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Synthesis and evaluation of hybrid drugs for a potential HIV/AIDS-malaria combination therapy

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ABSTRACT

Malaria and HIV are among the most important global health problems of our time and together are responsible for approximately 3 million deaths annually. These two diseases overlap in many regions of the world including sub-Saharan Africa, Southeast Asia and South America, leading to a higher risk of co-infection. In this study, we generated and characterized hybrid molecules to target *Plasmodium falciparum* and HIV simultaneously for a potential HIV/malaria combination therapy. Hybrid molecules were synthesized by the covalent fusion of azidothymidine (AZT) with dihydroartemisinin (DHA), a tetraoxane or a 4-aminoquinoline derivative; and the small library was tested for antiviral and antimalarial activity. Our data suggests that compound **7** is the most potent molecule in vitro, with antiplasmodial activity comparable to that of DHA (IC_{50} = 26 nM, SI >3000), a moderate activity against HIV (IC_{50} = 2.9 μM; SI >35) and not toxic to HeLa cells at concentrations used in the assay (CC_{50} >100 μM). Pharmacokinetics studies further revealed that compound **7** is metabolically unstable and is cleaved via O-dealkylation. These studies account for the lack of in vivo efficacy of compound **7** against the CQ-sensitive *Plasmodium berghei* N strain in mice, when administered orally at 20 mg/kg.

1. Introduction

Malaria caused by the human malaria parasite *Plasmodium falciparum* is responsible for close to one million deaths annually. It represents a fundamental threat to human health worldwide, and its chemotherapeutic treatment is increasingly complicated by frequent drug resistance.[1] In order to gain control of drug-resistant malaria, guidelines were released by WHO, which emphasized the use of an artemisinin-based combination therapy (ACT) involving drugs with independent targets.[1] In addition, co-infection with other infectious diseases further complicates the treatment in many regions of the world where malaria is endemic. It is therefore important to understand the effect of co-infections on patients and the consequences on treatment to design an effective drug regimen that could be used to control mixed infections.

On the other hand Acquired immunodeficiency syndrome (AIDS) is a disease of the human immune system caused by the human immunodeficiency virus (HIV). The disease is a major health problem in many parts of the world. In 2009, WHO estimated 2.0 million annual deaths due to AIDS.[2] Malaria and HIV/AIDS, taken together are two of the most devastating diseases of our time, especially in sub-Saharan Africa. Given the increasing number of people becoming infected with HIV, and the omnipresence of malaria in areas where the two diseases co-exist, the prevalence of co-infections is on the rise.

Over the past 10 years, research interest in malaria-HIV/AIDS co-infections has increased and data on the effect of these co-infections on patient health, implications for treatment and control programs are accumulating. HIV infection, through immunosuppression, affects the acquisition and persistence of immune response to malaria. The highest proportion of co-infection cases in adults are expected in areas with a high prevalence of HIV infection and a low occurrence of malaria because natural antimalarial

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immunity is not acquired.[3] It has been observed that HIV-infected people, in areas of malaria transmission, have more frequent episodes of symptomatic parasitemia and higher parasitemia than those without HIV.[4,5] Additionally, HIV infected people have an increase in viremia during episodes of malaria,[6,7] leading to a potential increase in the risk of HIV transmission. Finally, it appears that malaria and HIV co-infections may work synergistically, amplifying the prevalence and intensifying both in regions where they co-exist.

The concept of hybrid drugs, which typically involve covalent fusion of two or more existing drugs or pharmacophores to create a single molecule with multiple, but not necessarily simultaneous pharmacological targets,[8] is relatively new and has shown some success in cancer therapy.[9] In addition, promising hybrid antimalarials are currently under development.[10] A related approach is now being developed to generate anti-HIV hybrid drugs to target the virus' reverse transcriptase (RT) and integrase, the newest validated target against AIDS and retroviral infections.[11–14] The design of hybrid molecules to target HIV and *P. falciparum* simultaneously has not been reported; however, the intrinsic anti-HIV activity of some antimalarials and the antimalarial activity of HIV protease inhibitors (PIs) already reported could be exploited within this concept.[15–17]

