Carbamoyl Triazoles, Known Serine Protease Inhibitors, Are a Potent New Class of Antimalarials

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S *Supporting Information*
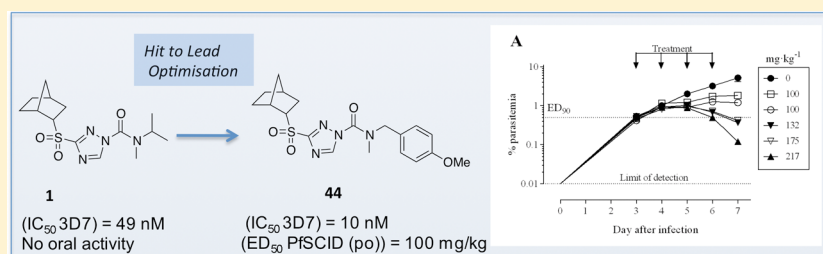
ABSTRACT: Screening of the GSK corporate collection, some 1.9 million compounds, against *Plasmodium falciparum* (*Pf*), revealed almost 14000 active hits that are now known as the Tres Cantos Antimalarial Set (TCAMS). Followup work by Calderon et al. clustered and computationally filtered the TCAMS through a variety of criteria and reported 47 series containing a total of 522 compounds. From this enhanced set, we identified the carbamoyl triazole TCMDC-134379 (**1**), a known serine protease inhibitor, as an excellent starting point for SAR profiling. Lead optimization of **1** led to several molecules with improved antimalarial potency, metabolic stabilities in mouse and human liver microsomes, along with acceptable cytotoxicity profiles. Analogue **44** displayed potent in vitro activity (IC_{50} = 10 nM) and oral activity in a SCID mouse model of *Pf* infection with an ED_{50} of 100 and ED_{90} of between 100 and 150 mg kg^{-1}, respectively. The results presented encourage further investigations to identify the target of these highly active compounds.

■ INTRODUCTION

Malaria, the disease caused by parasites of the *Plasmodium* genus, results in approximately 0.7 million deaths per annum and is responsible for some 660 million clinical cases worldwide.[1] Malaria affects some of the most vulnerable people in the world, particularly children and pregnant women in resource-poor regions such as sub-Saharan Africa and South East Asia. The current frontline treatments for *falciparum* malaria, the most deadly species, are artemisinin based combination therapies.[2] These consist of the coadministration of a highly efficacious but rapidly cleared artemisinin derivative[3] (dihydroartemisinin, artemether, or artesunate) with another, longer acting secondary agent, typically lumefantrine or piperaquine.[2] A current area of global concern in malaria is the emergence of resistance to antimalarial drugs, including the artemisinins, which renders them ineffective.[4−8] Recent reports have confirmed that parasites with reduced susceptibility to artemisinins, reflected in a slow parasite clearance rate, have

been discovered in Southeast Asia.[9−12] The issue of resistance coupled with the demand of a newly accepted set of antimalarial target product profiles[13] has prompted the search for antimalarials with novel mechanisms of action that can be deployed for treatment of all malaria species for prophylaxis and transmission blocking.

Screening of the GSK corporate collection, some 1.9 million compounds, against *Plasmodium falciparum* (*Pf*), revealed almost 14000 active hits that are now known as the Tres Cantos Antimalarial Set (TCAMS).[14] Further work by Calderon et al. filtered the TCAMS through a variety of criteria (activity, physicochemical properties, similarity indices, molecular weight, functional groups, scaffold novelty, etc.) and reported 47 series containing a total of 522 compounds.[15] From these compounds, we chose the carbamoyl triazole TCMDC-

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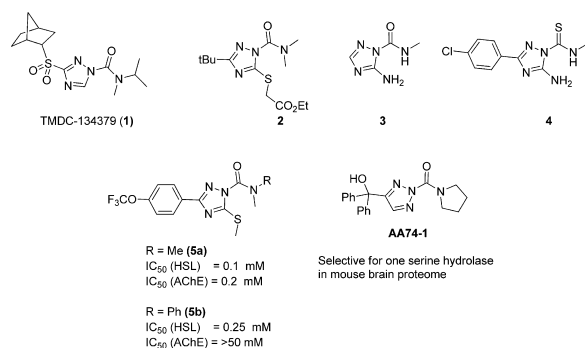134379 (**1**, Figure 1) as a starting point for further development. Carbamoyl triazoles have been extensively

Figure 1. Biologically active carbamoyl triazoles.

