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CONCISE ARTICLE

Squaric acid: a valuable scaffold for developing antimalarials?†

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We describe here the synthesis of a library of thirty-eight squaric derivatives and the evaluation of activity against papain-, falcipain-2- and a chloroquine-resistant strain of *P. falciparum*. The most active compounds combine significant antiplasmodial activity with minimal cytotoxicity.

Introduction

Malaria is one of the most widespread infectious diseases of our time, causing nearly 800 000 deaths each year.[1] The most lethal of the *Plasmodium* species that infects humans—*Plasmodium falciparum*—has acquired resistance to most clinically used antimalarials.[2] Furthermore, the first cases of putative resistance to artemisinin-based combination therapy (ACT) have been reported. For that reason, there is an urgent need for novel antimalarial drugs. In fact, a major challenge is to make sure that each new generation of molecules provides antiparasitic activity against the resistant strains.[3]

Following our studies on Michael acceptors as cysteine protease inhibitors we become interested in the squaric acid scaffold.[4–8] Our interest was triggered by reports showing that dimethyl squarate reacts efficiently with thiols to give the corresponding addition–elimination products,[9] suggesting that a similar process might also occur within the active site of a cysteine protease. Moreover, since the first synthesis of squaric acid a number of derivatives have been reported in the fields of bioorganic and medicinal chemistry. This scaffold has already been used with success to develop metalloenzyme inhibitors.[10–13] However, the potential of this scaffold in antimalarial agents remained to be explored. With this in mind, we developed a library of squaramide derivatives by incorporating substituents with different stereoelectronic properties and lipophilicities at C-3 and C-4 (Fig. 1) in order to perform a structure–activity study for this scaffold.

Meanwhile, in 2010, GlaxoSmithKline's chemical library for inhibitors of *P. falciparum* was made public to encourage additional drug lead identification efforts and further research into
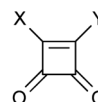

Fig. 1 General structure of squaric acid derivatives synthesized.

malaria. In that library were included four compounds containing a squaric moiety, with activities ranging from 62 to 878 nM against *P. falciparum* strain 3D7 (Fig. 2).[14] Two of these squaric acid derivatives were annotated as antagonists of serotonin receptor, a drug target without obvious orthologue in the malarial genome. More extended structure–activity data are required to evaluate the usefulness of the squaric acid scaffold as a starting point to develop novel antimalarials and to gain a better understanding of their putative targets.

Results and discussion

To explore the potential of the squaric acid scaffold as an antiplasmodial agent we synthesized a series of compounds
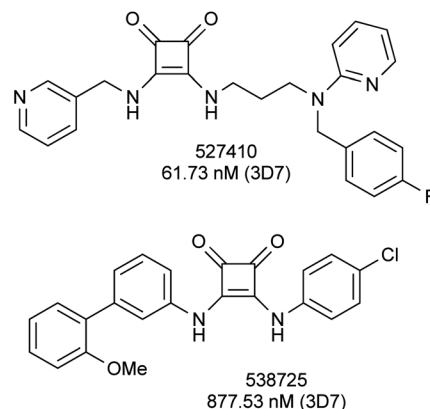

Fig. 2 Examples of squaric derivatives from the GSK library.

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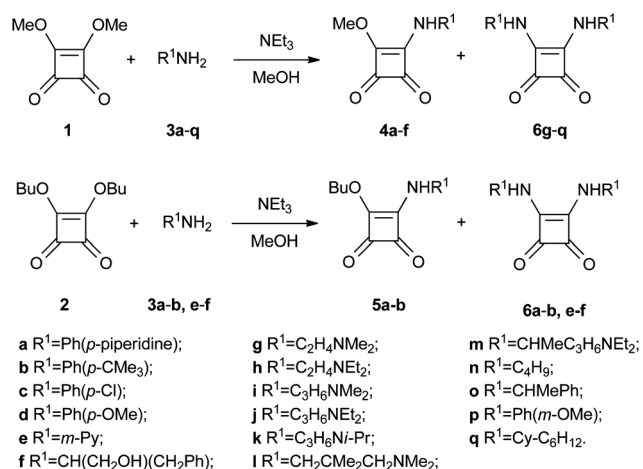
Scheme 1 Synthesis of squaramides **4a–f**, **5a–b**, **6a–b** and **6e–q**.