Herein we report the design and synthesis of three groups of hybrid molecules (Fig. 1) either using the available antimalarials dihydroartemisinin (DHA) and scaffolds derived from clinically used drugs such as chloroquine (CQ) or antimalarial experimental drugs such as the tetraoxanes, in combination with the anti-HIV drug azidothymidine (AZT). The first set of hybrids was inspired by the findings of Boelaert et al.[18] who reported an additive effect on HIV when CQ is administered in combination with azidothymidine (AZT) or another nucleoside reverse transcriptase inhibitor (NRTI). Therefore, a hybrid molecule was generated by covalent linkage of CQ-based 4-aminoquinoline diamines and AZT. The second set was based on the hypothesis that covalent association of DHA with AZT may yield a single molecule with an improved in vivo half-life, and hence a better pharmacokinetic (PK) profile compared to DHA, which is known to have a less favorable PK profile. Furthermore, and in an analogous manner to artemisinin derivatives such as artemether and artesunate, the biologically unstable glycosidic bond was used as a linker in artemisinin-based hybrids and was hypothesized to release dihydroartemisinin (DHA) and AZT upon cleavage in vivo. The third set was deduced from the second set and DHA was replaced by a tetraoxane-based peroxide molecule. We evaluated the antimalarial and anti-HIV

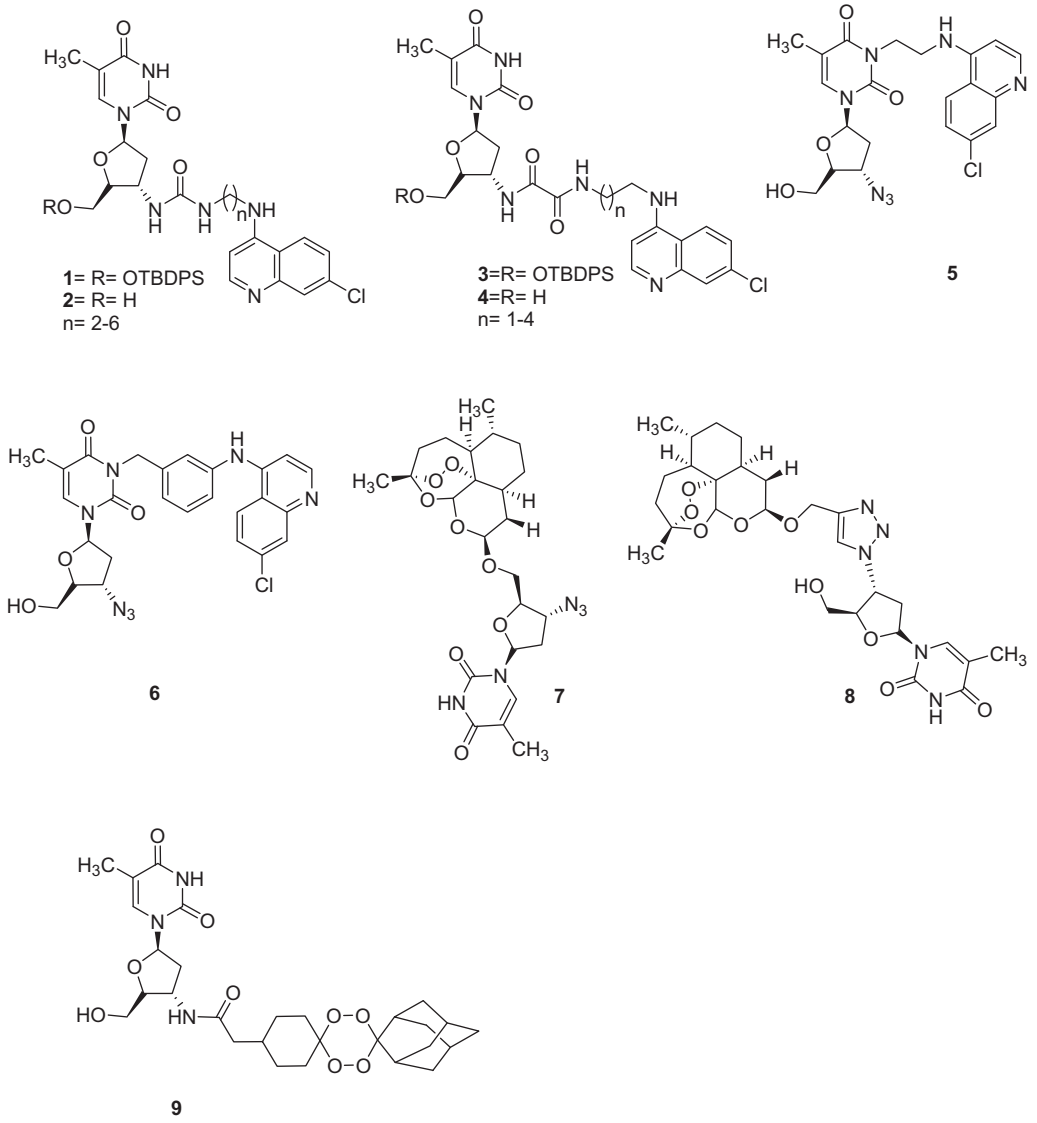


Figure 1. General structure of the target bifunctional hybrids of AZT.

activity of the hybrids and further studied the PK and metabolism properties of the most active hybrid.

