-aniline (**55a–c**) and 3-hydroxy-aniline (**56a–c**). Both substitution patterns resulted in compounds with low nM activity in the 72 h assay against *P. falciparum*. Despite significant improvement in the activity of these hybrid compounds compared to the original series in the 24 h assay, they still exhibited a difference between their IC_{50} after 24 and 72 h of incubation.

To assess the potency towards a resistant strain of *P. falciparum*, the IC_{50} values of a set of compounds was determined against the *P. falciparum* K1 strain (chloroquine-resistant). All compounds measured did not show an activity difference between the NF54 and the K1 strain (Table 2, Supplementary data). This series therefore has the potential to be active against chloroquine resistant parasites.

In order to evaluate the potential of the hybrid series in vivo, compound **52d** was selected for testing in the *P. berghei* infected mouse model.[15] The activity of the compound was measured after a single oral dose of 1×100 mg/kg. Compound **52d** showed a reduction of 87% of parasitemia compared to the non-treated mice (Fig. 9). Taking into account the modest IC_{50} of **52d** against *P. berghei*, this result confirms the potential of this hybrid series in the quest for new anti-malarials.

In conclusion, we have developed a new series of compounds based on a hydroxy-ethyl-amine scaffold. The compounds are highly potent against *P. falciparum* parasites in the 72 h assay, show only a low reduction in activity upon addition of plasma to the assay medium and the potential for low in vivo clearance. The incorporation of a *Mannich* base pharmacophore resulted in improved speed of action. Further results will be reported in due course.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.118.

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