reported as herbicides,[16] fungicides,[17] and insecticides,[18] particularly in the patent literature. Triazamate (Aphistar, **2**) is a commercial insecticide used in the control of aphids on various crops and kills insects by inhibition of acetylcholine esterase (AChE). Carbamoyl triazoles such as **3** and the related thiocarbamoyl triazole **4** were shown to be effective at reducing sephadex-induced eosinophilia in rats when administered both po and ip.[19] **4** was shown to have low toxicity (LD$_{50}$ > 2000 mg kg^{-1}) in mice. Jacobsen et al. reported carbamoyl triazoles were potent inhibitors of hormone sensitive lipase (HSL), an esterase responsible for regulation of fatty acid metabolism.[20] They found that, although selective over hepatic, lipoprotein, pancreatic, and butyrylcholine esterases, the compounds were still inhibitors of acetylcholine esterase (AChE). Given **5**'s similariy to **2**, this is hardly surprising. However, by exchanging an *N*-Me (**5a**) for *N*-Ph (**5b**), they were able to improve the selectivity for HSL over AChE by more than 2 orders of magnitude, demonstrating that designing in selectivity is possible for these compounds. A pseudoreversible mechanism of inhibition involving acylation of the active site serine is proposed. Cravatt et al. showed that carbamoyl triazoles are remarkably selective for the serine protease class of enzyme.[21] Furthermore, they were able to demonstrate excellent selectivity for a single serine protease in the mouse brain proteome after synthesizing a series of carbamoyl 1,2,3-triazole analogues. **AA74−1** was able to completely and selectively inhibit the serine hydrolase APEH in vivo at ip doses of 0.4 mg kg^{-1}. These studies show the potential of the carbamoyl triazoles to be developed as selective and potent pharmaceutical compounds.

■ RESULTS AND DISCUSSION

1 was attractive for several reasons. It is highly potent in vitro (IC$_{50}$ = 49 nM) against *Pf*, and it represents a chemical scaffold that is unique to known antimalarial compounds (Table 1). **1** is a potent inhibitor of the serine protease dipeptidyl peptidase IV (DPP-IV) (IC$_{50}$ = 9 nM).[22] Serine proteases are a relatively unexplored class of enzyme for antimalarial compounds, so there is potential to exploit novel enzyme targets in this class.[23−28] The likely mechanism of action is irreversible inhibition by carbamoylation of nucleophilic serine residues in the active site. Carbamoyl triazoles are reported to increase glucose tolerance in mice, through DPP-IV inhibition, with oral doses as low as 1 mg kg^{-1}.[22] **1** has physicochemical properties favorable for oral drugs (ChromlogD = 4.9, solubility

Table 1. In Vitro and In Vivo Data for Compound 1

measurement	value
Cl$_{int}$ mL^{-1} min^{-1} g^{-1} (m/h)[a]	18.2/1.6
in vitro $t_{1/2}$ min (m/h)	3.8/>30
HSA[b] binding (%)	58
CYP3A 4VG/4VR IC$_{50}$ (μM)	>50/31.6
ChromlogD (PFI)[c]	4.82 (5.8)
Tox$_{50}$ HepG2 (μM)	>100
P. berghei, 2 doses,[d] 50 mg kg^{-1}	ED$_{50}$ > 50 mg kg^{-1}

[a](mouse/human). [b]Human serum albumin. [c]PFI = ChromlogD + no. of aromatic rings. [d]p.o.

(chemoluminescent nitrogen detection kinetic solubility (CLND) ≥ 500 μM), passive permeability (PAMPA) >300 nm/s). In vitro pharmacological profiling which involves the screening of **1** against a broad range of human targets (receptors, ion channels, enzymes, and transporters) in order to identify specific molecular interactions that may cause adverse drug interaction in humans revealed no major concerns. Cytotoxicity against HepG2 cells was shown to have IC$_{50}$ > 100 μM. A QPatch assay was negative for hERG inhibition. High clearance in mouse liver microsomes (Cl$_{int}$ = 18.2 mL min^{-1} g^{-1}) demonstrated that metabolic stability was likely to provide an optimization challenge in this series. However, it should be noted that the values in human liver microsomes were significantly lower (Cl$_{int}$ = 1.6 mL min^{-1} g^{-1}). From a medicinal chemistry point of view, the compound was also attractive as it offers several sites for modification with high synthetic tractability.

There was, however, some cause for concern with respect to plasma/blood stability and consequent in vivo efficacy given the instability of the hit compound in both plasma and blood (Figure 2). Addition of protease/esterase inhibitors showed
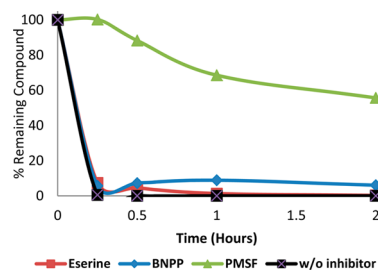

Figure 2. Mouse blood stability assays for **1** with and without various esterase/protease inhibitors.

that PMSF, a known, nonselective serine protease inhibitor, was able to significantly increase the mouse blood stability of the hit compound. This evidence indicates that proteases/esterases are the likely culprits for the instability.

