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Targeted delivery of compounds to *Trypanosoma brucei* using the melamine motif

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ABSTRACT

There is an urgent need for the development of new drugs for the treatment of human African trypanosomiasis. The causative organism, *Trypanosoma brucei,* has been shown to have some unusual plasma membrane transporters, in particular the P2 aminopurine transporter and related permeases, which have been used for the selective targeting of trypanocidal compounds to the organism. In this paper, we report the addition of melamine-based P2-targeting motifs to three different classes of compound in order to try and improve activity through increased selective uptake. The classes reported here are fluoroquinolones, difluoromethylornithine and artesunate derivatives.

1. Introduction

There is an urgent need for new drugs for the treatment of human African trypanosomiasis (HAT), a disease which is endemic in sub-Saharan Africa. We are interested in utilising nutrient transporters as a way to selectively target compounds to the parasite *Trypanosoma brucei* that causes HAT, as a potential method for drug discovery for this organism. In particular, *T. brucei* is auxotrophic for purines from the human host and imports them through different nucleoside/nucleobase transporters.[1,2] The P2 transporter, an unusual adenosine transporter found only in *T. brucei,* mediates the uptake of adenosine and adenine under physiological conditions. Unusually, it can also take up other motifs such as melamines (e.g., melamine-arsenical drugs) and benzamidines (e.g., pentamidine and berenil) (Scheme 1).[3–5] Thus, anti-trypanosomal agents such as the melamine-based arsenicals and pentamidine are selectively concentrated in *T. brucei* through the P2 transporter as well as through other trypanosome transporters. Pentamidine is taken up by at least two other transporters in addition to the P2 transporter; these have been designated the high affinity pentamidine transporter (HAPT1) and the low affinity pentamidine transporter (LAPT1), although their physiological roles are unknown.[6] Furthermore, experiments indicate that melamine-arsenicals also have alternative uptake routes in addition to the P2 transporter, and HAPT1 has been proposed to play such a role.[7] Thus, melamines and benzamidines are potentially substrates of several parasite transporters; the advantage of being substrates of several parasite-specific transporters is that moieties should still be taken up, even if loss of one transporter occurs through resistance.

Around 100 years ago, Paul Ehrlich developed the haptophore-toxophile biphasic theory of drug action.[8] In this theory, drugs can be described as having two moieties: the *haptophore* (anchorer) which directs a molecule towards a specific cell receptor; and the *toxophile* (poisoner) which is responsible for the cytotoxic activity by chemically combining with the target. Melarsoprol adheres to this theory; the haptophore being the melamine moiety, which is specifically recognised by the P2 (and other) trypanosome transporters, with the arsenic atom being the toxophile, which presumably reacts with thiols in the parasite. We have an interest in using these transporter systems, which recognise melamines and benzamidines, to selectively deliver trypanocidal agents to *T. brucei,* and have previously reported approaches to selectively deliver trypanocidal molecules to the parasite via the P2 transporter using Ehrlich's approach, employing the melamine moiety as the *haptophore,* and a variety of molecules as the *toxophile* (Scheme 2).[9]

In the first series of compounds we reported, the *toxophiles* or trypanotoxic moieties selected were structural analogues of polyamines, since perturbations to polyamine metabolism are known to be toxic to trypanosomes. When coupled to melamine, these compounds showed potent activity against *T. brucei.*[10,11] A second series of compounds used nitrofurans as the trypanotoxic moiety.

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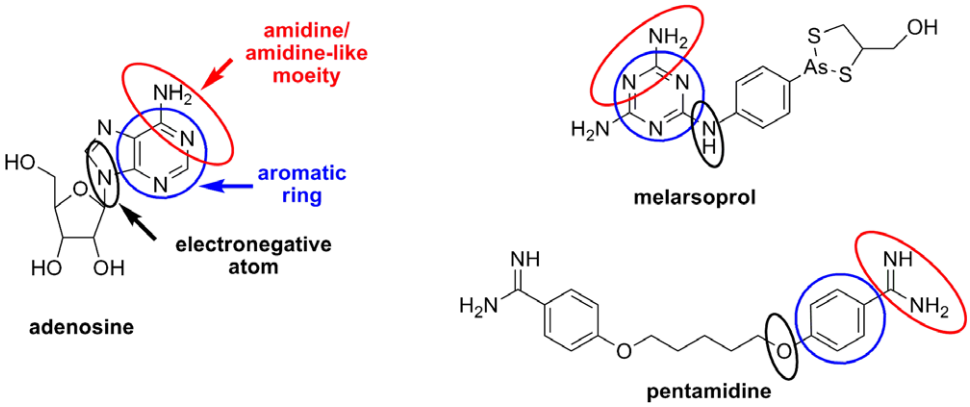
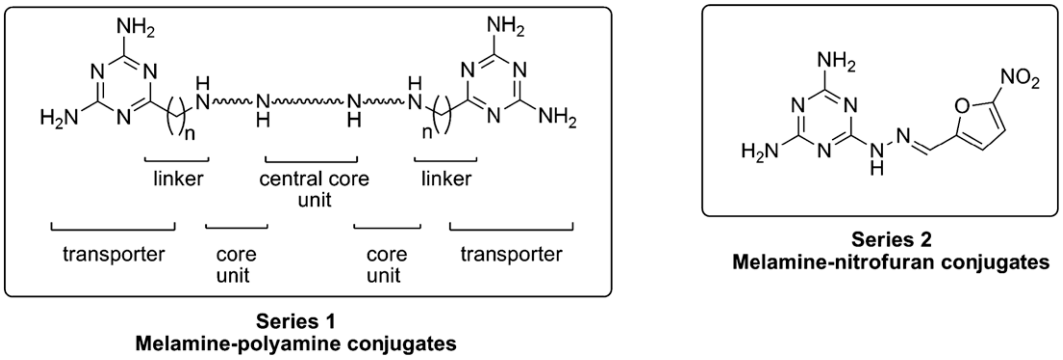


Scheme 1. Requirements for uptake through the P2 transporter.



Scheme 2. Melamine–polyamine conjugates and melamine–nitrofuran conjugates: **2** series of compounds selectively delivered to the parasite through the P2 transporter.

This has led to the identification of some very potent compounds, including compounds that were curative of trypanosome infections in mice.[12,13]

Having succeeded in developing a series of trypanocides in this way, we wanted to see if we could extend the range of compounds that can be delivered in this manner, focusing on compounds that have limited intrinsic activity against this parasite, with the expectation that potency can be improved markedly by inducing accumulation of these compounds via the P2 transporter that is known to accumulate its substrates by several orders of magnitude over extracellular concentrations by employing the trans-membrane proton motive force. Here, we report the attachment of three classes of known anti-microbial agent to the melamine moiety. The agents chosen, fluoroquinolones, artesunate and DFMO all present only relatively weak activity (with IC_{50} values in the micromolar range) against *T. brucei*. Our goal was to see if by selectively concentrating the compounds in the parasites we could significantly enhance activity, or whether the compounds have a low intrinsic level of activity against this organism, which cannot be overcome by selective concentration. In addition, we investigated the use of both a non-cleavable linker (an amide) and one that is potentially cleavable by esterases (an ester) (Scheme 3) to link the *haptophore* to the *toxophile*.

Eflornithine (DFMO) is used clinically against late-stage *T. brucei gambiense* infections; it targets ornithine decarboxylase (ODC). The main issues related to this drug are its high cost and the large doses required for administration. As it is an equally effective suicide inhibitor of mammalian ODC, differences in turnover rates between host and parasites have been evoked to explain its selectivity. But another reason advanced to explain the efficacy of eflornithine is the fact that mammalian cells could induce high affinity polyamine transporters in response to a polyamine starvation, while *T. brucei* has a limited capacity to scavenge the low abundance polyamines present in serum.[14,15] A formulation or derivative of eflornithine that was accumulated to high levels in *T. brucei* through active transport may have significant potential for improving this agent.

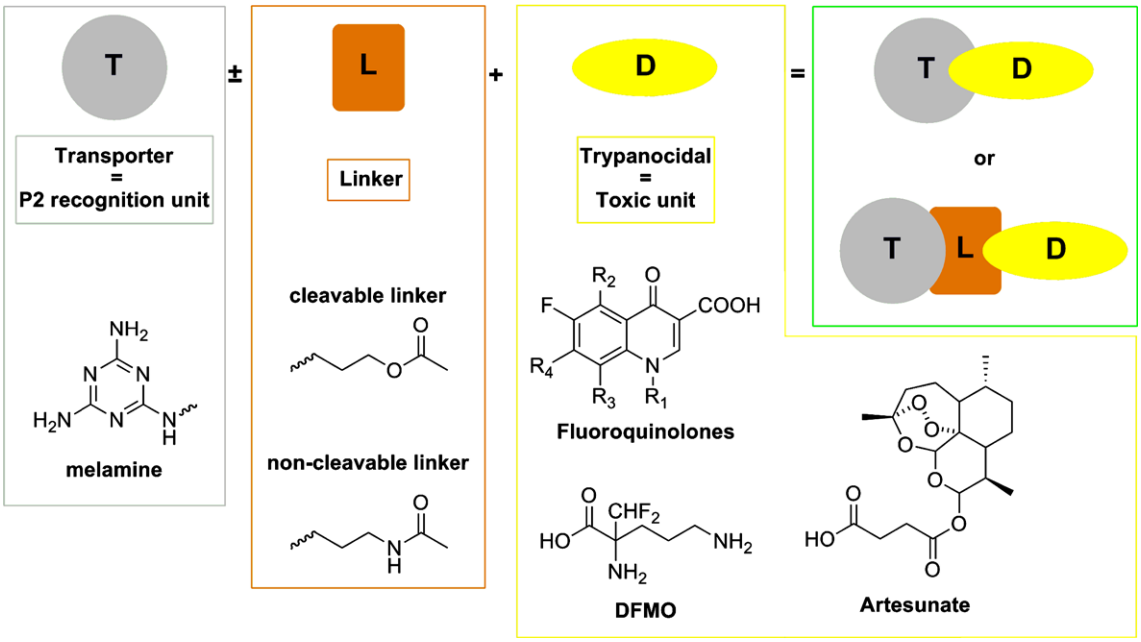
Fluoroquinolones are a class of DNA gyrase (prokaryotic topoisomerases II) inhibitors, used as antibacterials. *T. brucei* has a unique and complex type of mitochondrial DNA, demanding an abundant topoisomerase II activity and making inhibition of topoisomerases an interesting drug target.[16,17] Some fluoroquinolones have been shown to have moderate activity against *T. brucei*[16,18–20] and we wished to determine whether enhancing concentrative uptake by employing the P2 transporter would increase activity. Fluoroquinolones have the advantage of having very high selectivity for DNA gyrase, unlike other topoisomerase II inhibitors. Targeted delivery of fluoroquinolones to *T. brucei* may increase their potency.

