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Discovery of Oxybisbenzoylamides as a New Class of Antimalarial Agents

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We have previously described several potent dual inhibitors of *Plasmodium falciparum* (*Pf*) growth characterized by the presence of statin, a β-hydroxyl amino acid able to inhibit parasite's plasmepsins (PLM). While investigating the mechanism of action of these inhibitors new compounds deprived of statin were synthetized which lost the ability to inhibit PLM, but retained a significant *Pf* growth inhibition. Further structural simplifications showed that the inhibition of *Pf* viability was to ascribe to a new pharmacophore never described before as antimalarial

Introduction

Malaria has a massive impact on human health, being one of the major infectious in the world causing 219 million clinical cases and about 660 000 deaths in 2010, mainly children under the age of five.[1] The most deadly parasite among the five Plasmodium species that causes human malaria is *Plasmodium falciparum* (*Pf*).

Artemisinin-combination therapies (ACTs) represent the major forms of intervention against malaria; however, the spread of drug resistance, as recently reported for artemisinin derivatives[2,3] may become a major public health problem, hindering malaria treatment. Spreading of resistance to first line drugs over the past few decades has led to intensification of the monitoring of their efficacy, to ensure proper management of clinical cases and early detection of changing patterns of resistance in order to revise national malaria treatment policies. This issue, combined with few commercially available drugs and an increased focus in limiting malaria transmission, is the basis for the urgent need of new antimalarials with innovative mechanism of action to fight against the onset of cross-resistance and expand physician tools for malaria control and eventually elimination.

Plasmepsins (PLMs) are aspartic proteases that, in cooperation with other proteases, participate to the haemoglobin digestion in the parasitic food vacuole.[4] This process is crucial for the development of the parasite and its blockage leads to the *Plasmodium* death. Although the haemoglobin digestion process appears to be a good pharmacological target, molecules with a useful in vivo efficacy have not been obtained, yet. During our studies interesting results were generated by applying the "double-drug" approach:[5-9] we synthesized molecules characterized by the presence of statin, a β-hydroxyl amino acid able to inhibit aspartic proteases, such as PLMs, joined with the 8-aminoquinolinic ring system (**1**) derived from primaquine (PQ) or a hepatic schizonticidal atovaquone (**2**) or the 4-aminoquinolinic ring system (**3**) derived from chloroquine (CQ). (Figure 1)

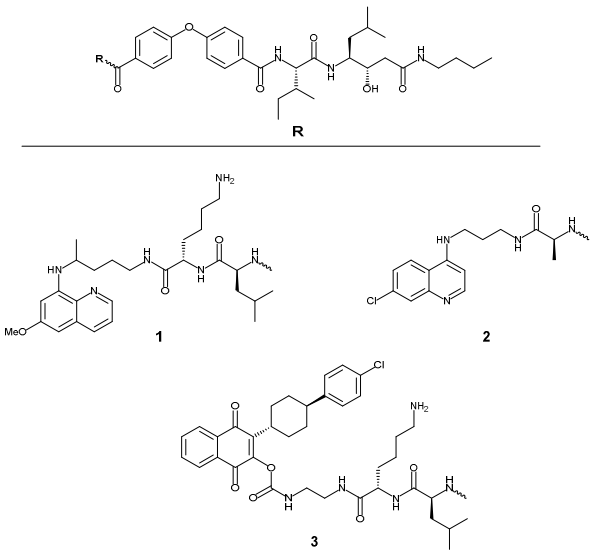

Figure 1: Previously synthesized double-drugs

These compounds were potent PLMs inhibitors and showed an antimalarial activity at concentrations significantly lower than any PLMs inhibitor previously reported. The antimalarial activity was also accompanied by low cytotoxicity and high

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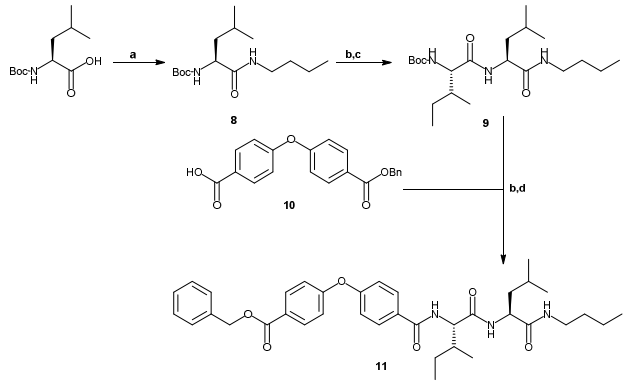
selectivity towards human proteases.[6] The compounds shown in Figure 1 are the most active representative for each class.