A preliminary evaluation of the in vivo efficacy (p.o. administration) of **1** against a *P. berghei* murine model of malaria infection showed a lack of oral efficacy (ED$_{50}$ > 50 mg kg^{-1}). Given the efficacy of related compounds with respect to glucose tolerance and the high potency of **1** in vitro against Pf, this result was surprising. The reason for this discrepancy could be explained by the different sites of action for glucose tolerance and antiplasmodial effects. DPP-IV is responsible for the deactivation of glucagon-like peptide-1 (GLP-1).[29] GLP-1 stimulates insulin and inhibits glucagon secretion. It follows that inhibition of DPP-IV increases the level of active GLP-1

and, through the action of GLP-1 on insulin and glucagon, lowers blood glucose (increased glucose tolerance). The interaction of DPP-IV/GLP-1 takes place predominantly on the brush border epithelium of the small intestine.[30] As a consequence, orally administered DPP-IV inhibitors do not have to enter the systemic circulation to exert their effect on glucose tolerance. On the other hand, to effectively affect malaria parasites, compounds must pass through the brush border epithelium, into the systemic circulation and finally into erythrocytes where the *Plasmodium* targets are located. Given that **1** is a likely irreversible inhibitor of DPP-IV (and other proteases) and shows low stability in blood, it can be seen that the difference in oral efficacy between plasmodial infection and glucose tolerance is explicable through the differing sites of action. The challenge was, therefore, to moderate the reactivity of the carbamoyl group in such a way that the antiplasmodial activity is retained, plasma/blood stability is increased, and clearance is reduced. This could conceivably be achieved by altering the steric and electronic properties of the carbamoyl group.

Further in vitro assays allowed us to gain more information with regard to the antiplasmodial effects of **1**. The parasite reduction ratio (PRR) assay is used to measure the killing kinetics of compounds against *Plasmodium falciparum*.[31] Several parameters can be determined from the assay and the killing profile compared to known antimalarial compounds previously reported. The assay is run at $10 \times IC_{50}$ of the compound to be studied. In vitro PRR represents the decrease in viable parasites by a drug treatment over one life cycle (48 h for *Pf*), and it is a direct measurement of the killing rate of an antimalarial drug. Figure 3 shows that, for **1**, $\log_{10} PRR = 3.1$, $PCT_{99.9\%} = 48$ h,
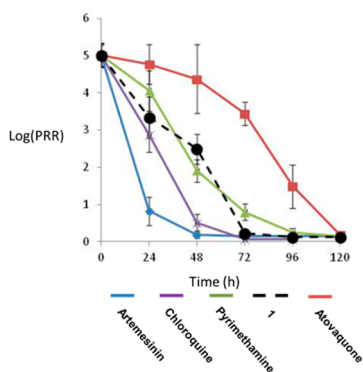
Figure 3. PRR assay of **1** (black) compared to that of artemesinin (blue), atovaquone (red), pyrimethamine (green), and chloroquine (purple).

and there is no lag phase. **1** has a profile between that of previously reported profiles for pyrimethamine and chloroquine[31] and is classified as a moderate killer.

To determine the SAR, a range of analogues were synthesized and tested in vitro against *Pf*. Three main areas were identified for SAR exploration (Figure 4): (i) The secondary amide substituents of the carbamoyl group, R' and R''. Various combinations of aryl, alkyl, and benzyl were considered in order to control the steric and electronic properties of the carbonyl group thought to be key to the serine protease inhibiting activity. (ii) The linker group (Y) separating the triazole ring from the varying R groups. Varying between the electron donating sulfide and electron withdrawing sulfone allows dramatic changes in the electronic properties
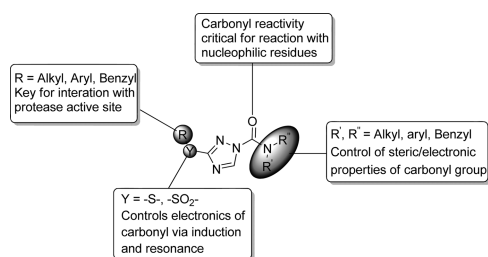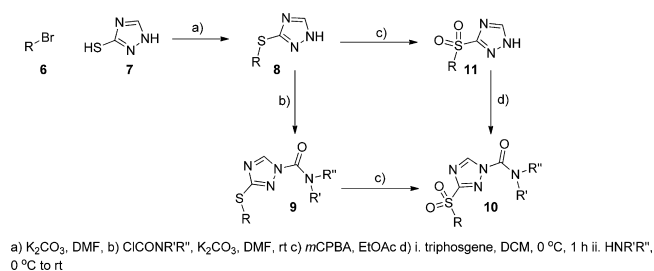
Figure 4. Key SAR features of carbamoyl triazoles.