Artesunate is a highly potent antimalarial, used in large quantities worldwide.[21] Its mechanism of action has not been understood so far but its activity is associated with generation of reactive oxygen species, and with an inhibition of the *P. falciparum* endoplasmic reticulum calcium (SERCA) pump.[22,23] Some artemisinin derivatives, including artesunate, show moderate activity against *T. brucei*.[24]

2. Chemistry

2.1. Synthesis of transporter recognition motifs

In order to link the melamine motif to the potential trypanocidal units, we generated two melamine derivatives bearing linkable side chains. These compounds are extremely useful as the terminal hydroxyl (**2a**) and amino (**2c**) groups can be linked with a large



Scheme 3. Concept and design of new trypanocidal drugs.

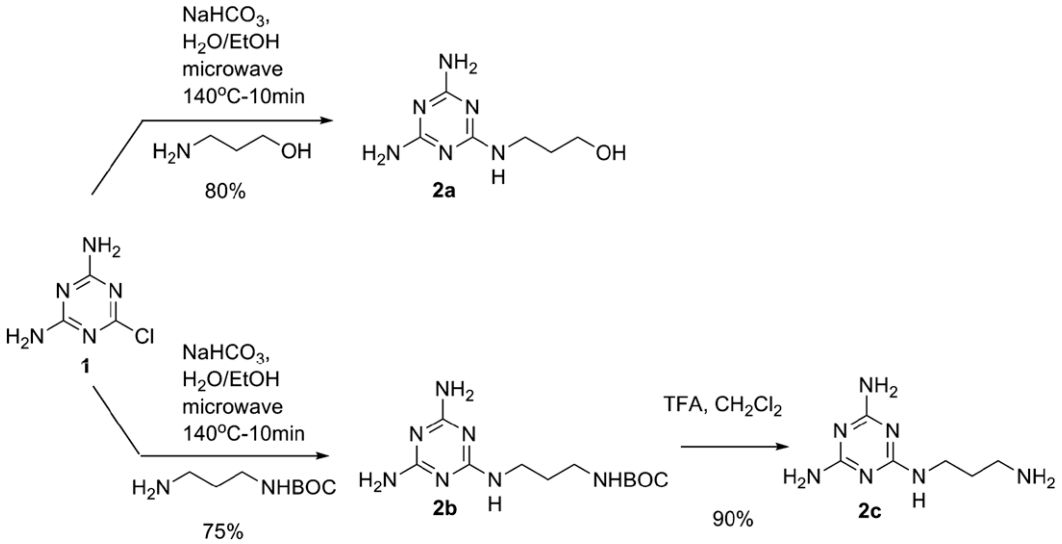
variety of chemical functions. Hydroxyl-triazine **2a** was directly obtained in good yield by microwave-assisted SN_{Ar} of 2,4-diamino-6-chlorotriazine **1** with aminopropan-1-ol (80%). The synthesis of amino-triazine **2c** was achieved in two steps. Microwave-assisted SN_{Ar} of 2,4-diamino-6-chlorotriazine **1** with BOC-1,3-diaminopropane led to BOC-protected amino-triazine **2b** in good yield (75%); then BOC deprotection of the crude, using TFA, led to **2c** (Scheme 4). Products **2a** and **2c** were purified by reverse phase flash chromatography.

2.2. Synthesis of melamine–DFMO conjugates

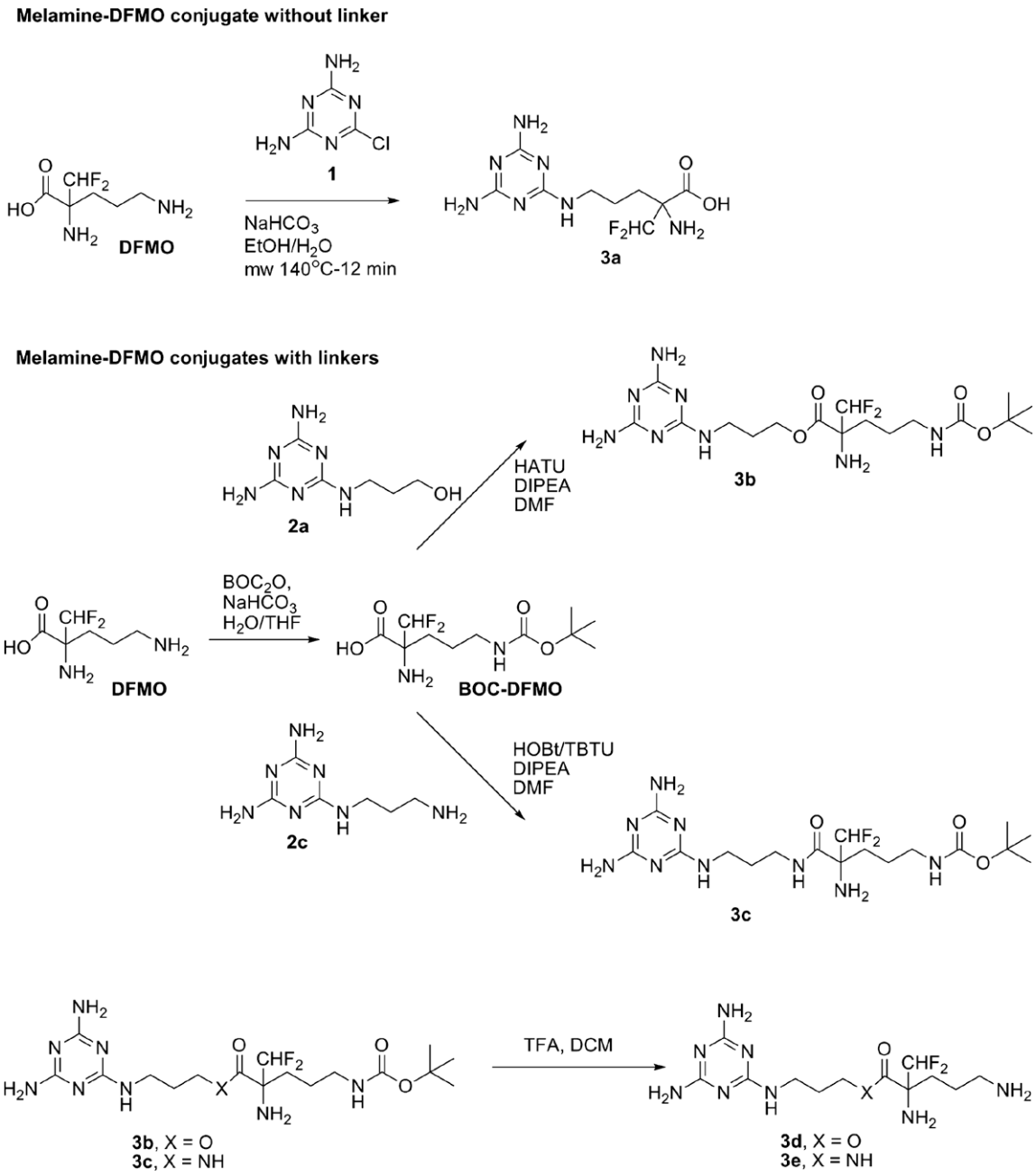
Initially, synthesis of melamine–DFMO conjugates without a linker was considered. A first compound was directly generated from the substitution of 2,4-diamino-6-chlorotriazine 1 by the γ-amino group of DFMO (Scheme 5). The reaction was successful although the pure final compound **3a** was obtained in very low yield (10%) after several attempts at purification (e.g., trituration with ethanol). The purification was eventually achieved by reverse phase flash chromatography, eluting with ethanol/water. However, this could be greatly improved by separating the mixture directly by reverse phase chromatography and by acidifying the mobile phase with TFA.

In a second approach, melamine–DFMO conjugates with a linker were synthesised through a coupling reaction between the carboxylic acid of DFMO and the hydroxyl/amine functionalities of triazine derivatives **2a**/**c** (Scheme 5).

For this purpose, BOC protection of the free amino groups of DFMO was required. The BOC–DFMO derivative was obtained in good yield from DFMO in the presence of BOC anhydride in THF/H_2O. Only the monoprotection of the γ-amino group of DFMO was observed; the α-amino group was remarkably un-reactive, probably due to the inductive effect of the carboxylic acid and steric hindrance.



Scheme 4. Synthesis of transporter/P2 recognition units **2a** and **2c**.



Scheme 5. Synthesis of melamine–DFMO conjugates without (**5a**) or with (**5b**–**e**) a linker.

It was decided to link the DFMO through the carboxylic acid. A compound linked through an ester should be hydrolysed by esterases to release DFMO, hence acting as a prodrug. Esterification between hydroxyl-triazine **2a** and BOC-DFMO was then attempted with different coupling agents. Use of DCC led to the melamine–DFMO conjugate **3b** but it was not possible to purify the products. Attempts with EDCI, polymer-supported DCC and the Mukaiyama coupling conditions using chloromethylpyridinium were unsuccessful. However, HATU successfully led to **3b**. Pre-activation of the carboxylic acid function of BOC–DFMO before addition of hydroxyl-triazine **2a** followed by direct purification of the crude by preparative HPLC gave the highest yield (30%) of the conditions investigated.

Coupling of the triazine to the carboxylate of the DFMO through an amide bond was also carried out to give the melamine–DFMO conjugate **3c**, using HOBt and TBTU to pre-activate the carboxylic acid, followed by addition of the amino-triazine **2c**.

As **3b** and **3c** contained the BOC-protected form of DFMO, their BOC deprotection was carried out with HCl in ethyl acetate, to afford the two analogues **3d** and **3e**. It was important that **3d** was maintained in its HCl salt form, as it appeared to be unstable with the free terminal amino group.

2.3. Synthesis of melamine–fluoroquinolone conjugates

The fluoroquinolones could be coupled to the melamine transporter moiety in two possible ways; either through the amine or through the carboxylic acid. We decided to investigate the effects of both. From commercially available fluoroquinolones, Ciprofloxacin, Norfloxacin, Lomefloxacin and Sparfloxacin (Scheme 6) were selected to be coupled directly with the melamine moiety via a microwave-assisted method (see Scheme 7).

SN_{Ar} of 2,4-diamino-6-chlorotriazine **1** by piperazine-free-amine was successfully carried out with ciprofloxacin and nor-

floxacin to give derivatives **4a** and **4b**, respectively. However, the lomefloxacin analogue was detected, but we were unable to obtain compound of sufficient purity for assay. Sparfloxacin did not react, presumably due to the steric hindrance afforded by the two methyl groups in the alpha position relative to the piperazine-free-amine that may have affected the nucleophilic attack.