containing different aromatic and aliphatic amines (Scheme 1). Commercially available squarates **1** and **2** and amines **3a–q** were used as starting materials. Squaramates **4a–f** and squaramides **6g–q** were obtained from dimethyl squarate **1** using one or two equivalents of the appropriate amines. In contrast, when we used the butyl squarate **2** and one equivalent of amines **3e–f** as the starting materials, we obtained squaramides **6e–f** instead of the corresponding squaramates **5**. Squaramide **6a** was obtained from reaction of two equivalents of butyl squarate **2** and amine **3a**.

Interestingly, compound **6b** was obtained by reflux of a mixture of one equivalent of butyl squarate **2** and amine **3b**. The same reaction at room temperature led to compound **5b**.

Compounds **4a–f**, **5a–b**, **6a–b** and **6e–q** were then screened against papain-, falcipain-2- and the chloroquine-resistant W2 strain of *P. falciparum* (Table 1). Most of the compounds were devoid of activity against *P. falciparum*. However, compounds **5a–b** and **6a–b** had low micromolar antiplasmodial activity, with IC$_{50}$ values ranging from 0.99 to 4.16 μM. Except for compounds **6a–b** and **6p**, all compounds were inactive against papain, the prototype for clan CA cysteine proteases. In fact, we already observed a similar result for squaric vinyl sulfones.[6]

Based on our previous results showing IC$_{50}$ values of around 1 μM for squaric-Leu-azaPhe-VSPh derivatives,[6] we tried to improve the activity of these compounds, by synthesizing two unsymmetrical squaramides. Ph(*p*-CMe$_3$) and CH(CH$_2$OH)-(CH$_2$Ph) were chosen as substituents due to the good activities of compounds **5b** and **6b** and to test the presence of a hydroxyl group, respectively. However, compounds **8b** and **8f** were devoid of antiplasmodial activity (IC$_{50}$, >10 μM) (Scheme 2).

The next step was to evaluate the potential of the squaric acid scaffold in the design of selective falcipain inhibitors, by incorporating the appropriate recognition moiety at the double bond. A series of compounds containing an aza-dipeptide recognition moiety were synthesized in which the squaric moiety acts as a replacement of the P$_1$ carbonyl group. Gly, Phe and homoPhe residues were selected for P$_1$. The recognition moiety was obtained starting with *N*-BocLeu and the corresponding hydrazine.

Table 1 Papain inhibition, falcipain-2 inhibition and antiplasmodial activity for compounds **4a–f**, **5a–b** and **6a–q**

Compounds	R^1	Papain IC$_{50}$/μM	Falcipain-2 IC$_{50}$^{a}/μM	*P. falciparum* W2 IC$_{50}$/μM
4a	Ph(*p*-piperidin-1-yl)	>10	ND	>10
4b	Ph(*p*-CMe$_3$)	>10	ND	>10
4c	Ph(*p*-Cl)	>10	NDs	>10
4d	Ph(*p*-OMe)	>10	ND	>10
4e	*m*-Py	>10	ND	>10
4f	CH(CH$_2$OH)(CH$_2$Ph)	>10	>50	>10
5a	Ph(*p*-piperidin-1-yl)	>10	ND	3.13 ± 0.35
5b	Ph(*p*-CMe$_3$)	>10	>50	2.65 ± 0.19
6a	Ph(*p*-piperidin-1-yl)	2.8 ± 1.3	>50	4.16 ± 0.54
6b	Ph(*p*-CMe$_3$)	8.1 ± 0.4	24.3 ± 3.98	0.99 ± 0.23
6e	*m*-Py	>10	ND	>10
6f	CH(CH$_2$OH)(CH$_2$Ph)	>10	ND	>10
6g	C$_2$H$_4$NMe$_2$	>10	ND	>10
6h	C$_2$H$_4$NEt$_2$	>10	>50	>10
6i	C$_3$H$_6$NMe$_2$	>10	ND	>10
6j	C$_3$H$_6$NEt$_2$	>10	>50	>10
6k	C$_3$H$_6$N*i*-Pr	>10	>50	>10
6l	CH$_2$CMe$_2$CH$_2$NMe$_2$	>10	ND	>10
6m	CHMeC$_3$H$_6$NEt$_2$	>10	ND	>10
6n	C$_4$H$_9$	>10	ND	>10
6o	CHMePh	>10	ND	>10
6p	Ph(*m*-OMe)	5 ± 1	>50	>10
6q	Cy-C$_6$H$_{12}$	>10	ND	>10
E−64		0.12 ± 0.02	0.06 ± 0.01	1.94 ± 0.004