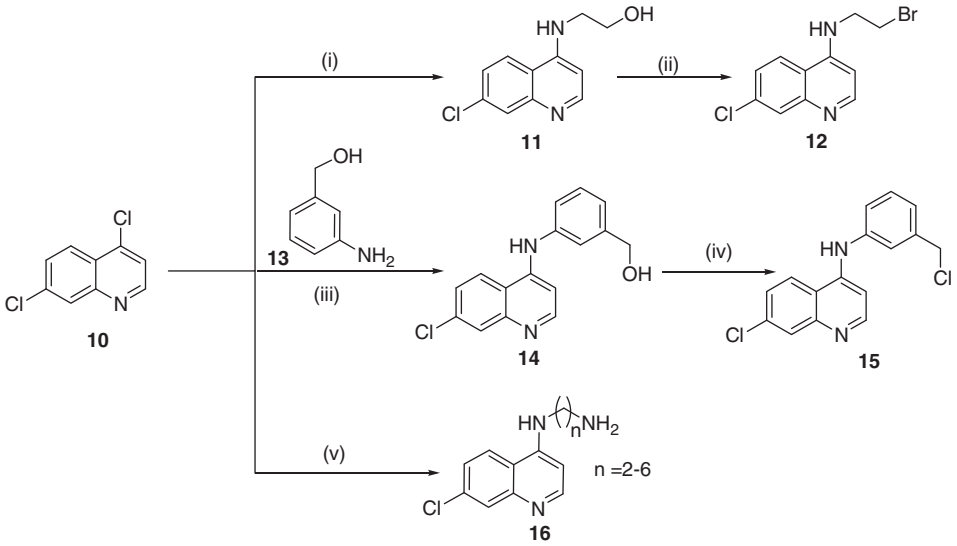
2. Synthesis

2.1. AZT–CQ hybrids

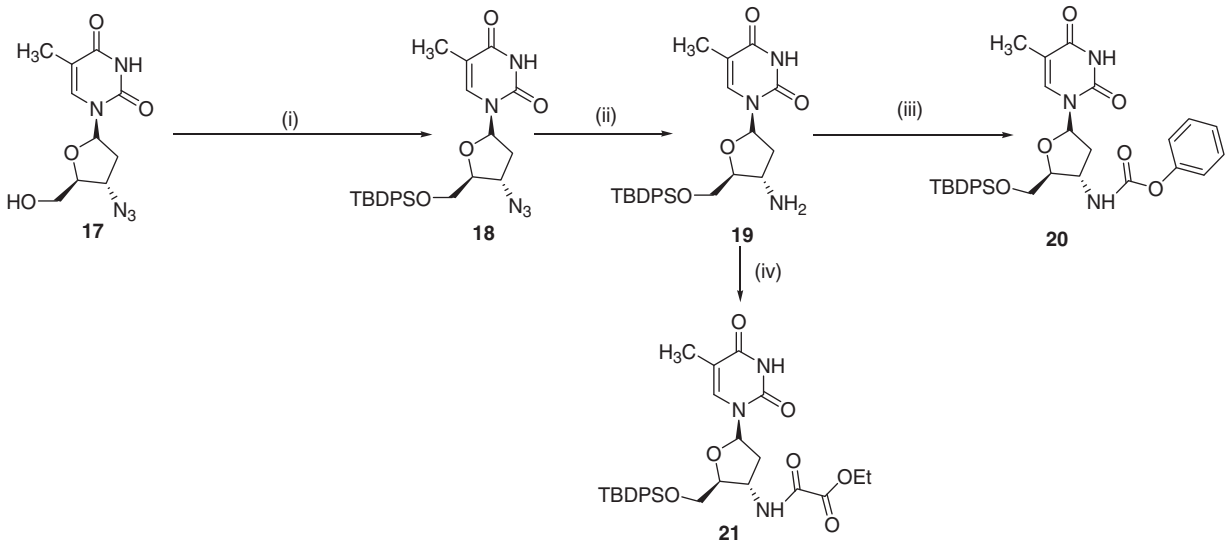
The 4,7-dichloroquinoline based intermediates required for the desired synthesis (Scheme 1) were obtained as mentioned in the reported procedures.[19,20] The intermediate **12** was prepared by an initial reaction of ethanolamine with 4,7-dichloroquinoline **10** to produce **11** which was heated at 165 °C in the presence of HBr and H_2SO_4. Similarly, the intermediate **15** was obtained by refluxing 4,7-dichloroquinoline **10** and 3-aminobenzyl alcohol **13** to yield **14**, which was further reacted with thionyl chloride at 0 °C to obtain **15**. Synthesis of a range of 4-aminoquinoline diamines **16** was realized by refluxing 4,7-dichloroquinoline and diamines.

