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Atovaquone-Statine "Double-Drugs" with High Antiplasmodial Activity

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Malaria is one of the most widespread parasitic infections in the world; the unavailability of a vaccine and the spread and intensification of drug resistance over the past 15–20 years have led to a dramatic decline in the efficacy of the most affordable antimalarial drugs.[1–3] The reported presence of sporadic cases of artemether resistance indicates the need for increased vigilance and a coordinated and rapid deployment of drug combinations.[4]

Plasmepsins are a family of proteases comprising aspartic proteases Plasmepsins I, II, and IV (PLM I, PLM II, and PLM IV) and an histoaspartic protease (HAP) localized in the digestive vacuole of *Plasmodium falciparum* (*Pf*) together with other proteases (that is, falcipains). These proteases are involved in the degradation of haemoglobin during the intraerythrocytic cycle of *Pf*. Haemoglobin digestion is a crucial process for the survival of the parasite in the red blood cell (RBC) both for providing amino acids for protein biosynthesis and for reducing the osmotic pressure in the RBC. The inhibition of these enzymes has been proposed as a promising target for the development of a new antimalarial therapeutic approach.[5] The protease machinery developed by the parasite to accomplish this task is quite redundant and the role of each enzyme needs further investigation. The developed PLM inhibitors, including molecules with a statine-based core and peptidomimetic structures, showed a limited effectiveness in inhibiting the intraerythrocytic parasite growth (IC_{50} range 1–20 μM), although their K_i values against PLMs were in the nanomolar range.[6] Their low potency against cultured parasites may be explained by the redundancy of proteases which implies that the blockade of one or two PLMs may not be lethal.[7]

The "double-drug" approach combines two different inhibitors into a single chemical entity by a linker, with the aim of improving the physicochemical characteristics of the individual compounds.[8] We have previously reported the high antiplasmodial activity of double-drug **1**, designed by linking a statine-based inhibitor of PLMs, with primaquine, a known antimalarial drug active against the hepatic stage of *Plasmodium*.[9,10] We describe herein the synthesis and biological evaluation of a series of ATQ–statine double-drugs. ATQ is a very potent antimalarial drug used in association with proguanil (Malarone, GSK) to reduce the risk of inducing resistance. ATQ shows subnanomolar IC_{50} values against the intraerythrocytic parasite growth in vitro and acts through inhibition of the cytochrome bc1 complex (Complex III).[11] Basic carbamate prodrug **2** of ATQ was previously described[12] and was designed to improve water solubility and the rapid release of ATQ by a pH-dependent mechanism.[13]

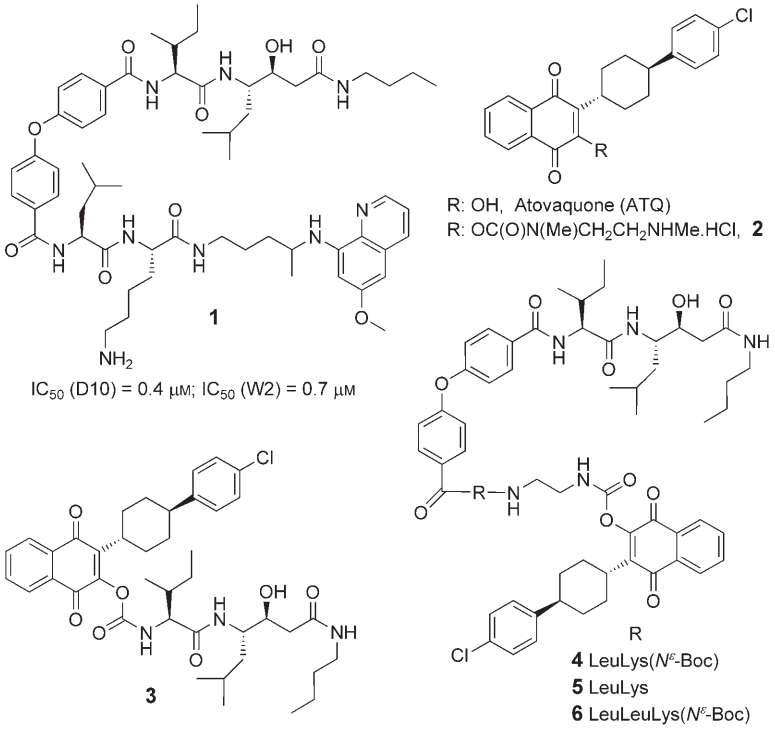

Figure 1. Atovaquone-statine double drugs described in this study.