During our studies, discrepancies in the relationships between the inhibition of PLM2 and of *Pf* growth become evident. By comparing compounds **1**, **2** and **4** that present quinolinic substituents (Table 1), **2** and **4** showed very similar values of PLM2 inhibition. However compound **2**, having the quinolinic ring system derived from CQ, presented an antimalarial activity significantly higher (4-7 times) than compound **4** against the CQ-resistant, W2 strain. Furthermore, compound **4** was the best PLM2 inhibitor and the worst antimalarial.

To test the contribution of PLM2 inhibition to the antimalarial activity of the double drugs, we designed compounds **5-7** using compounds **1**, **2** and **4** as model, replacing statin, responsible for PLM2 inhibition, with leucine. (Table 1)

Results and discussion

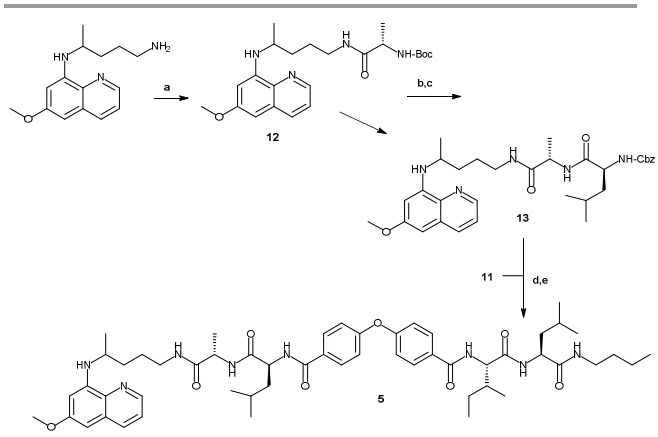
Compounds **5-7** were prepared using the dipeptidic benzyl ester derivative of oxybisbenzoic acid **11** (Scheme 1) that was synthesized by coupling Boc-Leu with butylamine leading to amide **8**, that after acidic cleavage of Boc was reacted with Boc-Ile generating dipeptide **9**. After deprotection, dipeptide **9** underwent a coupling reaction with 4-(4-((benzyloxy)carbonyl)phenoxy)benzoic acid **10** leading to compound **11** (Scheme 1).



Scheme 1. Reagents and conditions: (a) n-Butylamine, N,N,N′,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-Hydroxybenzotriazole(HOBt), 4-Methylmorpholine (NMM); (b) HCl 4N, dioxane; (c) BocIleOH, HBTU, HOBt, NMM; (d) HBTU, HOBt, NMM

Compound **5** was obtained by coupling the dipeptide derivative of primaquine **13** with oxybisbenzoic ester **11**, after simultaneous deprotection by hydrogenolysis. (Scheme 2).

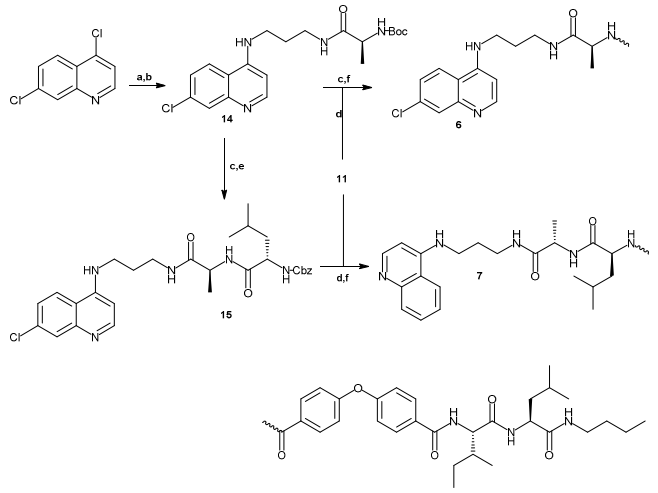
Compounds **6** and **7** were prepared according to scheme 3. A nucleophilic substitution of chlorine in position 4 of 4,7-dichloroquinoline with a large excess of 1,3-diaminopropane followed by coupling with Boc-Ala led to intermediate **14**. The Boc deprotected intermediate **14** underwent a further coupling reaction with Cbz-Leu to generate dipeptide **15** (Scheme 3).



Scheme 2. Reagents and conditions: (a) BocAlaOH, HBTU, HOBt, NMM; (b) HCl 4N, dioxane; (c) CbzLeuOH, HBTU, HOBt, NMM; (d) Pd/C, H2, MeOH; (e) HBTU, HOBt, NMM.