The synthesis of AZT based bifunctional hybrids (Fig. 1) was initiated by an initial silylation of AZT **17** using *tert*-butyldiphenylsilylchloride and imidazole in dry methylene chloride followed by azide reduction using the zinc-ammonium chloride protocol to yield the corresponding *O*-silyl protected amine **19** in excellent yields.[21,22] The silylated amine **19** was then converted to carbamate **20** by reacting with phenyl chloroformate in the presence of dry pyridine in dry dichloromethane. Similarly **21** was obtained by treatment of **19** with ethyloxalyl chloride in the presence of dry dichloromethane at 0 °C. The progress of the reaction at each time was monitored by tlc and on completion, the reaction mixture was concentrated and chromatographed using a ethyl acetate: hexane solvent system to obtain silylated intermediates **20** and **21** in good yields (Scheme 2). The structures of the compounds have been assigned with the aid of spectral data.

The purified 4-aminoquinoline based intermediates were reacted with silylated azidothymidine intermediates **20** and **21** in



Scheme 1. Reagents and conditions: (i) ethanolamine, reflux, 90%; (ii) HBr/H_2SO_4, 165 °C, 74%; (iii) **13**, ethanol reflux, 78%; (iv) $SOCl_2$, dry DMF, 76%; (v) diamino alkanes, reflux, quantitative.



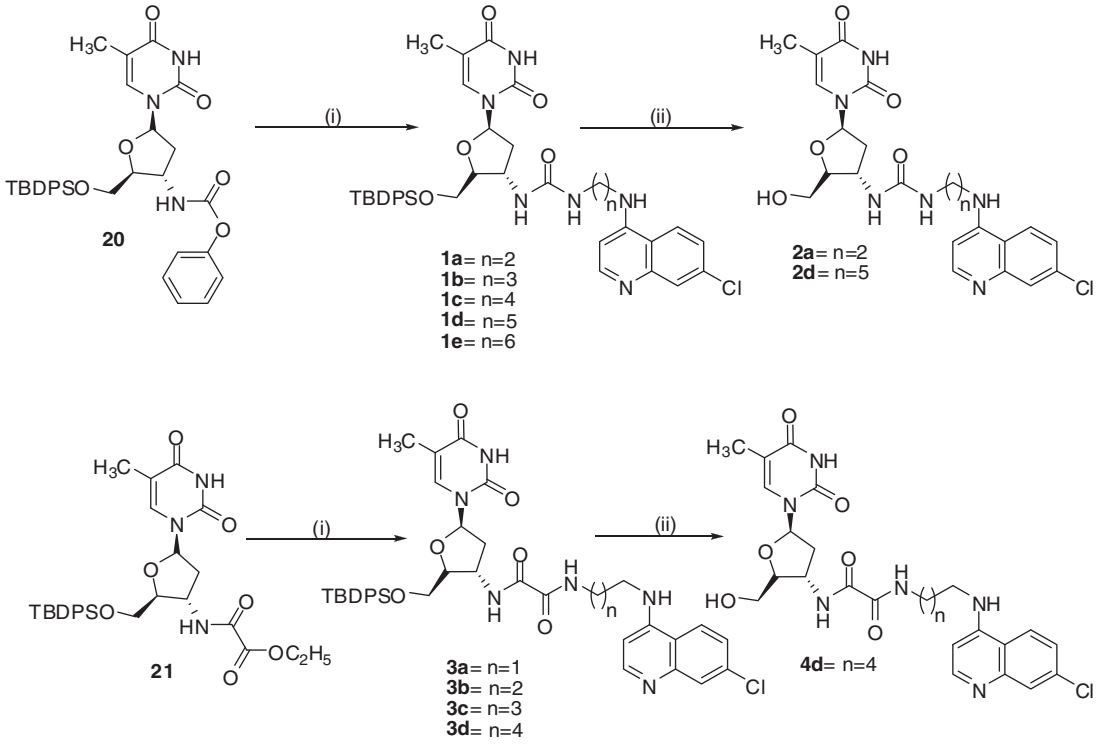
Scheme 2. Reagents and conditions: (i) *tert*-butyldiphenylsilylchloride, imidazole, dry dichloromethane, N_2 atm, 88%; (ii) zinc–ammonium chloride, C_2H_5OH/H_2O reflux, 81%; (iii) PhOCOCl, pyridine, $CHCl_3$ reflux, 82%; (iv) ethyloxalylchloride, DCM, 0 °C, 87%.