We assumed that by replacing primaquine with ATQ a further advantage could be obtained as both ATQ and statine are active during the intraerythrocytic stage of *Pf*. Double-drugs **3**–**6** were therefore designed (Figure 1): compound **3** is characterized by ATQ bound directly to IleSta through a carbamate

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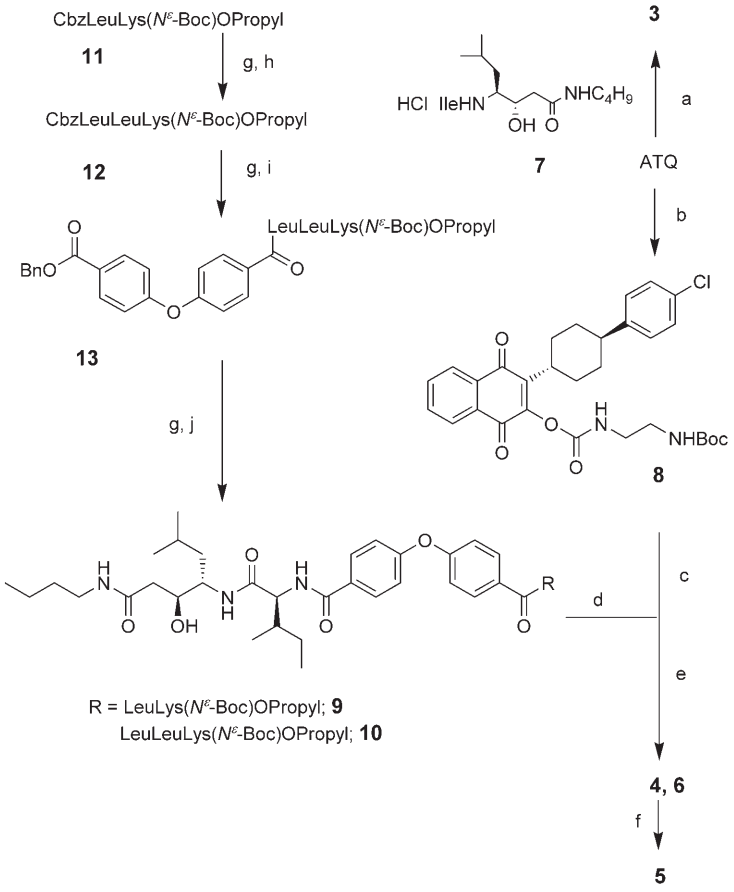
group; compounds **4** and **5** are characterized by the carbamate of ATQ bound to the Lys residue of **1** through an ethylenamine bridge; in compound **6** an additional Leu residue was included to increase the distance between the bulky ATQ and statine.

Compound **3** was prepared by reaction of ATQ with IleStatine hydrochloride **7**[10] and triphosgene[14] (Scheme 1). Compounds **4**–**6** were prepared by removal of the Boc group of carbamate **8**, obtained from the reaction of ATQ with *N*-Boc-ethylenediamine and triphosgene, followed by coupling using HATU with carboxylic acids obtained from basic hydrolysis of peptidic esters **9**[10] or **10** (Scheme 1). Tripeptidic ester **10** was prepared in three coupling steps starting from dipeptide **11**;[10] first CbzLeucine was introduced followed by the monobenzylester of 4,4′-oxy(bisbenzoic acid), and finally by IleStatine hydrochloride **7**[10]. Phosphate **5** was obtained by treatment of **4** with TFA followed by H_3PO_4.