and, therefore, reactivity of the carbamoyl group. This change is also attractive from a tractability point of view as the sulfones are accessible from the sulfides via simple oxidation. (iii) The R group bonded to linker Y. Although remote from the carbamoyl group and unlikely to influence the reactivity directly, the R group should have profound effects on activity by way of mediating the interaction with protease active sites. The S_1/S_1' sites of proteases play a vital role in substrate recognition/binding, and therefore groups remote to the reactive center are key to inhibitor activity.

As mentioned previously, **1** was attractive from a medicinal chemistry point of view due to the high tractability offered by its structure. Scheme 1 shows the synthetic approach used to
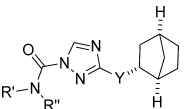
Scheme 1. Overview of Chemistry for Synthesis of 1 and Analogues

a) K₂CO₃, DMF, b) ClCONR'R'', K₂CO₃, DMF, rt c) *m*CPBA, EtOAc d) i. triphosgene, DCM, 0 °C, 1 h ii. HNR'R'', 0 °C to rt

prepare the analogues tested. Nucleophilic substitution of aryl bromides **6** by mercaptotriazole **7** with potassium carbonate in DMF yielded the sulfides **8** in good to excellent yields. Reaction of **8** with various carbamoyl chlorides yielded the carbamoyl sulfide analogues of type **9**, which were converted to sulfones **10** in good yield by oxidation with *m*CPBA. Alternatively, the sulfides **8** were oxidized to the triazole sulfones **11** with *m*CPBA in EtOAc. Reaction of **11** with triphosgene in DCM generates a carbamoyl chloride that was reacted in situ with secondary amines to yield analogues of sulfone **10**.

Table 2 shows the results obtained for a series of analogues exploring the steric properties around the carbamoyl group. However, the N-Me ^iPr substitution pattern was found to be optimal (**1**, vide supra). Substitutions with low steric demand such as those in **13−15** exhibited nanomolar levels of in vitro activity. Sterically demanding N-substituents such as those in **16−18** were inactive in vitro, most likely due to a decreased reactivity toward the target enzymes. The pyrrolidine analogue **19** showed a similar activity to **1**, while piperidine **21** was less active. The N-benzyl compound **22** has a moderate level of activity, **23** and **24**, containing a N-phenyl groups had poor activity.

The sulphonyl group is strongly electron withdrawing and as such imparts enhanced leaving group ability onto the triazole. The oxidation level of the sulfur atom, therefore, presents an

Table 2. In Vitro *Pf* (3D7) IC$_{50}$s for a Range of Carbamoyl Triazoles[a]

-NR'R''	Y		*Pf* IC$_{50}$ (µM) (SD)	-NR'R''	Y	*Pf* IC$_{50}$ (µM) (SD)
(structure)	-S-	12	0.071 (± 0.024)	(structure)	-S-	20	0.178 (±0.021)
(structure)	-SO$_2$-	13	0.047 (± 0.015)	(structure)	-SO$_2$-	21	0.626 (±0.114)
	-S-	14	0.141 (±0.005)	(structure)	-SO$_2$-	22	0.256 (±0.075)
(structure)	-SO$_2$-	15	0.093 (±0.006)		-SO$_2$-	23	2.69 (±0.879)
(structure)	-SO$_2$-	16	>5 (NA)	(structure)	-S-	24	>5 (NA)
(structure)	-SO$_2$-	17	>5 (NA)	(structure)	-SO$_2$-	25	0.585 (±0.013)
	-S-	18	1.42 (±0.006)		-S-	26	2.240 (±0.210)
(structure)	-SO$_2$-	19	0.084 (±0.015)	(structure)	-SO$_2$-	27	0.145 (±0.031)

[a]Atovaquone was used as positive control with IC$_{50}$ = 0.001 µM

opportunity to temper the reactivity of the carbamoyl group. A range of sulfide analogues were synthesized and their in vitro activities compared with those of the corresponding sulfones. Although differences in activity were observed, it was not possible to establish any SAR conclusions regarding the preferred oxidation state of the sulfur atom from these results.