The synthesis of melamine–fluoroquinolone conjugates linked with esters and amides through the carboxylic acid of the fluoroquinolone was then carried out. For this purpose ofloxacin was selected, as the presence of a methyl group on the piperazine will prevent it from interfering with the coupling reactions (Scheme 8). Coupling of both the hydroxyl-triazine derivative **2a** and the amino-triazine derivative **2b** was carried out using HATU as the coupling agent, followed by purification using semi-preparative HPLC, to give the ester (**4e**) and amide (**4f**) derivatives, respectively.

2.4. Synthesis of melamine–artesunate conjugates

Couplings between the carboxylic acid of artesunate and hydroxyl/amine functions of triazine derivatives **2a**/**c** were carried out using the previously described conditions (Scheme 9). The melamine–artesunate conjugate **5a** was obtained by esterification with artesunate and hydroxyl-triazine **2a**, using HATU as the coupling agent. Similarly, the melamine–artesunate conjugate **5b** was obtained by coupling artesunate with amino-triazine **2b**, using HOBt/TBTU as coupling agents. Both reactions were carried out in DMF in the presence of DIPEA, with a pre-activation of the carboxylic functionality, and the products were directly purified by preparative HPLC.

3. Biological results

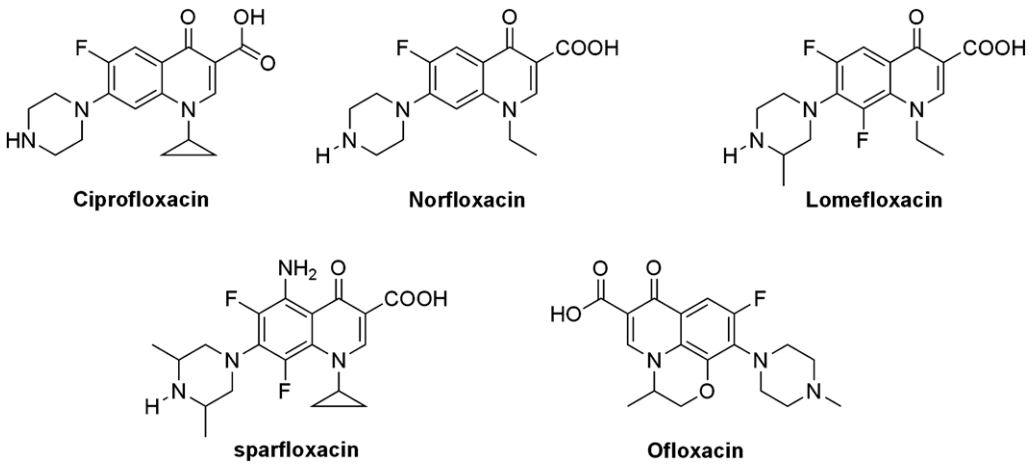
The motivation behind generation of the melamine-linked trypanocides described here was to increase accumulation of these compounds by their active transport through the P2 aminopurine transporter, and possibly through other transporters we believe to recognise melamine and other nitrogen heterocycles in *T. brucei*. For this purpose, three strains of *T. brucei* were used to test the activity of the different compounds: a wild-type strain of *T. brucei brucei* (s427) that is proficient in P2 activity, a mutant lacking the P2 transporter (*TbAT1* knockout) and a mutant lacking the P2 transporter and further selected for pentamidine resistance which has lost the HAPT1 carrier (B48).[7] The results are summarised in Tables 1–4.

Firstly, the transporter motifs themselves (**2a** and **2c**) showed no intrinsic activity (Table 1), so any activity observed is not from them.

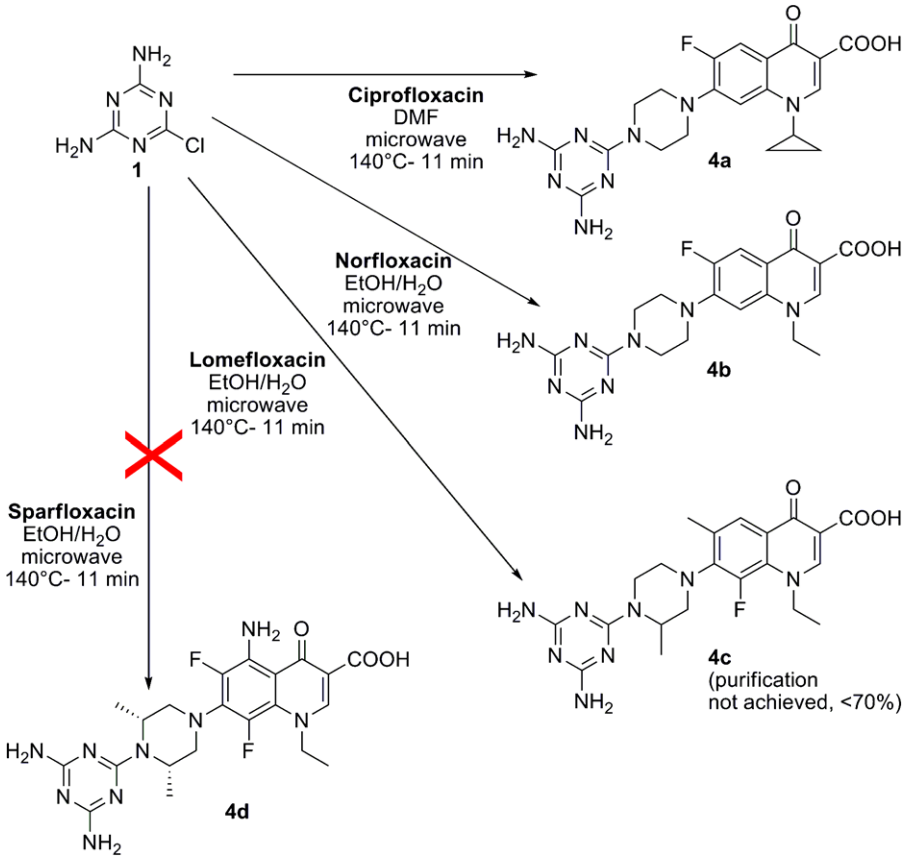
Data for the DFMO conjugates are presented in Table 2. DFMO itself showed approximately the same low level of activity for all three strains of trypanosomes ($IC_{50} \sim 50\ \mu M$). There is no active uptake of DFMO through either the P2 or HAPT1 transporters, which is to be expected given that eflornithine is an amino acid analogue most likely carried via one of the parasite's amino acid permeases. BOC-DFMO and melamine-DFMO conjugate **3a** did not show any trypanocidal activity. As DFMO acts as a suicide inhibitor of ODC, we can suppose that the free γ-amino group of DFMO is necessary for its interactions with ODC. This can be seen in the crystal structure (2tod), where the γ-amino group is tightly bound by interactions with the side chain of aspartic acid, the main chain carbonyl of tyrosine and various water-mediated interactions.[25] Results obtained for melamine–DFMO conjugates with an ester (**3c, 3d**) or an amide (**3b, 3e**) show essentially no activity. The lack of activity of these compounds suggests that DFMO cannot tolerate substitution on the carboxylate functionality. Interestingly, crystal structures of ornithine decarboxylase (2tod, 1njj, 1f3t) suggest that the active site is open, where the carboxylic acid is situated. Presumably, the substituents on the carboxylate either block an important interaction between the carboxylate and the enzyme, or there is a steric clash between the substituent and the enzyme. The ester derivatives (**3c, 3d**) were designed to be cleaved by esterases. However, it is possible that this does not occur due to the steric hindrance around this position. More work needs to be done to investigate this.

Data for the melamine-fluoroquinolone conjugates are given in Table 3. Fluoroquinolones, on their own, possess a variety of trypanocidal activities. Toxicity against the w.t. strain of *T. brucei* was moderate for ciprofloxacin, norfloxacin and sparfloxacin (IC_{50} = 17–40 μM), poor for lomefloxacin (IC_{50} = 197 μM) and not measurable for ofloxacin (IC_{50} > 100 μM). Literature data suggest that fluoroquinolones in general have activity in the low micromolar space against *T. brucei*. The melamine-ciprofloxacin conjugate **4a** gave an activity in the millimolar range (IC_{50} = 4.9 mM), and was 300-fold less potent than ciprofloxacin against w.t. strain of *T. brucei*.

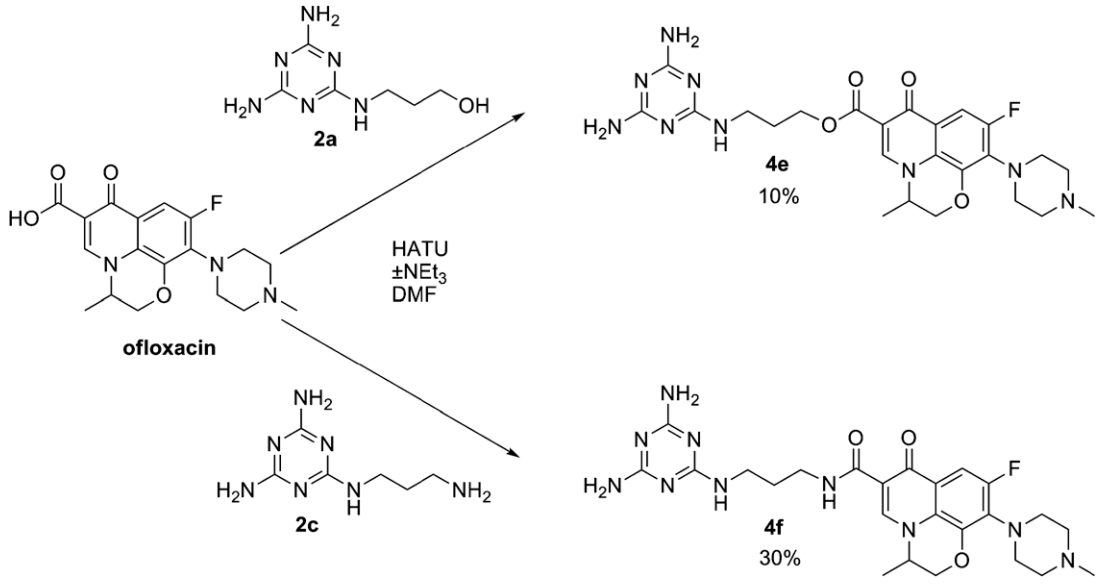
The melamine-norfloxacin conjugate **4b** was much more interesting. Compound **4b** was eight times more active against the w.t. strain than norfloxacin (IC_{50} = 5.2 μM), although sensitivity for **4b** was significantly lower in TbAT1-KO and B48 strains compared to the w.t. strain (resistance factors, respectively, 6.3 and 5.3), returning activities to levels similar to wild type. The melamine-



Scheme 6. Fluoroquinolones selected as potential trypanocidal moieties.