^{a} ND = not done.
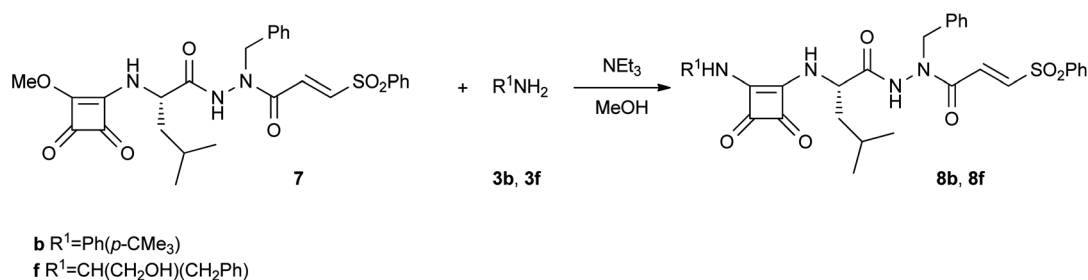
Scheme 2 Synthesis of squaramides **8b** and **8f**.
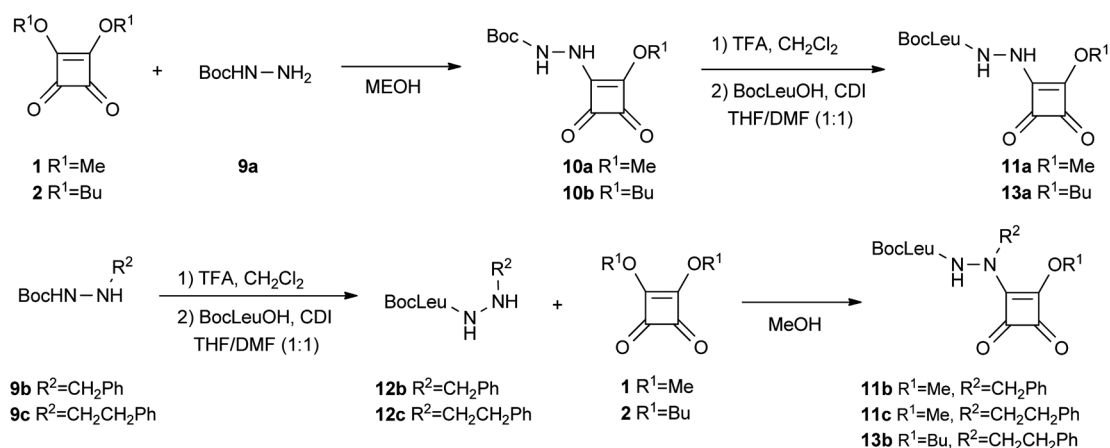
Scheme 3 Synthesis of aza squaric derivatives **11a–c** and **13a–b**.