Compound **7** was obtained by coupling reaction after simultaneous one-pot deprotection of benzyl ester **11** and dipeptide **15**. As expected, during catalytic hydrogenation, the chlorine atom in position 7 was removed[10] (Scheme 3).

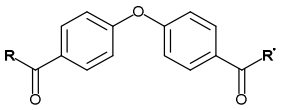
Compound **6** was obtained by coupling reaction between deprotected intermediates **14** and **11** (Scheme 3).

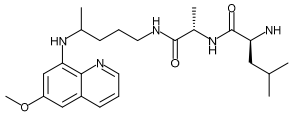
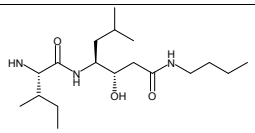
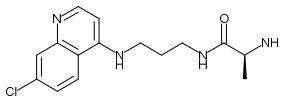
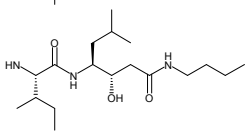
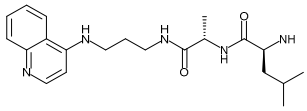
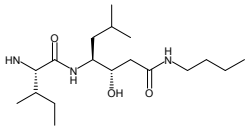
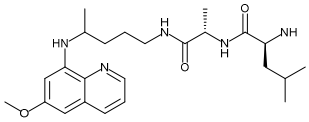
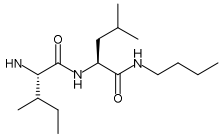
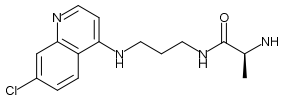
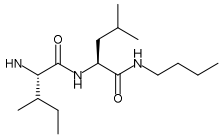
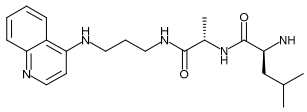
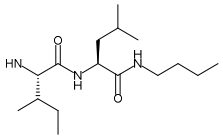


Scheme 3. Reagents and conditions: (a) Propane-1,3-diamine, 140°C; (b) BocAlaOH, HBTU, HOBt, NMM; (c) HCl 4N, dioxane; (d) Pd/C, H_2, MeOH (e) CbzLeuOH, HBTU, HOBt, NMM;. (f) HBTU, HOBt, NMM.

Compounds **5-7** were tested against W2, CQ-resistant and D10, CQ-sensitive *Pf* strains,[11] as well as for PLM2 inhibition and the results are shown in Table 1. As expected compounds **5-7** were unable to inhibit PLM2 proteolytic activity, but they showed an antimalarial activity significantly higher than their statin analogues against both *Pf* strains.

Table 1: Enzyme inhibition and antiplasmodial activity of compounds **1-2** and **4-7**



Entry	R	R'	PLM2 Ki (nM)	D10 IC_{50}(nM)[c]	W2 IC_{50}(nM)[c]
1[b]			26.3	200	300
2[c]			3.8	114	64
4[c]			2.6	161	474
5			n.a.	37	104
6			n.a.	43	39
7			n.a.	63	54
CQ			n.t.	23	412

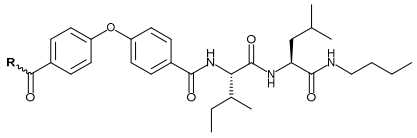
[a]Results are the mean of two experiments performed in duplicate. [b]From reference 6. [c]From reference 9. n.a.: No inhibition observed at 1μM. n.t.: Not tested

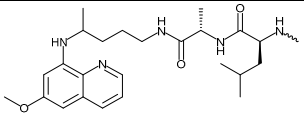
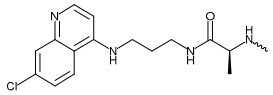
Interestingly, while compound **4** and **5** showed cross-resistance with CQ, compounds **6** and **7** demonstrated a similar activity on both D10 and W2 strains. Compound **6** being the most active was also tested for cytotoxicity against normal human fibroblast (FDH)[6] and against a macrophage cell line (HMEC-1), showing an effect only in the micromolar range (FDH, IC_{50}: 17.99 ± 8.7 μM; HMEC-1, IC_{50}: 17.58 ± 7.8 μM) with a good therapeutic index (approx. 450).

Therefore, compounds **5-7** are potent inhibitors of *Pf* growth, but they do not act through PLMs inhibition. To verify if these compounds share the same mechanism of action of CQ, we assessed the ability of compounds **5** and **6** to inhibit β-haematin formation using the BHIA method previously described.[12]

Table 2 shows that compound **6**, characterised by the 4-aminoquinoline ring system, inhibits β-haematin formation better than CQ (IC_{50} 0.79 vs 1.36 of CQ), whereas compound **5** is inactive as PQ.