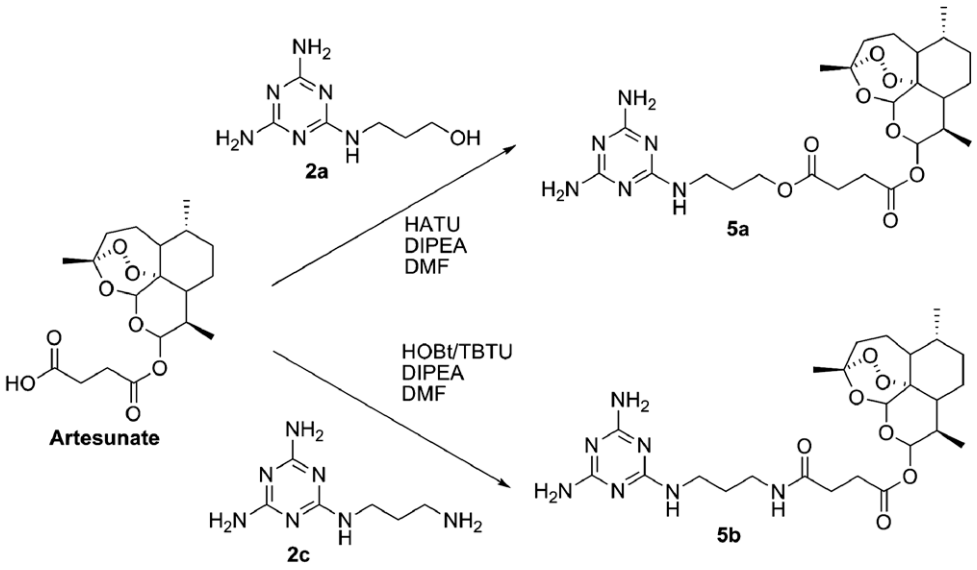
Scheme 7. Synthesis of melamine–fluoroquinolones conjugates **3a**–**d** without a linker.



Scheme 8. Synthesis of melamine–ofloxacin conjugates **4a**–**b** with a linker.

ofloxacin conjugate **4f** followed the same trend as **4b**, although at a lower range of activity. Specifically, **4f** exhibited an IC_{50} in the high micromolar range (i.e., 112 μM) against the w.t. strain, while ofloxacin was totally inactive. Moreover, its efficacy against the *TbAT1*-KO and B48 appeared to diminish as it was not measurable. Interestingly, a different behaviour is observed with the melamine-ofloxacin conjugate **4e**. Although it is 3.5 times more active than **4f** against the w.t. strain (32 μM), its efficacy is the same against *TbAT1*-KO and B48 strains. These data strongly suggest that compounds **4b, 4e** and **4f** are taken up and concentrated in the parasite, giving more potent compounds than the parent. The data also indicate that uptake of **4b** and maybe **4f** may principally be via the P2 transporter, although the intake of **4e** does not appear to be exclusively related to the P2 transporter.

Interestingly, the compounds were designed with an expectation that they would inhibit topoisomerase activity associated with



Scheme 9. Synthesis of melamine–artesunate conjugates **6a**–**b** with a linker.

Table 1
Activity of transporter units against *T. brucei*

Cpmd	*T. brucei brucei* EC_{50} (μM)			Resistance factor KO/wt	Resistance factor B48/wt
	Wild type	*TbAT1* KO	B48 (*TbAT1* KO + Pent res)		
2a	>100	>100	>100	–	–
2c	>100	>100	>100	–	–

Table 2
Activities of DFMO and conjugates

Cmpd	*T. brucei brucei* EC_{50} (μM)			Resistance factor KO/wt	Resistance factor B48/wt
	Wild type	*TbAT1* KO	B48 (*TbAT1* KO + Pent res)		
DFMO	44.6	54.9	50.3	1.2	1.1
BOC-DFMO	>100	>100	>100		
3a	>100				
3b	382	400.4	888	1.0	2.3
3c	355	104.7	>100	0.3	
3d	>100	>100	>100		
3e	>100	>100	>100		

Table 3
Activities of fluoroquinolones and analogues

Name	*T. brucei brucei* EC_{50} (μM)			Resistance factor KO/wt	Resistance factor B48/wt
	Wild type	*TbAT1* KO	B48 (*TbAT1* KO + Pent res)		
Ciprofloxacin	17.2				
4a	4940				
Norfloxacin	39.9				
4b	5.2	32.7	27.7	6.3	5.3
Lomefloxacin	197				
Sparfloxacin	18.5				
Ofloxacin	>100				
4e	31.6	35.7	31.5	1.1	1.0
4f	112	>100	>100		

the trypanosome's mitochondrial genome, the kinetoplast. It was hypothesised, therefore, that cell death might be associated with loss of DNA replication. To test this, we analysed the status of cellular DNA (nuclear and kinetoplast) in trypanosomes treated with the most potent of the fluoroquinolone derivatives (**4b**) after 24 h at its EC_{50} concentration. DAPI (4′,6-diamidino-2-phenylindole) was used to stain the DNA-containing organelles in situ. Rather than witnessing the kinetoplast disintegrate as has been seen in cells treated with trypanocidal diamidine drugs[26] the parasites became multi-kinetoplastic, indicating that the effect of this melamine-fluoroquinolone derivative was not related to inhibition of DNA replication but to some other cellular event that uncoupled DNA replication and kinetoplast-kinesis (Fig. 1). This phenomenon was observed in the majority of the cells treated with **4b**. In con-

Table 4
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Name	*T. brucei brucei* EC_{50} (μM)			Resistance factor KO/wt	Resistance factor B48/wt
	Wild type	*TbAT1* KO	B48 (*TbAT1* KO + Pent res)		
Artesunate	12.8	15.0	27.5	1.2	2.1
5a	4.0	5.0	12.1	1.2	3.0
5b	2.6	2.9	7.1	1.1	2.7

trast, no such effect was seen with the norfloxacin when used at its EC_{50} concentration for 24 h.

Data for artesunate and the artesunate–melamine conjugates are given in Table 4. Artesunate presented a moderate activity with no significant difference between the wild-type and the two knockout strains of *T. brucei* (EC_{50} = 13–27 μM), suggesting that artesunate, as expected, is not accumulated by either the P2 or HAPT1 transporters. The melamine–artesunate conjugates **5a–b** were 4–5 times more active than artesunate on the w.t strain. This may indicate some selective uptake of these compounds or it could also indicate that addition of the melamine group alters intracellular activity. There was no variation in the activity observed between the w.t. and *TbAT1*-KO strains with **5a-b**, indicating that carriers other than the P2 transporter are also contributing to uptake. Interestingly **5a** and **5b** both exhibited threefold less trypanotoxic effect against B48 than w.t. strain. A role for the HAPT1 transporter, that has been reported as absent in B48 and involved in diamidine uptake, is thus possible.

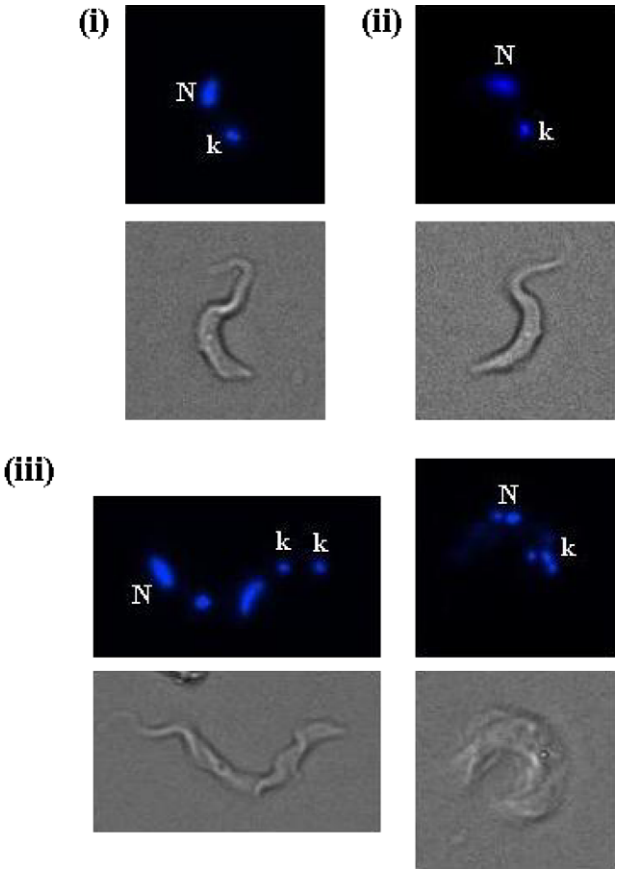

Figure 1. Fluorescence microscopy of *T. brucei* wild-type strain after 24-hour treatment with norfloxacin (ii) and **4b** (iii), respectively. Untreated cells (i) were used as negative control. Cellular DNA (N = nucleus, k = kinetoplast) was stained with DAPI and viewed under 40× magnification.

4. Conclusion

In this study, we took three compound classes that have weak anti-trypanosomal activity (DFMO, EC_{50} = 45 μM; fluoroquinolones, EC_{50} = 17–>1000 μM; artesunate, EC_{50} = 13 μM), and investigated whether coupling them to motifs known to allow selective concentration into trypanosomes would enhance their potency. There was a slight increase in potency for some fluoroquinolones and for artesunate when coupled to the melamine functionality. No increase in potency was found for DFMO. In this latter case, it was suspected that the position of the substituent melamine group would prevent binding of DFMO to ODC hence we created an ester-linked DFMO–melamine conjugate. In this case, the steric bulk α- to the carboxylate group of this compound probably protected the ester link from cellular esterases that otherwise might cleave it to release free eflornithine, rendering this conjugate inactive.

Our previous work has shown that potency of various trypanocides can be enhanced by attaching compounds to the melamine moiety, recognised by the P2 and other related transporters. In the cases presented here, a significant increase in potency is not evident. A number of reasons could explain this. The case of DFMO has been discussed above. Some fluoroquinolones are known to be active against *T. brucei* in the low micromolar range.[18] We observed a similar level of potency in our experiments with unconjugated fluoroquinolones. However, fluoroquinolones are selectively active against bacteria, as they inhibit bacterial DNA gyrase and bacterial topoisomerase IV, which are distinct from mammalian DNA topoisomerase II.[27] Although trypanosome mitochondrial DNA is, like all other mitochondrial DNA, bacterial in origin, the trypanosome kinetoplast topoisomerase II enzymes are of the eukaryotic type rather than prokaryotic type,[28] accounting for the low activity of fluoroquinolones against *T. brucei*. Our fluorescence microscopy experiments reveal that DNA replication proceeds in treated cells which supports the idea that fluoroquinolones are not inhibiting topoisomerases in trypanosomes, at least in the case of the melamine-coupled derivative described here.