Quantum mechanical calculations were performed in order to investigate if differences in antimalarial activity were attributable to the oxidative state of the sulfur atom [(II) and (VI)] (Table 2). Given the proposed mechanism of action of this chemotype, we hypothesized that electronic structure calculations might be able to capture aspects of a compound's reactivity and relate those to measured IC$_{50}$s. Several descriptors derived from the calculations were investigated including LUMO, electronegativity, and softness (see Supporting Information (SI) for a complete list). A good linear correlation ($r^2 = 0.79$) was found between the "softness" of the molecules and the measured in vitro IC$_{50}$ values (Figure 5). The model passed tests for validity (see SI) and had a good 5-fold cross validation r^2 value of 0.65 ± 0.07 indicative of a robust model. The softness of a molecule is a measure of a
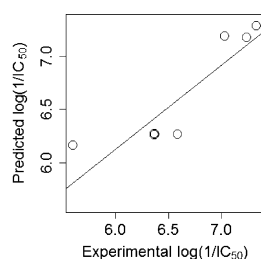
Figure 5. Plot of experimental log(1/IC$_{50}$) vs predicted log(1/IC$_{50}$) using calculated molecular softness.

compound's polarizability and electronegativity and is consistent with the proposed antimalarial mechanism of action, i.e., carbonyl reactivity with protein nucleophiles.

The *S*-substituent was also varied from the original *exo*-norbornane (Table 3). Cyclohexyl substitution provided **28**, a

Table 3. In Vitro *Pf* (3D7) IC$_{50}$s for Carbamoyl Triazoles Containing Different *S*-Substituents[a]

Compound		*Pf* IC$_{50}$ (µM)	Compound		*Pf* IC$_{50}$ (µM)
(structure)	28	0.028	(structure)	32	1.895
(structure)	29	0.008	(structure)	33	0.164
(structure)	30	0.571	(structure)	34	0.418
(structure)	31	0.019	(structure)	35	1.438

[a]Atovaquone was used as positive control with IC$_{50}$ = 0.001 µM

compound endowed with slightly improved potency. The bulky, lipophillic 2-adamantyl group provided the highly potent **29**. Aromatic groups attached directly to sulfur **30−33** resulted in lower activity except the highly potent **31**. The *S*-benzyl compounds **34** and **35** were considerably less active than the corresponding norbornyl analogues **19** and **20**.

The C-5 of the triazole also offers an opportunity to increase steric demand around the carbamoyl group; however, the 5-Me compound **36** proved to be inactive (Figure 6).

36

Pf IC$_{50}$ >4
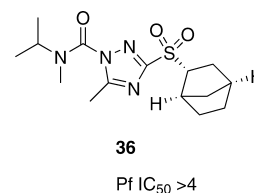
Figure 6. In vitro *Pf* (3D7) IC$_{50}$ for **36**.

To assist in analyzing and interpreting the SAR associated with the compounds synthesized, QSAR models were developed for the quantitatively measured IC$_{50}$ values. Initially, models were built using the quantum mechanical approach as described above, however, none of the models explored using a genetic algorithm multiple linear regression approach were satisfactory (data not shown). An alternative nonlinear approach was employed in which models were constructed using random forests[32] within a multiple cross-validation scheme[33] to select the optimal parameter (mtry) to use with the molecular descriptors[34] (for further computational details, see the SI). We adopted this approach of using multiple training and test sets as every sample may be needed for the

model,[32] and previous studies have indicated the inadequacies of using single test set to assess predictive power.[35] Indeed, often the performance estimates superior to a single test set because they evaluate many alternate versions of the data.[33] The multiple cross validation approach showed that an mtry value of 109 was optimal with ($r^2 = 0.94 \pm 0.20$). Applying this model gave an excellent overall fit with the antimalarial activity (Figure 7A, $r^2 = 0.92$). The top 10 most influential descriptors
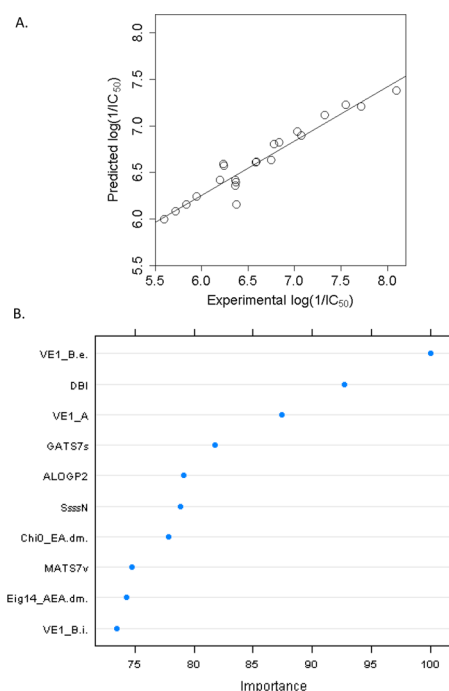
Figure 7. (A) Plot of experimental $\log(1/IC_{50})$ vs predicted $\log(1/IC_{50})$ of the entire quantitative data set. (B) Relative importance of the top 10 most influential descriptors in the model.

are shown in Figure 7B (definitions of descriptors are provided in the SI). Most of these descriptors are challenging to interpret directly, however the utility of the robust QSAR developed would be of value in future virtual screening studies to inform and prioritize synthesis and testing.