Scheme 1. Reagents and conditions: a) **7**, Triphosgene, DIEA; b) $BocNH(CH_2)_2NH_2$ Triphosgene, DIEA; c) HCl 4N, dioxane; d) NaOH 2N, dioxane/H_2O; e) HATU, HOBt; f) TFA, H_3PO_4; g) H_2, Pd/C; h) CbzLeuOH, HBTU, HOBt; i) Monobenzylester of 4,4′-oxybisbenzoic acid, HBTU, HOBt; j) **7**, HBTU, HOBt.

The inhibition of *Pf* growth in RBC by compounds **3**–**6**, **9**, and ATQ was evaluated against chloroquine (CQ) sensitive D10 and CQ resistant W2 strains (see Table 1). Compound **3**, in which ATQ has been directly bound to the residue of isoleucine, despite the limited inhibition of PLM II, demonstrates a good inhibition of *Pf* growth in both strains. This result is remarkable and it could be due to an effect of the molecule as a whole or to the release of ATQ in the watery environment. Compounds **4** and **6**, characterized by a peptide spacer between ATQ and statine (Lys(N^{ε}-Boc)-Leu and Lys(N^{ε}-Boc)-Leu-Leu respectively), show similar inhibition for both PLM II and *Pf* growth. This suggests that the addition of a residue of leucine does not improve the activity of this class of inhibitors. Compounds **4** and **6** show a ninefold increase in PLM II inhibitory activity in comparison to compound **3**, however the inhibitory activity against *Pf* growth diminished (3–5-fold), suggesting that these two activities are not correlated. Removal of the Boc group from compound **4** gave compound **5**, isolated as a phosphate salt. Compound **5** was three to five times less active against PLM II compared to compound **4**, but showed similar activity against *Pf* in comparison to compound **3**. The deleterious effect of the Boc group on the inhibition of *Pf* growth is not of simple interpretation and it could be linked to the excessive dimensions reached by these derivatives. For compound **9** the inhibitory activity of *Pf* drastically diminished thus confirming that ATQ is responsible for the activity of compounds **3**–**6**.

Table 1. Effect of compounds **3**–**6**, **9**, and ATQ on PLM II activity and on growth of CQ sensitive (D10) and CQ resistant (W2) *P. falciparum* strains.

Compd.	PLM II K_i [nM] ± S.D.	D10(CQ-S) IC_{50} [nM]	W2(CQ-R) IC_{50} [nM]
3	142.4 ± 0.8	0.95	0.61
4	17.3 ± 0.6	4.8	5.3
5	27.0 ± 2.3	0.69	1.05
6	15.1 ± 5.7	3.3	3.4
9	11.6 ± 0.4	120	140
ATQ	> 1 μM	0.3	0.43

Stability of compounds **3**–**6** at 37 °C in phosphate buffer at pH 7.4 and in acetate buffer at pH 4.0 was determined conveniently by HPLC analysis. In every case at pH 7.4 after 5 min there was complete conversion of the starting material into ATQ, whereas all compounds were stable at pH 4.0. ATQ-derived basic carbamate prodrug **2** has been previously synthesized[12] and this molecule spontaneously decomposes in water[13] releasing ATQ by a pH-dependent mechanism that does not rely on enzyme activity and occurs very rapidly at physiological pH (7.4). This mechanism involves the basic group present in the molecule. We tend to exclude that ATQ could be released from compounds **3**, **4**, and **6** according to the mechanism described above, as these compounds do not have basic groups. Rapid release of ATQ (5 min at pH 7.4) could be due to the anchimeric assistance of the oxygen in *ortho* position to the carbamate.[15]

The most promising inhibitor of *Pf* growth (compound **3**), when tested on human fibroblasts cell line, showed cytoxicity

(IC_{50}: 5 μM) at concentrations much higher than those required for *Pf* inhibition, indicating that this compound interferes with mechanisms unique for the parasite.

In conclusion, we have found inhibitors that possess a very good effectiveness against *Pf* growth, these compounds are therefore good candidates for further studies on ATQ-resistant *Pf* strains.

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