Artesunate and other artemisinin derivatives are used as antimalarials. Their mode(s) of action are thought to include generation of radicals, depolarisation of the mitochondrial membrane potential and inhibition of the SERCA pumps.[24] Various artemisinin derivatives have been shown to have activity against *T. brucei* in the low micromolar range,[24] and we report similar activity here. The mode of action of these compounds against *T. brucei* is not known; however, the compounds are clearly much less active than against *Plasmodium falciparum,* which could suggest an alternate mode of action. Recent evidence from Kaiser et al appears to indicate a very different mode of action in *T. brucei* compared to *P. falciparum.*[29] They reported relatively little difference between the activities of peroxides and their deoxy analogues against *T. brucei*, which is in marked contrast to their effects against *P. falciparum,* where the peroxide analogues were significantly more active than their deoxy counterparts. We were able to enhance trypanocidal activity in this case by employing efforts to increase uptake, but only by a small amount.

In conclusion, the approach of attaching directing groups, such as the melamine functionality, to trypanotoxic compounds appears

not to impart high trypanocidal activity on all compounds, although it has been shown to work extremely well in some instances, most notably highly trypanocidal nitrofuran derivatives. The lack of significant improvement in potencies in the case of the fluoroquinolones and artemisinin derivatives may be due to their low intrinsic activity to *T. brucei*.

5. Experimental

5.1. General information

Mass spectra were recorded at a Mariner mass spectrometer or at a MicroTOF mass spectrometer from Bruker Daltonics, where ionisation was achieved in the positive and negative electrospray modes using a 1:1 mixture of acetonitrile and water plus 0.2% formic acid or a 95:5 mixture of methanol and water plus 0.2% formic acid as a mobile phase. *m/z* values are given together with the assignment of ion peaks. High resolution mass spectra were recorded by the National Mass Spectrometry Service Centre in Swansea. High resolution mass spectra were also recorded at a MicrOTOF mass spectrometer from Bruker Daltonics, University of Dundee. Accurate mass measurement was performed by peak matching.

^{1}H NMR spectra were recorded at a Bruker 300 MHz NMR spectrometer or at a Bruker 500 MHz NMR spectrometer using the applied solvent simultaneously as internal standard. Chemical shifts (δ) are given in ppm together with the multiplicity, relative frequency, assignment and the coupling constants (n*J*(H,H)/MHz) of the observed signals. Chemical shifts for AB systems are directly deduced from Mestre-C free software or Bruker Topspin programme. (For NMR values given s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, br = broad). DMSO-d_6/DCl was used in some experiments as solvent for some insoluble compounds and represents a mixture of DMSO-d_6, in which two drops of DCl were added.

For some peaks, the * after the number was used to describe the extra peak associated to tautomerism.

In some cases, the numbering and the nomenclature system used for the drawn structures in the following chapters are not referring to IUPAC nomenclature in order to aid the NMR interpretation. Numbering of artemisinin derivatives **5a**–**b** is presented according to the usual numbering of artemisinin as indicated in the text.

^{13}C NMR spectra were recorded at a Bruker 300 MHz spectrometer or at a Bruker 500 MHz spectrometer using the applied solvent simultaneously as internal standard. Chemical shifts (δ) are given in ppm. Chemical shifts for AB systems are directly deduced from Mestre-C free software or Bruker Topspin programme.

Purification by column chromatography was performed on Sorbosil C60A silica gel-40–60 μm from Merck. Flash chromatography was performed using either the Flashmaster II from Jones Chromatography or Combiflash Companion system and Presearch® propylene columns, which were prepacked with silica. Qualitative thin layer chromatography (TLC) was done on precoated aluminium sheets Silica gel 60 F254 from Merck. Compounds were detected with either nihydrin, $KMnO_4$ or 254 nm UV light.

Reverse phase prepacked silica columns (Presearch) were equilibrated before usage with Combiflash companion system. MeOH was previously run through the column with medium pressure settings followed by an increased concentration of either H_2O or CH_3CN up to the initial conditions of the running program. The column after usage was washed and stored wet with high concentration of organic solvent (H_2O or CH_3CN > 90%).

Microwave-assisted reactions were carried out using the automated Biotage Initiatior System.

Melting points were determined with a Gallenkamp melting point apparatus, and are not corrected.

Solvents and reagents were purchased from Sigma–Aldrich or Fluka, and were used without further purification. Dry solvents were purchased in sure sealed bottles stored over molecular sieves. Deuterated solvents were purchased from Goss.

5.2. 3-(4,6-Diamino-1,3,5-triazin-2-ylamino)propan-1-ol (2a)

Diaminochlorotriazine (1 g, 6.87 mmol), propanolamine (627 μL, 8.25 mmol) and $NaHCO_3$ (701 mg, 8.25 mmol) were suspended in water/MeOH (10/10 mL) and heated in the microwave for 25 min at 140 °C. The solvents were evaporated, and the crude mixture was purified by reverse phase chromatography (Elution: H_2O/MeOH, gradient 9:1 to 1:1) to obtain 3-(4,6-diamino-1,3,5-triazin-2-ylamino)propan-1-ol 2a as a white solid (1 g, 80%). LRMS (ES^+): *m/z* 185.13 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_6H_{13}N_6O)^+$, 185.1145; found, 185.1151. ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.59 (p, *J* = 6.5 Hz, 2H, CH_2–*CH_2*–CH_2), 3.23 (q, *J* = 6.5 Hz, 2H, *CH_2*–NH–), 3.42 (t, 2H, *J* = 6.5 Hz, *CH_2*–OH), 4.54 (s, 1H, *OH*), 5.98 (s, 2H, N*H_2*), 6.15 (s, 2H, N*H_2*),6.42 (t, 1H, *J* = 6 Hz, –CH_2–CH_2–*NH*–). ^{13}C NMR (500 MHz; DMSO-d_6) δ 32.6 (CH_2–*CH_2*–CH_2), 36.9 (*CH_2*–NH–), 58.4 (*CH_2*–OH), 166.3 (triazine *C*), 166.8 (triazine *C*), 167.1 (triazine *C*).

5.3. *tert*-Butyl 3-(4,6-diamino-1,3,5-triazin-2-ylamino)propylcarbamate (2b)

Diaminochlorotriazine (500 mg, 3.43 mmol), BOC-aminopropylamine (718 mg, 4.12 mmol) and $NaHCO_3$ (350 mg, 4.12 mmol) were suspended in water/MeOH (8/8 mL) and heated in the microwave for 10 min at 140 °C. The solvents were evaporated, and the crude mixture was washed with EtOH (6 × 5 mL). The filtrate was concentrated and recrystallised in dichloromethane to afford **2b** as a white sticky solid (75%). LRMS (ES^+): *m/z* 284.19 ($(M+H)^+$, 100%). ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.37 (s, 9H, –C–(*CH_3*)$_3$), 1.53 (t, *J* = 6.0 Hz, 2H, CH_2–*CH_2*–CH_2), 2.93 (q, *J* = 6.0 Hz, 2H, *CH_2*–NH–), 3.16 (q, *J* = 6.0 Hz, 2H, *CH_2*–NH–), 5.97 (s, 2H, N*H_2*), 6.11 (s, 2H, N*H_2*), 6.40 (t, 1H, *J* = 6 Hz, –*NH*–) 6.84 (s, 1H, –*NH*–). ^{13}C NMR (500 MHz; DMSO-d_6) δ 28.2 (–C–(*CH_3*)$_3$), 32.8 (CH_2–*CH_2*–CH_2), 37.1 (*CH_2*–NH–), 37.5 (*CH_2*–NH–), 77.4 (–*C*–$(CH_3)_3$) 155.6 (C═O) 166.3 (triazine *C*), 166.9 (triazine *C*), 167.2 (triazine *C*).

5.4. *N*2-(3-Aminopropyl)-1,3,5-triazine-2,4,6-triamine (2c)

Crude triazine derivative **2b** (1.1 g, 3.9 mmol) was neutralised with HCl (0.2 M) and then suspended in DCM (20 mL). TFA (1.5 mL, 20.2 mmol) was added to the mixture which caused the suspension to be solubilised. The mixture was left stirring overnight until complete reaction of starting material. The reaction mixture was stirred for 2 days at room temperature. The solvent was evaporated and the crude mixture was purified by reverse phase chromatography (Elution: H_2O/MeOH + 0.1% triethylamine, gradient 95:5 to 8:2) to obtain **2c** as a white solid (673 mg, 90%). LRMS (ES^+): *m/z* 184.13 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_6H_{14}N_7)^+$, 184.1305; found, 184.1312. ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.78 (d, *J* = 6.0 Hz, 2H, –CH_2–*CH_2*–CH_2–), 2.82 (s, 2H, –*CH_2*–NH–), 3.32 (t, *J* = 6.0 Hz, 2H, –*CH_2*–NH–), 7.80–8.20 (br s, 7H, –*NH*–, 3-*NH_2*). ^{13}C NMR (500 MHz; DMSO-d_6) δ 25.1 (*CH_2*), 36.0 (NH–*CH_2*), 36.3 (NH–*CH_2*), 113.4–120.56 (q, *CF_3*) 158.4 (*$C_{triaz.}$*), 158.7 (*$C_{triaz.}$*), 158.9 (*$C_{triaz.}$*).

5.5. BOC-DFMO

DFMO (726 mg, 4.19 mmol) was dissolved in H_2O/THF. BOC_2O (1.371 g, 6.28 mmol) and $NaHCO_3$ (1.126 g, 13.41 mmol) were

added to the solution, and the mixture was stirred overnight at room temperature. The solvents were evaporated, and the crude compound was directly purified by chromatography (Elution: CH_2Cl_2/MeOH + 0.1% Net_3, gradient 95:5 to 9:1) to obtain BOC-DFMO as a colourless oil (929 mg, 80%). LRMS (ES^+) *m/z* 283.15 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_{11}H_{21}F_2N_2O_4)^+$, 283.1464; found, 283.1464. ^{19}F NMR (500 MHz, DMSO-d_6) δ −126.0 (dd, J = 54.0 Hz, J = 272.0 Hz, 1F, CHF_2), -131.1 (dd, J = 57.0 Hz, J = 272.0 Hz, 1F, CHF_2). ^1H NMR (500 MHz, DMSO-d_6) δ 1,1 (m, 1H, –CH_2–CH_2–CH_2–NH–), 1,19, (s, 10H, –C–$(CH_3)_3$, –CH_2–CH_2–CH_2–NH–), 1.33 (m, 1H, –CH_2–CH_2–CH_2–NH–), 1.49 (m, 1H, CH_2–CH_2–CH_2–NH–), 2.67 (m, 2H, –CH_2–CH_2–CH_2–NH–), 5.93 (t, J = 55 Hz, 1H, –CHF_2), 6.64 (s, 1H, –COOH). ^{13}C NMR (500 MHz, DMSO-d_6) δ 23.8 (–CH_2–CH_2–CH_2–NH–), 28.23 (–C–$(CH_3)_3$), 30.1 (–CH_2–CH_2–CH_2–NH–), 39.9 (–CH_2–CH_2–CH_2–NH–), 63.2 (d, J = 15.5 Hz, –C–CHF_2), 77.3 (–C–$(CH_3)_3$), 117.8 (t, J = 245.0 Hz, CHF_2), 155.5 (–C(O)–O–C–(CH_3)), 168.3 (–COOH).