Further insight into the behavior of the carbamoyl triazoles comes from observations regarding the 48 vs 72 h IC_{50} values, and the results are shown in Table 4. However, none of the compounds tested showed any significant difference in IC_{50} between the two time points.

Table 4. In vitro *Pf* IC_{50} at 48 and 72 h for Carbamoyl Triazoles[a]

	Pf IC_{50} (μM) (SD)	
compd	48 h	72 h
1	0.016 ($\pm$0.001)	0.028 ($\pm$0.004)
17	>5 (NA)	> 5 (NA)
18	1.428 ($\pm$0.006)	0.537 ($\pm$0.185)
19	0.037 ($\pm$0.002)	0.037 ($\pm$0.002)
22	0.247 ($\pm$0.086)	0.379 ($\pm$0.196)
23	2.686 ($\pm$0.879)	2.713 ($\pm$0.237)

[a]Atovaquone was used as positive control with 48/72 h IC_{50}s: 0.001 μM (48 h)/0.0003 μM (72 h)

A screen of several, structurally diverse analogues was then undertaken to establish the metabolic stability in both mouse and human liver microsomes (Table 5) The di-isopropyl-
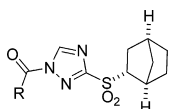
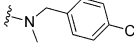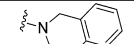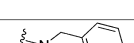**Table 5. In Vitro Clearance and Half Lives in Mouse and Human Liver Microsomes for Carbamoyl Triazoles**
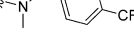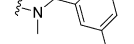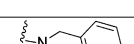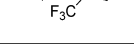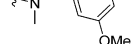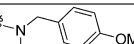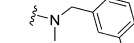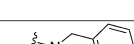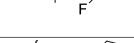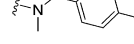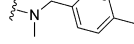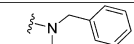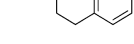
	mouse		human	
compd	in vitro Cl_{int} (mL min^{-1} g^{-1})	in vitro $t_{1/2}$ (min)	in vitro Cl_{int} (mL min^{-1} g^{-1})	in vitro $t_{1/2}$ (min)
18	22.0	<5	3.1	22.3
19	>30	<5	1.0	>30
20	>30	<5	1.5	>30
22	4.0	19.4	1.5	>30

containing **18** showed an in vitro intrinsic clearance in mouse liver microsomes of 22.0 mL^{-1} min^{-1} g^{-1}, very similar to that of **1**. The pyrrolidine analogues **19** and **20** both exhibit higher clearance (>30 mL min^{-1} g^{-1}) than **1**. These values all correspond to in vitro $t_{1/2}$ values of <5 min. This data suggests that high clearance in mouse liver microsomes is a common feature across the series of compounds containing exclusively aliphatic *N*-substituents. However, all of these compounds showed much lower clearance in human liver microsomes. **22** showed an improved clearance in the mouse microsomes of 4.0 mL min^{-1} g^{-1} which corresponds to an in vitro $t_{1/2}$ of almost 20 min.

With the improved metabolic stability and reduced clearance exhibited by **22**, it was decided to investigate the SAR around the benzyl group with a view to obtaining higher potency. A series of benzyl analogues were synthesized, and the results are shown in Table 6. Addition of a chlorine atom in the 4- (**37**) or 2- (**38**) position led to a slight reduction in activity compared to **22**, 3-Cl analogue **39** maintained the same activity. Trifluoromethyl substitution (**40**−**42**) led to a reduction in activity with **40** and **39** being significantly less active than **22**. 3-OMe analogue **43** showed an improvement in potency over **22** and the 4-OMe **44** was extremely potent in the low nanomolar range (10 nM). The 2,4-dimethoxy substituted **45** was also highly potent (30 nM). Fluoro substituted compounds **46**−**48** showed similar activity to **22** with the position of F-substitution having little effect on the activity. Introduction of a heterocycle such as pyridine in **49** (86 nM) was well tolerated and slightly more potent than **22**. While 4-SO$_2$Me had negligible impact on potency (**50**), 4-Ac and 4-*t*Bu analogues were both highly active displaying IC_{50} values of 50 and 16 nm, respectively. The 3-CN benzyl substituted **53** and the bicyclic tetrahydroisoquinoline **54** were inactive. It was previously observed that the diisopropyl analogue **17** was significantly more stable in blood compared to **1** (Figure S2), presumably a result of the highly sterically demanding substitution around the carbamoyl group. Because of the potency exerted by the 4-OMe benzyl group and the stabilizing ability of bulky aliphatic groups, **55** and **56** were synthesized containing cyclopropyl and isopropyl groups, respectively. However, the in vitro activities of these compounds were too low to warrant further investigation.