The aza-Gly compound was obtained from reaction of Boc protected hydrazine **9a** with dimetyl squarate **1**. Deprotection of the Boc group, followed by coupling with BocLeu, led to aza squaric derivative **11a** containing Gly in P_1 (Scheme 3).
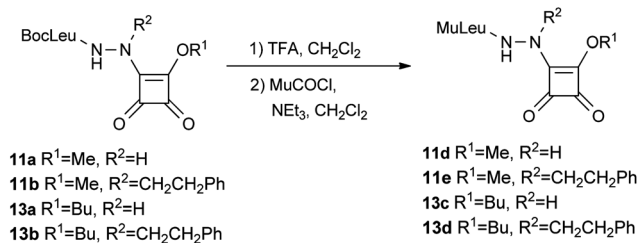
The aza squaric acid derivatives containing Phe and homoPhe in P_1 were synthesized starting from the corresponding Boc protected hydrazines **9b–c**. Deprotection of the Boc group with TFA, followed by coupling with BocLeuOH, led to amino acid hydrazides **12b–c**. Finally, coupling with the dimetyl squarate **1** led to aza squaric derivatives **11b–c** in 42–47% yields (Scheme 3). All of the proposed structures were established by NMR (^{1}H, ^{13}C, COSY and HMQC), IR, and MS.
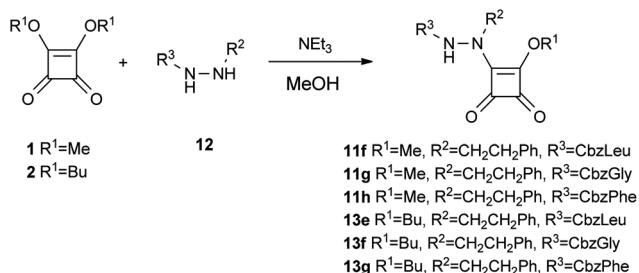
Table 2 Papain inhibition and antiplasmodial activity for compounds **11d–h** and **13a–g**

Compounds	R^1	R^2	R^3	Aza-dipeptide	Papain C_{50}/µM	*P. falciparum* W2 IC$_{50}$/µM
11a	Me	H	BocLeu	BocLeu-azaGly	>10	8.53 ± 0.71
11b	Me	CH$_2$Ph	BocLeu	BocLeu-azaPhe	>10	>1[a]
11c	Me	CH$_2$CH$_2$Ph	BocLeu	BocLeu-azahomoPhe	>10	6.36 ± 0.78
11d	Me	H	MuLeu	MuLeu-azaGly	>10	>10
11e	Me	CH$_2$CH$_2$Ph	MuLeu	MuLeu-azahomoPhe	>10	>10
11f	Me	CH$_2$CH$_2$Ph	CbzLeu	CbzLeu-azahomoPhe	>10	2.99 ± 0.23
11g	Me	CH$_2$CH$_2$Ph	CbzGly	CbzGly-azahomoPhe	>10	9.36 ± 0.1
11h	Me	CH$_2$CH$_2$Ph	CbzPhe	CbzPhe-azahomoPhe	>10	3.14 ± 0.07
13a	Bu	H	BocLeu	BocLeu-azaGly	>10	>10
13b	Bu	CH$_2$CH$_2$Ph	BocLeu	BocLeu-azahomoPhe	>10	>10
13c	Bu	H	MuLeu	MuLeu-azaGly	>10	9.45 ± 0.02
13d	Bu	CH$_2$CH$_2$Ph	MuLeu	MuLeu-azahomoPhe	>10	>10
13e	Bu	CH$_2$CH$_2$Ph	CbzLeu	CbzLeu-azahomoPhe	>10	4.51 ± 0.25
13f	Bu	CH$_2$CH$_2$Ph	CbzGly	CbzGly-azahomoPhe	>10	5.17 ± 0.04
13g	Bu	CH$_2$CH$_2$Ph	CbzPhe	CbzPhe-azahomoPhe	>10	4.69 ± 0.13
E−64					0.3 ± 0.2 (nM)	1.94 ± 0.004

[a] Insoluble, saturated solution of 1 µM was used.

11a R^1=Me, R^2=H
11b R^1=Me, R^2=CH$_2$CH$_2$Ph
13a R^1=Bu, R^2=H
13b R^1=Bu, R^2=CH$_2$CH$_2$Ph

11d R^1=Me, R^2=H
11e R^1=Me, R^2=CH$_2$CH$_2$Ph
13c R^1=Bu, R^2=H
13d R^1=Bu, R^2=CH$_2$CH$_2$Ph

Scheme 4 Synthesis of *N*-Mu aza squaric derivatives **11d–e** and **13c–d**.