Table 2. Inhibition of β-haematin formation[12]



Entry	R	BHIA[a]
5		> 8
6		0.79
CQ		1.36
PQ		> 10

[a]The IC_{50} represents the molar equivalents of test compounds relative to haematin required to inhibit β-haematin formation by 50%.

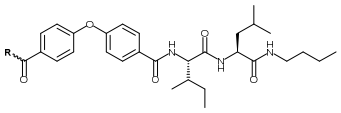
This is consistent with previous data showing that the 8-aminoquinolinic ring system is not able to interfere with β-haematin formation.[12]

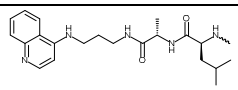
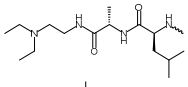
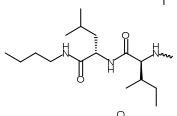
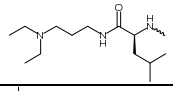
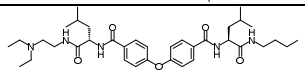
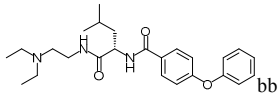
The lack of inhibition of β-haematin formation and the loss of PLM2 inhibition paired with the good antiplasmodial activity of compound **5**, led us to suppose that this new class of *Pf* inhibitors may owe its activity to a different mechanism of action.

To verify this hypothesis, compounds **16-18** were synthetized replacing the quinolinic substructure with a short alkyl chain. The basic tertiary amine was introduced in compound **16** to improve aqueous solubility otherwise extremely low. Compound **17** shows the same substituent on both sides and compound **18** presents a one amino acid shortened peptide chain. The influence of the dipeptide IleLeu on the other side of the oxybisbenzoic scaffold was evaluated by removing Ile (**19**) and the entire substituent (**20**), thus achieving further reduction of the peptide character and molecular weight.

Compounds **16-18** were synthesized similarly to compound **5** (scheme 2) using in the first step the appropriate amine (see supplementary information) while for the preparation of compounds **19** and **20** a slightly different synthetic route was adopted (see supplementary information). Compounds **16-20** were tested against W2, CQ-resistant and D10, CQ-sensitive *Pf* strains and results are shown in Table 3.

Table 3: Antiplasmodial activity of compounds **7**, **16-20**.



Cmp	R	D10 IC_{50}(nM)[c]	W2 IC_{50}(nM)[c]
7		63	54
16		345	143
17		1981	1620
18		121	103
19		357	314
20		NA*	NA*

***NA: IC_{50} > 5 μg/ml**

Compounds **16**, **18** and **19** showed an antiplasmodial activity in nanomolar range while compound **17** resulted considerably less active compared to compound **7**, probably due to its limited acqueous solubility. Compound **20** showed very limited antiplasmodial activity. Compounds **18** and **19** were also tested for cytotoxicity against normal human fibroblast (FDH)[6] ((**18**) IC_{50}: 16.3 ± 4.9 μM; (**19**) IC_{50} : 23.0 ± 7.3 μM) showing an effect similar to compound **6**. Compound **18** showed a good therapeutic index (> 100).

To assess the attractiveness of compounds **5-7**, **16, 18, 19** as new hits the values of ligand lipophilicity efficiency (LLE) were calculated.[13] Table 4 shows that the substitution of the quinolinic ring system with a tertiary amine resulted in a clear improvement of LLE. Compounds **18 and 19** are particularly attractive because show very similar IC_{50} values against CQ-sensitive and CQ-resistant *Pf* strains and the highest LLE value.

Table 4: LLE* values for compounds **5-7**, **16**, **18, 19**.

Entry	LLE (pH 7.4) D10	LLE (pH 7.4) W2
5	0.52	0.07
6	1.59	1.63
7	1.80	1.87
16	2.74	3.12
18	3.35	3.41
19	2.99	3.04

*LLE[13] = pIC_{50} – clogD(pH 7.4)[14]

Conclusions

On the basis of these results and the fact that **18** and **19** do not present any known antimalarial pharmacophoric group, it is likely that these molecules own their activity to a new mechanism of action.

Considering that, any new antimalarial drug needs to be effective against drug-resistant strains, but also affordable for those in need in developing countries, it is essential to develop molecules with an inexpensive synthesis. The removal of the non-natural β-hydroxyl amino acid statin greatly facilitated the synthesis reducing the inherent costs. Therefore, we believe that compounds **18** and **19** are good starting points to develop a new class of antimalarial agents.

Acknowledgements

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