The two most potent compounds, **44** and **52**, were then evaluated for microsomal stability. **44** (Cl_{int} = 3.0 mL min^{-1} g^{-1}; $t_{1/2}$ = 22.6 min) showed a similar clearance value to that of **22** (vide supra) in mouse liver microsomes, and **52** had a lower metabolic stability under the same conditions (Cl_{int} = 7.8 mL min^{-1} g^{-1}; $t_{1/2}$ = 9.0 min). The ChromLogD (pH 7.4) for **44** and **52** were 5.72 and 7.69, respectively. **44** was significantly

Table 6. In Vitro IC$_{50}$s for *N*-Benzyl Substituted Carbamoyl Triazoles vs the 3D7 Strain of *Plasmodium falciparum*[a]

R	Compound	*Pf* IC$_{50}$ (μM) (SD)	R	Compound	*Pf* IC$_{50}$ (μM) (SD)
(4-Cl benzyl)	37	0.602 (± 0.084)	(3-F benzyl)	47	0.373 (±0.015)
(2-Cl benzyl)	38	0.725 (± 0.162)	(2-F benzyl)	48	0.180 (±0.029)
(3-Cl benzyl)	39	0.307 (±0.089)	(pyridyl)	49	0.086 (±0.013)
(4-CF$_3$ benzyl)	40	0.554 (±0.008)	(4-SO$_2$Me benzyl)	50	0.290 (±0.065)
(3-CF$_3$ benzyl)	41	1.862 (±0.368)	(4-Ac benzyl)	51	0.050 (±0.015)
(2-CF$_3$ benzyl)	42	1.263 (±0.157)	(4-t-Bu benzyl)	52	0.016 (±0.01)
(3-OMe benzyl)	43	0.036 (±0.004)	(3-CN benzyl)	53	3.43 (±0.511)
(4-OMe benzyl)	44	0.010 (±0.002)	(tetrahydroisoquinoline)	54	>5 (NA)
(2-OMe,5-OMe benzyl)	45	0.040 (±0.011)	(4-OMe benzyl, cyclopropyl)	55	1.023 (±0.118)
(4-F benzyl)	46	0.197 (±0.079)	(4-OMe benzyl, isopropyl)	56	0.84 (±0.05)

[a]Atovaquone was used as positive control with 48 h IC$_{50}$: 0.001 μM.

more soluble (CLND) than **52**, where maximum concentrations were measured at 125 and 28 μM, respectively. Cytotoxicity assays against HepG2 cells revealed IC$_{50}$ > 100 μM for **44** and = 75.2 μM for **52**.

Given the slightly higher potency, reduced in vitro clearance, adequate toxicological profile, and superior physicochemical properties of **44**, an in vivo oral efficacy study was initiated with this compound. The experiment was carried out in the GSK *Pf* humanized mouse model of infection in a standard four-day assay as described by Jiménez-Díaz.[36] In this assay, **44** was efficacious against *P. falciparum* in vivo showing an ED$_{90}$ ≈ 128 mg kg^{-1} (Figure 8A) and clear phenotypic effects on parasites remaining in blood (Figure 8B) were observed. Interestingly, **44** was not detected in peripheral blood of infected humanized mice. This apparent disconnect between the in vivo efficacy and levels in blood of **44** could be related to the instability of the compound in blood.

To further characterize the profiles of **1** and **44**, we conducted a phenotypic assay to assess the effects of the two compounds on the parasite life cycle (Figure 9). In the presence of **1** and **44**, the parasites could be found as primarily young trophozoites at both 24 and 48 h after drug treatment. Parasites are arrested in young trophozoite stage comparable to atovaquone-treated parasites included as control.