1 R^1=Me
2 R^1=Bu

12

11f R^1=Me, R^2=CH$_2$CH$_2$Ph, R^3=CbzLeu
11g R^1=Me, R^2=CH$_2$CH$_2$Ph, R^3=CbzGly
11h R^1=Me, R^2=CH$_2$CH$_2$Ph, R^3=CbzPhe
13e R^1=Bu, R^2=CH$_2$CH$_2$Ph, R^3=CbzLeu
13f R^1=Bu, R^2=CH$_2$CH$_2$Ph, R^3=CbzGly
13g R^1=Bu, R^2=CH$_2$CH$_2$Ph, R^3=CbzPhe

Scheme 5 Synthesis of *N*-Cbz aza squaric derivatives **11f–h** and **13e–g**.

Compounds **11a–c** were then screened against the W2 strain of *P. falciparum* (Table 2). The results show that the recognition moiety seems to be essential for antiplasmodial activity, as compounds **4a–f** and **6e–q**, lacking the aza-dipeptidyl moiety, were devoid of activity. In contrast, compounds **11a** and **11c** had good antiplasmodial activity, with IC$_{50}$ values of 9 and 6 μM, respectively. Probably FP-2 inhibition is not the primary mode by which derivatives **11** exert their antiplasmodial activities, since compound **11c** was not active against falcipain-2 for the tested concentrations. In fact, all compounds were inactive against papain.

To further explore the potential of the aza-dipeptidyl squaric acid derivatives as antimalarials we completed our structure–activity study by synthesizing other derivatives of compounds **11a** (aza-Gly) and **11c** (aza-homoPhe).

We synthesized a new series of compounds containing Cbz-protected amino acids in P$_2$. Gly, Leu and Phe residues in P$_2$ were selected in order to modulate the interaction with P$_2$ (Scheme 4).

Two compounds were also synthesized with a 4-morpholine-carbonyl (Mu) protected Leu in P$_2$ and an aza-Gly or aza-homoPhe in P$_1$ (Scheme 4). Commercially available methyl **1** and butyl **2** squarates were used as starting materials.

The BocLeu butoxy squaric derivatives **13a** and **13b** were synthesized using the same methodology applied for the synthesis of compounds **11a** and **11c**, respectively (Scheme 3). Compounds **11d–e** and **13c–d** were obtained from the corresponding BocLeu aza squaric acid derivatives with 69–76% yields, by deprotection of the Boc group with TFA, followed by reaction with 4-morpholinecarbonyl chloride (Scheme 4). The *N*-Cbz protected aza squaric acid derivatives were synthesized from the corresponding amino acid hydrazides in 71–83% yields (Scheme 5).

Compounds **11d–h** and **13a–g** were then tested against papain and against the W2 strain of *P. falciparum* (Table 2). Inspection of the data in Table 2 allows the following observations:

(1) As observed before for compound **11c**, azadipeptidyl squaric derivatives did not inhibit papain at the tested concentrations.

(2) For OMe compounds containing an aza-homoPhe or an aza-Gly in P$_1$ and Leu in P$_2$, the presence of a Mu protective group resulted in the loss of activity against *P. falciparum* (*i.e.* compounds **11d** and **11e** *versus* their Boc-protected counterparts **11a** and **11c**). However, the opposite result was observed for the inhibitor containing an aza-homoPhe in P$_1$ and a Cbz-Leu in P$_2$ (*i.e.* compound **11f**). In this case, activity was increased *ca.* 2-fold higher than that of its Boc-protected counterpart **11c**.

(3) The order of inhibitory activity against *P. falciparum* for the methoxy series depended on the nature of the P$_2$ residue and varied in the order Leu > Phe > Gly. However, this effect had no major impact on activity for the butoxy series (**13e** *vs.* **13f** *vs.* **13g**).

(4) Related to the Cbz series, substitution of the OMe group for an OBu group in the squaric moiety improved the activity *ca.* 2-fold only for the inhibitors containing a CbzGly amino acid (*i.e.* compound **13f** *versus* the OMe counterpart **11g**).