5.6. 2-Amino-5-(4,6-diamino-1,3,5-triazin-2-ylamino)-2-(difluoromethyl) pentanoic acid (3a)

6-Chloro-2,4-dichlorotriazine **1** (84 mg, 0.55 mmol) was mixed with DFMO (100 mg, 0.55 mmol) and $NaHCO_3$ (92.41 mg, 0.55 mmol). The mixture was dissolved in EtOH/H_2O (4 mL 1/1) and the sealed vial was placed in biotage microwave reactor at 140 °C for 12 min. The suspension became a clear solution. LCMS showed the formation of the expected product. The solvent was removed under vacuum and the solid was triturated with EtOH and then purified by column flash chromatography (EtOH/H_2O = 100:0 to 85/15). The fractions were collected together and concentrated under vacuum. The solid was dried over high vacuum to afford **5a** as a pure white solid (13 mg, 8%). LCMS (ES^+) *m/z* 292.14 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_9H_{16}F_2N_7O_2)^+$, 292.1328; found, 292.1329. ^1H NMR (500 MHz; D_2O) δ 1.20 (m, 2H, –CH_2–CH_2–CH_2–NH–), 1.58 (m, 2H, CH_2–CH_2–CH_2–NH–), 2.79 (m, 2H, –CH_2–CH_2–CH_2–NH–), 6.08 (bm, 4H, 2 NH_2), 6.40 (t, 1H, CHF_2). ^{13}C NMR (500 MHz; D_2O) δ 18.5 (–CH_2–CH_2–CH_2–NH–), 23.9 (–CH_2–CH_2–CH_2–NH–), 32.3 (–CH_2–CH_2–CH_2–NH–), 60.0 (–C–CHF_2), 119.6 (CHF_2), 166.2 ($C_{triazine}$) 167.0 ($C_{triazine.}$) 167.2 ($C_{triazine.}$).

5.7. *tert*-Butyl 4-((3-(4,6-diamino-1,3,5-triazin-2-ylamino)propoxy)carbonyl)-4-amino-5,5-difluoropentylcarbamate (3b)

BOC-DFMO (138 mg, 0.49 mmol) was dissolved in DMF (1.5 mL), then HATU (205 mg, 0.53 mmol) and DIPEA (170 μL, 0.98 mmol) were added to the solution. The mixture was stirred for 1 h at rt under Argon, and then triazine **2a** (108 mg, 0.59 mmol) was added. The reaction was stirred for 48 h, then diluted in toluene (10 mL) and concentrated under vacuum. The crude mixture was directly purified by preparative HPLC (Elution: CH_3CN/H_2O + 0.1% NH_3, gradient 5:95 to 95:5) to afford **3b** as a white pure solid (64 mg, 30%). Mp 60 ± 2 °C. LRMS (ES^+) *m/z* 449.23 ($(M+H)^+$, 100%). HRMS (ES^+) calcd mass for $(C_{17}H_{31}F_2N_8O_4)^+$, 449.2431; found, 449.2429. ^{19}F NMR (500 MHz; MeOD-d_4) δ −133.92 (dd, J = 56.0 Hz, J = 279.5 Hz, –CHF_2), -129.24 (dd, J = 56.0 Hz, J = 279.5 Hz, –CHF_2). ^1H NMR (500 MHz; MeOD-d_4) δ 1.32 (m, 1H, –C–CH_2–CH_2–CH_2–NH–), 1.44 (s, 9H, –C–$(CH_3)_3$), 1.65 (m, 2H, –C–CH_2–CH_2–CH_2–NH–), 1.88 (m, 1H, –C–CH_2–CH_2–CH_2–NH–), 1.95 (p, J = 6.5 Hz, 2H, –NH–CH_2–CH_2–CH_2–O–), 3.06 (m, 2H, –C–CH_2–CH_2–CH_2–NH–), 3.43 (t, J = 6.5 Hz, 2H, –NH–CH_2–CH_2–CH_2–O–), 4.28 (t, J = 6.5 Hz, 2H, –NH–CH_2–CH_2–CH_2–O–), 6.03 (t, J = 56.0 Hz, 1H, –CHF_2). ^{13}C NMR (500 MHz; MeOD-d_4) δ 25.0 (–C–CH_2–CH_2–CH_2–NH–), 28.8 (–C–$(CH_3)_3$), 29.8 (–NH–CH_2–CH_2–CH_2–O–), 32.5 (–C–CH_2–CH_2–CH_2–NH–), 38.3 (–NH–CH_2–CH_2–CH_2–O–), 41.2 (–C–CH_2–CH_2–CH_2–NH–), 64.7 (d, J = 20.0 Hz, –C–CHF_2), 65.0 (–NH–CH_2–CH_2–CH_2–O–), 80.0 (–C–$(CH_3)_3$), 117.9 (t, J = 246.0 Hz, CHF_2), 158.5 (–CO–O–C–$(CH_3)_3$), 167.6 ($C_{triazine}$), 172.3 (d, J = 6.0 Hz, –CH_2–O–CO–).

5.8. *tert*-Butyl 4-(3-(4,6-diamino-1,3,5-triazin-2-ylamino)propylcarbamoyl)-4-amino-5,5-difluoropentylcarbamate) (3c)

BOC-DFMO (187 mg, 0.66 mmol) was dissolved in DMF (2 mL), and then HOBt (134 mg, 0.99 mmol), TBTU (318 mg, 0.99 mmol) and DIPEA (345 μL, 1.98 mmol) were added to the solution. The mixture was stirred for 15 min at room temperature under Argon, and then triazine **2c** (157 mg, 0.86 mmol) was added. The reaction was stirred for 18 h, then diluted in toluene (20 mL) and concentrated under vacuum. The crude mixture was directly purified by preparative HPLC (Elution: CH_3CN/H_2O + 0.1% NH_3, gradient 5:95 to 95:5) to afford **3c** as a white pure solid (50 mg, 25%). LRMS (ES^+) *m/z* 448.28 ($(M+H)^+$, 100%). ^{19}F NMR (500 MHz; MeOD-d_4) δ −138.7 (dd, J = 57.0 Hz, J = 276.0 Hz, –CHF_2), −130.7 (dd, J = 56.0 Hz, J = 276.0 Hz, –CHF_2). ^1H NMR (500 MHz; MeOD-d_4) δ 1.36 (m, 1H, –C–CH_2–CH_2–CH_2–NH–), 1.47 (s, 9H, –C–$(CH_3)_3$), 1.53 (td, J = 3.0 Hz, J = 13.0 Hz, 1H, –C–CH_2–CH_2–CH_2–NH–), 1.64 (m, 2H, –NH–CH_2–CH_2–CH_2–NH–), 1.69 (m, 1H, –C–CH_2–CH_2–CH_2–NH–), 1.82 (td, J = 4.5 Hz, J = 13.0 Hz, 1H, –C–CH_2–CH_2–CH_2–NH–), 3.04 (m, 2H, –NH–CH_2–), 3.14 (m, 1H, –NH–CH_2–), 3.33 (m, 1H, –NH–CH_2–), 3.36 (m, 1H, –NH–CH_2–), 3.48 (m, 1H, –NH–CH_2–), 6.14 (t, J = 57.0 Hz, 1H, –CHF_2). ^{13}C NMR (500 MHz; MeOD-d_4) δ 25.0 (–C–CH_2–CH_2–CH_2–NH–), 28.8 (–C–$(CH_3)_3$), 31.2 (–NH–CH_2–CH_2–CH_2–NH–), 32.6 (–C–CH_2–CH_2–CH_2–NH–), 36.5 (–NH–CH_2–), 37.9 (–NH–CH_2–), 41.3 (–NH–CH_2–), 64.4 (t, J = 22.0 Hz, –C–CHF_2), 80.0 (–C–$(CH_3)_3$), 118.4 (t, J = 243.0 Hz, CHF_2), 158.5 (–CO–O–C–$(CH_3)_3$), 168.0 ($C_{triazine}$), 173.4(–CH_2–NH–CO–).

5.9. 3-(4,6-Diamino-1,3,5-triazin-2-ylamino)propyl 2,5-diamino-2-(difluoromethyl)pentanoate hydrochloride (3d)

Compound **3b** (68 mg, 0.15 mmol) was dissolved in EtOAc (0.5 mL), and HCl 1 N (0.5 mL) was added to the solution. After 2 h, the reaction mixture was concentrated and the crude was directly purified by preparative HPLC (Elution: CH_3CN/H_2O + 0.1% TFA, gradient 5:95 to 95:5) to afford **3d** as a white solid (30 mg, 60%). Mp 110±2 °C. LRMS (ES^+) *m/z* 349.20 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_{12}H_{23}F_2N_8O_2)^+$, 349.1907; found, 349.1907. ^{19}F NMR (500 MHz; MeOD-d_4) δ −132.8 (dd, J = 53.0 Hz, J = 286.0 Hz, –CHF_2), −127.5 (dd, J = 53.0 Hz, J = 286.0 Hz, –CHF_2). ^1H NMR (500 MHz; MeOD-d_4) δ 1.73 (m, 1H, –C–CH_2–CH_2–CH_2–NH_2), 1.96 (m, 1H, –C–CH_2–CH_2–CH_2–NH_2), 2.08 (m, 1H, –NH–CH_2–CH_2–CH_2–O–), 2.15 (td, J = 4.0 Hz, J = 13.5 Hz, 1H, –C–CH_2–CH_2–CH_2–NH_2), 2.29 (td, J = 4.0 Hz, J = 13.5 Hz, 1H, –C–CH_2–CH_2–CH_2–NH_2), 3.04 ((t, J = 7.5 Hz, 2H, –NH–CH_2–), 3.55 (td, J = 2.5 Hz, J = 6.5 Hz, 2H, –NH–CH_2–), 4.47 (m, 2H, –CH_2–O–), 6.63 (t, J = 52.5 Hz, 1H, –CHF_2). ^{13}C NMR (500 MHz; MeOD-d_4) δ 22.3 (–C–CH_2–CH_2–CH_2–NH_2), 29.1 (–NH–CH_2–CH_2–CH_2–O–), 29.5 (–C–CH_2–CH_2–CH_2–NH_2), 38.5 (–NH–CH_2–), 40.0 (–NH–CH_2–), 65.7 (t, J = 20.0 Hz, –C–CHF_2), 67.0 (–CH_2–O–), 158.3, 160.9, 166.6 (–CH_2–O–CO–, $C_{triazine}$), (–CHF_2 and 1$C_{triazine}$ not observed).