The final part of our study was focused on establishing if this chemotype had the potential to target the best characterized malarial serine proteases examined to date, PfSUB1 (*Pf* subtilisin like serine protease-1). This enzyme is released into the parasitophorous vacuole where it activates a family of protease-like proteins called SERA, leading to rupture of the vacuole and host cell membrane.[37−39] Inhibition of PfSUB1 is a promising approach to block propagation of a malarial infection by preventing the egress of merozoites from the red blood cell.[39] **1**, **12**, **18**, **34**, and **44** were screened against this target, and none of the azole compounds showed inhibitory activity against this serine protease. The positive control NF2114 compound showed good inhibition, with an IC$_{50}$ of ≈3 μM (SI). This result indicates that the novel molecules prepared here, and the known serine protease inhibitor carbamoyl triazole **1** are likely targeting novel uncharacterized plasmodial serine protease enzymes.

■ CONCLUSIONS

The carbamoyl triazole series (**1**) belonging to the TCAMS is a promising new compound class for antimalarial agents that potentially work by inhibition of novel serine protease targets other than PfSUB1. Analogue synthesis led to several compounds with low nanomolar in vitro activity. The *N*-benzyl series contains several highly potent compounds with low nanomolar activity such as **44**, **45**, **51**, and **52**. These new molecules display a greatly improved metabolic stability over
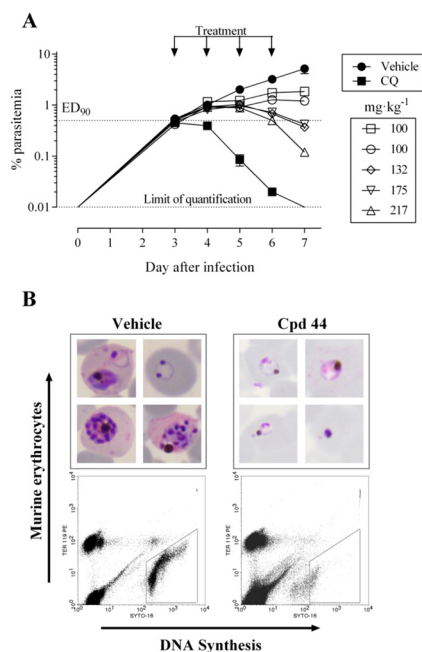
Figure 8. Efficacy of **44** against the human malaria parasite in the GSK *Pf* humanized mouse model. (A) Parasitemia in individual mice treated with different doses of **44** during the efficacy assay. Each open symbol represents an individual mouse treated with **44**. Solid symbols are the average of $n = 4$ mice treated with vehicle (solid circle) and $n = 2$ treated po with chloroquine at 10 mg·kg^{-1} (solid square). (B) Microscopic and flow cytometry analysis of *P. falciparum* present in peripheral blood of mice treated with vehicle or **44** at 217 mg·kg^{-1}. Samples were taken 96 h after starting the treatment. Flow cytometry dot plots from samples of peripheral blood show *P. falciparum*-infected human erythrocytes (polygonal region). Animals treated with vehicle show healthy nonsynchronized asexual stages, whereas the parasites present in **44**-treated animals are altered rings, abnormal trophozoites with hemozoin granules and pyknotic cells.
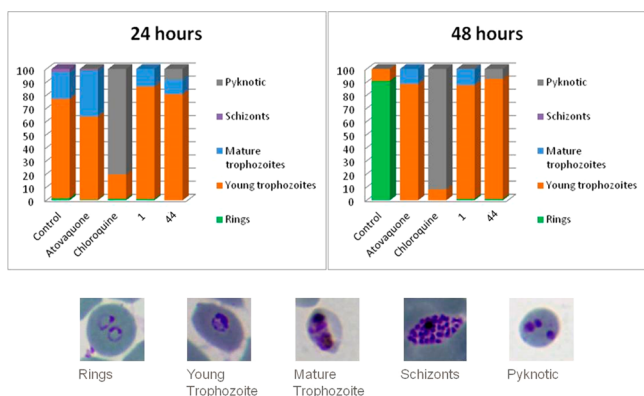

Figure 9. Phenotypic parasite populations examined by microscopy at 0, 24, and 48 h for **1** and **44**. In vitro *P. falciparum* phenotypic profile was determined for **1** and **44** at 10× their respective *Pf* IC$_{50}$, and standard antimalarials were also included as controls: atovaquone and chloroquine.

the hit compound **1** in mouse liver microsomes. **44** is orally efficacious in a SCID mouse model of *Pf* infection with an ED$_{50}$ and ED$_{90}$ of approximately 100 and 150 mg kg^{-1}, respectively. The results presented warrant further investigation to identify the target of these highly active compounds.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00434.

> Molecular formula strings (XLSX)
> General experimental procedures and characterization data for all synthesized compounds (PDF)

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Notes
The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents. All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.
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