(5) For compounds containing an aza-homoPhe or an aza-Gly in P$_1$ and BocLeu, the substitution of the OMe group for an OBu group in the squaric moiety resulted in the loss of activity against *P. falciparum* (*i.e.* compounds **13a** and **13b** *versus* their OMe counterparts **11a** and **11c**).

Table 3 Cytotoxicity against NIH 3T3 and Hek 293T cells for compounds **5a–b**, **6a–b**, **11a**, **11c**, **11f–h**, **13c** and **13e–g**

Compounds	W2 IC$_{50}$/μM	NIH 3T3 IC$_{50}$/μM	Hek 293T IC$_{50}$/μM	SI^a	log P^b
5a	3.13 ± 0.35	>100	>100	>32	3.48
5b	2.65 ± 0.19	17.51 ± 1.27	12.35 ± 1.20	4.7	4.07
6a	4.16 ± 0.54	>50	>50	>12	4.53
6b	0.99 ± 0.23	>100	>100	>101	5.70
11a	8.53 ± 0.71	>100	>100	>12	1.30
11c	6.36 ± 0.78	>100	>100	>16	2.85
11f	2.99 ± 0.23	>100	>100	>33	3.28
11g	9.36 ± 0.1	>100	>100	>11	1.82
11h	3.14 ± 0.07	>100	>100	>32	3.56
13c	9.45 ± 0.02	>100	>100	>11	1.03
13e	4.51 ± 0.25	>100	>100	>22	4.48
13f	5.17 ± 0.04	>100	>100	>19	2.97
13g	4.69 ± 0.13	>100	>100	>21	4.72

^{a} Selectivity index toward W2 strain, which is expressed by the ratio IC$_{50}^{Hek}$/IC$_{50}^{W2}$. ^{b} Estimated by the ALOGPS 2.1 algorithm.[15]

(6) For compounds containing the Mu-Leu-azaGly sequence (*i.e.* compounds **11d** and **13c**) substitution of the OMe for an OBu group was beneficial. In fact, compound **11d** was inactive but compound **13c** presented an IC_{50} of 9.5 μM.

The *in vitro* cytotoxicity of the most active compounds for *P. falciparum* (compounds **5a–b**, **6a–b**, **11a**, **11c**, **11f–h**, **13c** and **13e–g**) was evaluated using NIH 3T3 and Hek 293T cells. Remarkably, most of the squaric derivatives were non-cytotoxic up to 100 μM (Table 3). Only, squaramate **5b** led to some cytotoxicity when compared to the other squaric derivatives; but even in this case, the selectivity index (SI) is *ca.* 5, which indicates only modest toxicity.

Importantly, the calclogP values[15] for the most active compounds range from 1.0 to 5.7 (Table 3) suggesting that they present appropriate cell permeability properties.

Conclusions

We have synthesized a library of 38 squaric derivatives and evaluated activity against papain-, falcipain-2- and a chloroquine-resistant strain of *P. falciparum*.

The first series of compounds was obtained by reaction of dimethyl or dibutyl squaramates with the appropriate amines in moderate to good yields.

Aza-dipeptidyl squaric acid derivatives displayed good antiplasmodial activity against *P. falciparum*. The most active compounds contained an azahomoPhe moiety at the peptidic sequence and presented IC_{50} values of around 3 μM. No cysteine protease inhibition was observed for compounds containing a recognition moiety, suggesting that FP-2 inhibition is not the primary mode by which derivatives **11** and **13** exert antiplasmodial activities.

However, the recognition moiety seems to be important for the antiplasmodial activities of the squaric derivatives, as most of the non-peptidic compounds were inactive against *P. falciparum*. For this reason, one might speculate that the compounds are inhibiting other proteases involved in the parasite life cycle. In fact, *in silico* approaches have identified 92 putative proteases in the *P. falciparum* genome.[16] Further investigations are necessary to clarify the target of these compounds.

In conclusion, several squaric acid derivatives showed significant antiplasmodial activity against a CQ-resistant *P. falciparum* strain and minimal cytotoxicity. Overall, aza dipeptide squaric derivatives are promising lead compounds for the development of new agents against *P. falciparum* malaria.

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