5.10. *N*-(3-(4,6-Diamino-1,3,5-triazin-2-ylamino)propyl)-2,5-diamino-2-(difluoromethyl)pentanamide (3e)

Compound **3c** (50 mg, 0.11 mmol) was dissolved in EtOAc (0.5 mL), and HCl 1 N (0.5 mL) was added to the solution. After 1 h, the reaction mixture was concentrated and the crude was directly purified by preparative HPLC (Elution: CH_3CN/H_2O + 0.1%NH_3, gradient 5:95 to 95:5) to afford **3e** as a white solid (40 mg, 100%). Mp 110±2 °C. LRMS (ES^+) *m/z* 348.21 ($(M+H)^+$, 100%). HRMS (ES^+) *m/z* calcd mass for $(C_{12}H_{24}F_2N_9O)^+$, 348.2066;

found, 348.2064. ^{19}F NMR (500 MHz; MeOD-d_4) δ −131.2 (dd, J = 53.0 Hz, J = 286.5 Hz, –CHF_2), −128.3 (dd, J = 53.0 Hz, J = 286.5 Hz, –CHF_2). ^{1}H NMR (500 MHz; MeOD-d_4) δ 1.68 (m, 1H, –C–CH_2–CH_2–CH_2–NH_2), 1.91 (m, 3H, –C–CH_2–CH_2–CH_2–NH_2, –NH–CH_2–CH_2–CH_2–NH–), 2.12 (td, J = 4.0 Hz, J = 13.0 Hz, 1H, –C–CH_2–CH_2–CH_2–NH_2), 2.47 (m, 1H, –C–CH_2–CH_2–CH_2–NH_2), 3.05 ((t, J = 7.5 Hz, 2H, –NH–CH_2–), 3.35 (m, 1H, –NH–CH_2–), 3.45 (m, 3H, –NH–CH_2–), 6.68 (t, J = 53.0 Hz, 1H, –CHF_2). ^{13}C NMR (500 MHz; MeOD–d_4) δ 20.9 (–C–CH_2–CH_2–CH_2–NH_2), 27.9 (–C–CH_2–CH_2–CH_2–NH_2), 28.7 (–NH–CH_2–CH_2–CH_2–NH–), 37.8 (–NH–CH_2–), 38.5 (–NH–CH_2–), 39.1 (–NH–CH_2–), 65.2 (t, J = 19.0 Hz, –C–CHF_2), 114.3 (t, J = 249.5 Hz, CHF_2), 157.0, 160.0, 160.8, 164.1 (–CH_2–NH–CO–, 3$C_{triazine}$).

5.11. 1-Cyclopropyl-6-(4-(4,6-diamino-1,3,5-triazin-2-yl)piperazin-1-yl)-7-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (4a)

Ciprofloxacin (102.0 mg, 0.30 mmol) was mixed with 6-chloro-2,4-diaminotriazine (46 mg 95%, 0.30 mmol) and $NaHCO_3$ (63.6 mg 99%, 0.75 mmol). The mixture was dissolved in 3 mL of DMF and reacted at 160 °C for 6 min in biotage microwave reactor. LCMS analysis showed the formation of the expected product. The white solid was recrystallised from H_2O/DMF (200 mL 1/1). The solvent was filtered off and the solid dried over high vacuum to afford **4a** as a white pure solid (53 mg, 40%). LCMS (ES$^+$) m/z 441.18 ((M+H)$^+$, 100%). HRMS (ES$^+$) m/z calcd mass for $(C_{20}H_{22}FN_8O_3)^+$, 441.1793; found, 441.1798. ^{1}H NMR (500 MHz; DMSO) δ 1.19 (m, 2H, –CH–CH_2), 1.33 (m, 2H, –CH–CH_2), 3.32 (t, 4H, J = 4.5 Hz, 2 –$CH_{2\text{-}piperazine}$), 3.82 (m, 1H, –CH_2–CH), 3.88 (t, 4H, J = 4.5 Hz, 2 2 –$CH_{2\text{-}piperazine}$), 6.23 (br s, 4H, 2–NH_2), 7.61 (m, 1H, CH_{Ar}), 7.93 (d, 1H, J = 13.0 Hz, CH_{Ar}), 8.67 (s, 1H, HOOC–C=CH), 15.23 (br s, 1H, –$COOH$). ^{13}C NMR (125 MHz; DMSO-d_6): δ 7.5 (2 –CH–CH_2–), 35.9 (–CH–CH_2–), 42.2 (–$CH_{2\text{-}piperazine}$) 49.6 (–$CH_{2\text{-}piperazine}$), 106.7 (CH_{Ar}), 115.7 (CH_{Ar}), 136.7 (C_{Ar}), 144.8 (C_{Ar}), 157.2 ($C_{Ar}F$), 165.3 ($C_{triazine}$), 166.3 ($C_{triazine}$), 167.2($C_{triazine}$). 176.5 (–$COOH$)

5.12. 1-Ethyl-6-(4-(4,6-diamino-1,3,5-triazin-2-yl)piperazin-1-yl)-7-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (4b)

Norfloxacin (100 mg, 0.31 mmol) was mixed with 6-chloro-2,4-diaminotriazine (40 mg, 0.31 mmol) and $NaHCO_3$ (44 mg, 0.51 mmol). The mixture was dissolved in 5 mL of EtOH/H_2O and reacted at 140 °C for 11 min in biotage microwave reactor. LCMS analysis showed the formation of the expected product. The white solid was recrystallised from H_2O (200 mL). The solvent was filtered off and the solid dried over high vacuum giving **4b** as a yellow pure solid (50 mg, 45%).

LCMS (ES$^+$) m/z 441.18 ((M+H)$^+$, 100%). HRMS (ES$^+$) m/z calcd mass for $(C_{19}H_{22}FN_8O_3)^+$, 429.1793; found, 429.1803. ^{1}H NMR (500 MHz; DMSO-d_6) δ 1.39 (t, 3H, J = 7.0 Hz, –CH_2–CH_3), 3.39 (t, 4H, J = 5.0 Hz, 2-$CH_{2piperazine}$), 3.96 (t, 4H, J = 5.0 Hz, 2 –$CH_{2piperazine}$), 4.59 (q, 1H, J = 7.0 Hz, –CH_2–CH_3) 5.45 (br s, 4H, 2-NH_2), 7.22 (m, 1H, CH_{Ar}), 7.92 (d, 1H, J = 13.0 Hz, CH_{Ar}), 8.93 (s, 1H, HOOC–C=CH), 15.23 (br s, 1H, –$COOH$). ^{13}C NMR (500 MHz; DMSO-d_6) δ 14.4 (CH_3), 35.9 (–CH_2 –CH_3), 43.0 ($CH_{2piperazine}$), 49.0 ($CH_{2piperazine}$), 106.9 (CH_{Ar}), 115.9 (CH_{Ar}), 137.1 (C_{Ar}), 145.0 (C_{Ar}), 156.4 ($C_{Ar}F$), 165.9 (–$COOH$), ($C_{triazine}$ n.o.).

5.13. 3-(4,6-Diamino-1,3,5-triazin-2-ylamino)-propyl 9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-3,7-dihydro-2*H*-[1,4]oxazino[2,3,4-*ij*]quinoline-6-carboxylate (4e)

Ofloxacin (300 mg, 0.82 mmol) and HATU (321 mg, 0.82 mmol) were mixed together and suspended in dry DMF (7 mL) at rt and with argon atmosphere. The mixture was left stirring at rt for 1.30 h followed by the addition of triazine **2a** (180 mg, 0.98 mmol). The initial pale yellow colour of the mixture did not change after the different additions. LCMS showed the formation of a new product with MW = 372, which could correspond to the methyl ester derivative of ofloxacin generated during the ionisation process. The molecular ion of the expected product was also observed with low intensity compared to that of the methyl ester derivative. The mixture was directly purified by preparative HPLC. The different fractions were collected and concentrated under vacuum. LCMS of the white product showed a high intensity peak corresponding to the expected product **4e** (41 mg, 10%).

LCMS (ES$^+$) m/z 528.23 ((M+H)$^+$, 100%). HRMS (ES$^+$) m/z calcd mass for $(C_{24}H_{31}FN_9O_4)^+$, 528.2478; found, 529.2479. ^{19}F NMR (500 MHz; MeOD-d_4) −74.3 (d, 1F, F). ^{1}H NMR (500 MHz; MeOD-d_4) δ 1.29 (d, CH_3–CH–), 1.82 (t, 2H, –NH–CH_2–CH_2–CH_2–O–), 2.59 (m, 4H, $CH_{2\text{-}piperazine}$), 2.68 (m, 3H, –N–CH_3), 3.27 (m, 1H, CH_3–CH–), 3.32–3.37 (m, 6H, 2 $CH_{2\text{-}piperazine}$, –NH–CH_2–CH_2–CH_2–O–), 4.16 (m, 2H, –NH–CH_2–CH_2–CH_2–O–), 4.29 (m, 2H, –CH_2–O–), 4.44 (br s, 1H, –NH–), 7.36 (d, 1H, CH_{Ar}), 8.42 (s, 1H, N–, =CH–N–). ^{13}C NMR (125 MHz, DMSO-d_6,) δ 17.7 (CH_3–CH–), 28.5 (–NH–CH_2–CH_2–CH_2–O–), 36.9 (–NH–CH_2–CH_2–CH_2–O–), 45.4 (–N–CH_3), 49.5 (–$CH_{2\text{-}piperazine}$), 53.7 (CH_3–CH–), 54.9 (–$CH_{2\text{-}piperazine}$), 62.1 (CH_2–O–), 68.1 (–NH–CH_2–CH_2–CH_2–O–), 103.6 (CH_{Ar}), 108.8 (C_{Ar}), 128.8 (C_{Ar}), 130.4 (C_{Ar}), 140.4 (C_{Ar}), 146.2 (=CH–N–), 154.1 C_{Ar}–F), 156.1 (–O–CO–), 164.6 ($C_{triazine}$), 166.2 ($C_{triazine}$), 171.5 (–CO–).

5.14. *N*-(3-(4,6-Diamino-1,3,5-triazin-2-ylamino)propyl)-9-fluoro-3,7-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2*H*-[1,4]oxazino[2,3,4-*ij*]quinoline-6-carboxamide (4f)

Ofloxacin (200 mg, 0.55 mmol) and HATU (221 mg, 0.58 mmol) were suspended in DMF (3 mL). The suspension was stirred for 1 h at rt under argon, and then a solution of triazine **2c** (131 mg, 0.71 mmol) in DMF (3 mL) and NEt_3 (100 μL, 1.3 equiv) was added. The reaction mixture was stirred overnight at room temperature under argon, and then the LCMS still showed the presence of ofloxacin. An extra equivalent of triazine **2c** (131 mg, 0.71 mmol) and NEt_3 (100 μL, 1.3 equiv) was added and the reaction was let to stir for 1 day at rt under argon, and was then stopped. The suspension was diluted in MeOH and filtered. The filtrate was directly purified by preparative HPLC to afford **4f** as a white pure solid (90 mg, 30%). Mp. 195 ± 2 °C. LRMS (ES$^+$) m/z 527.24 ((M+H)$^+$, 100%). HRMS (ES$^+$) m/z calcd mass for $(C_{24}H_{32}FN_{10}O_3)^+$, 527.2637; found, 527.2644. ^{19}F NMR (500 MHz; MeOD-d_4) δ −122.8 (d, J = 12.5 Hz, F). ^{1}H NMR (500 MHz; MeOD-d_4) δ 1.52 (d, J = 7.0 Hz, 3H, CH_3–CH–), 1.77 (p, J = 6 Hz, 2H, –NH–CH_2–CH_2–CH_2–NH–), 2.35 (s, 3H, –N–CH_3), 2.57, (m, 4H, $CH_{2\text{-}piperazine}$), 3.35 (m, 4H, –$CH_{2\text{-}piperazine}$), 3.44 (m, 4H, –CH_2–NH–), 4.39 (dd, J = 2.5 Hz, J = 11.5 Hz, 1H, –CH_2–O–), 4.46 (dd, J = 2.5 Hz, J = 11.5 Hz, 1H, –CH_2–O–), 4.62 (m, 1H, CH_3–CH–), 7.41 (d, J = 12.5 Hz, 1H, CH_{Ar}), 8.46 (s, 1H, =CH–N–). ^{13}C NMR (500 MHz; MeOD-d_4) δ 18.2 (CH_3–CH–), 31.7 (–NH–CH_2–CH_2–CH_2–NH–), 37.1 (–CH_2–NH–), 38.2 (–CH_2–NH–), 46.5 (–N–CH_3), 51.3 (–$CH_{2\text{-}piperazine}$), 56.5 (CH_3–CH–), 56.7 (–$CH_{2\text{-}piperazine}$), 69.5 (CH_2–O-), 105.0 (d, J = 24.0 Hz, CH_{Ar}), 111.6 (C_{Ar}), 123.4 (d, J = 9.0 Hz, C_{Ar}), 125.9 (C_{Ar}), 132.7 (d, J = 14.5 Hz, C_{Ar}-F), 141.6 (d, J = 6.5 Hz, C_{Ar}), 145.8 (=CH–N–), 156.1, 158.1, 166.8, 168.8,(–NH–CO–, 3 $C_{triazine}$), 176.2 (–CO–).

5.15. 3-(4,6-Diamino-1,3,5-triazin-2-ylamino)-propyl artesunate-19-carboxylate (5a)

Artesunate (100 mg, 0.26 mmol) was dissolved in DMF (2 mL), and then HATU (104 mg, 0.27 mmol) was added to the solution. The mixture was stirred for 1 h at rt under Argon, and then triazine **2a** (64 mg, 0.34 equiv) was added. The reaction was stirred for 48 h, then diluted in toluene (10 mL) and concentrated under vacuum. The crude mixture was directly purified by preparative HPLC to afford **5a** as a white pure solid (25 mg, 20%). Mp 95 ± 2 °C. LRMS

(ES$^+$) *m/z* 551.29 ((M+H)$^+$, 100%). HRMS (ES$^+$) *m/z* calcd mass for $(C_{25}H_{39}N_6O_8)^+$, 551.2824; found, 551.2823. ^{1}H NMR (500 MHz; MeOD-d_4) δ 0.88 (d, J = 7.0 Hz, 3H, CH_3-15), 0.98 (d, J = 6.5 Hz, 3H, CH_3-16), 1.03 (m, 1H, H-7), 1.25 (m, 1H, H-5a), 1.33–1.55 (m, 3H, H-5, H-6, H-8), 1.37 (s, 3H, CH_3-14), 1.61 (m, 1H, H-8a), 1.68–1.8 (m, 2H, H-7′, H-8′), 1.9 (p, J = 6.5 Hz, 2H, –O–CH_2–*CH_2*–CH_2–NH–), 1.85–1.95 (m, 1H, H-5′), 2.04 (m, 1H, H-4), 2.34 (m, 1H, H-4′), 2.47 (m, 1H, H-9), 2.69 (m, 2H, –*CH_2*–CO–O–), 2.75 (m, 2H, –*CH_2*–CO–O–), 3.41 (dd, J = 6.5 Hz, 2H, –O–CH_2–CH_2–*CH_2*–NH–), 4.19 (t, J = 6.5 Hz, 2H, –O–*CH_2*–CH_2–CH_2–NH–), 5.54 (s, 1H, H–12), 5.78 (d, J = 10.0 Hz, H-10). ^{13}C NMR (500 MHz; MeOD-d_4) δ 11.6 (C-15), 19.8 (C-16), 22.0 (C-8), 24.9 (C-5), 25.0 (C-14), 29.0, 29.1, 29.9 (2 *CH_2*–CO–O–, –O–CH_2–*CH_2*–CH_2–NH–), 32.2 (C-9), 34.5 (C-7), 36.4 (C-4), 37.4, 37.5 (C-6, –O–CH_2–CH_2–*CH_2*–NH–), 45.7 (C-8a), 52.1 (C-5a), 62.8 (–O–*CH_2*–CH_2–CH_2–NH–), 80.5 (C-12a), 92.0 (C-12), 92.9 (C-10), 104.8 (C-3), 166.6 (*$C_{triazine}$*), 167.0 (*$C_{triazine}$*), 167.6 (*$C_{triazine}$*), 172.1 (CH_2-*CO*-O-), 173.1 (CH_2–*CO*–O–).

5.16. *N*-(3-(4,6-Diamino-1,3,5-triazin-2-ylamino)propyl)-artesunate-19-carboxamide (5b)

Artesunate (100 mg, 0.26 mmol) was dissolved in DMF (2 mL), and then HOBt (53 mg, 0.39 mmol), TBTU (125 mg, 0.39 mmol) and DIPEA (118 μL, 0.68 mmol) were added to the solution. The mixture was stirred for 15 min at room temperature under Argon, and then triazine **2c** (57 mg, 0.31 mmol) was added. The reaction was stirred for 18 h, then diluted in toluene (20 mL) and concentrated under vacuum. The crude mixture was directly purified by preparative HPLC (Elution: CH_3CN/H_2O + 0.1% NH_3, gradient 5:95 to 95:5) to afford **5b** as a white pure solid (50 mg, 35%). Mp. 125±2 °C. LRMS (ES$^+$) *m/z* 550.35 ((M+H)$^+$, 100%). HRMS (ES$^+$) *m/z* calcd mass for $(C_{25}H_{40}N_7O_7)^+$, 550.2984; found, 550.2974. ^{1}H NMR (500 MHz; DMSO-d_6) δ 0.76 (d, J = 7.0 Hz, 3H, CH_3-15), 0.89 (d, J = 6.5 Hz, 3H, CH_3-16), 0.94 (m, 1H, H-7), 1.17 (m, 1H, H-5a), 1.23–1.38 (m, 1H, H-5), 1.29 (s, 3H, CH_3-14), 1.38–1.50 (H-6, H-8), 1.50–1.66 (m, 5H, H-8a, H-7′, H-8′, –NH–CH_2–*CH_2*–CH_2–NH–), 1.81 (m, 1H, H-5′), 2.00 (m, 1H, H-4), 2.18 (td, J = 3.5 Hz, J = 14.5 Hz, 1H, H-4′), 2.28 (m, 1H, H-9), 2.39 (t, 2H, J = 6.5 Hz, –*CH_2*–CO–O–), 2.60 (m, 2H, –*CH_2*–CO–O–), 3.05 (q, J = 6.5 Hz, 2H, –CH_2–*CH_2*–NH–), 3.17 (q, J = 6.5 Hz, 2H, –CH_2–*CH_2*–NH–), 5.56 (s, 1H, H-12), 5.65 (d, J = 10.0 Hz, H-10), 5.94 (s, 2H, -*NH_2*), 6.11 (s, 2H, –*NH_2*), 6.40 (t, J = 6.0 Hz, 1H, –*NH*–), 7.89 (t, J = 6.0 Hz, 1H, –*NH*–). ^{13}C NMR (500 MHz; DMSO-d_6) δ 11.7 (C-15), 20.1 (C-16), 21.0 (C-8), 24.2 (C-5), 25.5 (C-14), 28.9, 29.5, (2 *CH_2*–CO–O–, –NH–CH_2–*CH_2*–CH_2–NH–), 31.6 (C-9), 33.7 (C-7), 35.9 (C-4, C-6), 36.2 (–CH_2–*CH_2*–NH–), 37.2 (–CH_2–*CH_2*–NH–), 44.5 (C-8a), 51.1 (C-5a), 79.8 (C-12a), 90.5 (C-12), 91.6 (C-10), 103.5 (C-3), 166.3 (*$C_{triazine}$*), 166.9 (*$C_{triazine}$*), 167.2 (*$C_{triazine}$*), 170.4 (CH_2–*CO*–), 171.2 (CH_2–*CO*–).

5.17. Parasite assays

Three strains of *T. b. brucei* (s427), wild-type, *TbAT1* knockout and B48, were cultured to the optimum density of 1–2 × 10^6 cells mL^{-1} in HMI-9 supplemented with 10% foetal calf serum (FCS) under environmental conditions of 37 °C and 5% CO_2. Solutions of test compounds were prepared in culture media at stock concentration of 200 μM and diluted serially (1:2) across the 96-well, flat-bottomed solid white plates to give a total of 11 decreasing concentrations (100 μL well^{-1}). The last well of each series was left blank, that is, 'drug-free' (negative control). Pentamidine and diminazene were applied as the positive controls. Cells were prepared at the concentration of 4 × 10^4 cells mL^{-1} and added to each of the respective compound series (100 μL well^{-1}). Plates were incubated at 37 °C/5% CO_2 for 48 h prior to the addition of Alamar Blue solution (20 μL well^{-1}, 0.49 mM in 1X PBS, pH 7.4) followed by a further 24-h incubation. Assay endpoints were measured fluorimetrically with the fluorescence spectrometer (FluoStar, BMG LabTech, Germany) and Optima programme set at $\lambda_{excitation}$ of 530 nm and $\lambda_{emission}$ of 590 nm. Data were analysed using Prism 5.0 software to obtain IC_{50} values. Experiment was performed in duplicate and repeated thrice.

5.18. Fluorescence microscopy

T. b. brucei (s427) wild-type was cultured in HMI-9 supplemented with 10% FCS and treated with 40 μM of norfloxacin and 5 μM of **4b** (i.e., respective IC_{50} values, Table 3) in separate culture flasks for 24 h. Non-treated cells were used as negative control. Each sample was spun down and resuspended to a cell density of 1 × 10^7 cells mL^{-1}. Cellular DNA was stained with 10 μM of DAPI (4′,6-diamidino-2-phenylindole) and observed using the fluorescence microscope (Axioplan2 Imaging, Carl Zeiss, Germany) under 40× magnification.

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