the presence of dry methanol as solvent to obtain silylated target compounds **1a–e** and **3a–d** following purification by column chromatography.[23] These silylated hybrids were then desilylated using K_2CO_3 in refluxing methanol,[23] to yield the corresponding **2a**, **2d** and **4d** as shown in Scheme 3.

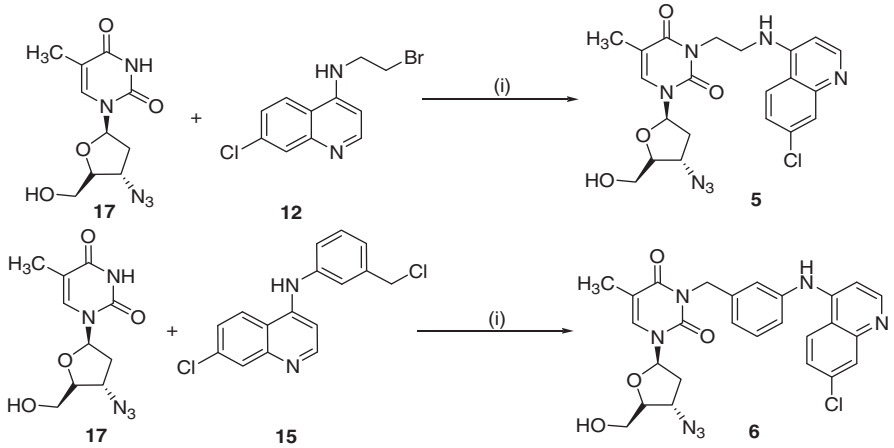
Quinoline-based hybrids **5** and **6** were obtained by substitution at the N-3 position of AZT, which was conducted in the presence of dry DMF using the synthetic sequence shown in Scheme 4.

2.2. AZT-artemisinin

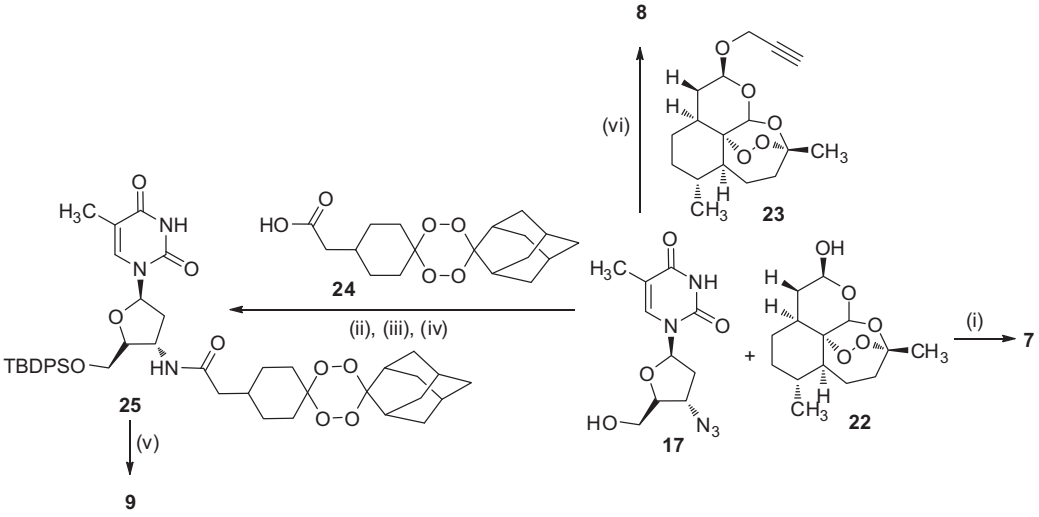
The targeted compound **7** was obtained by BF_3 etherate-mediate *O*-glycosylation of dihydroartemisinin (Scheme 5). For the synthesis of the acetylenic artemisinin intermediate **23,** a procedure reported by Li et al.[24] was used. It involved the reaction of dihydroartemisinin and propargyl alcohol in dry DCM at 0 °C and then 25 °C for 10 h in the presence of BF_3 etherate. The AZT–ART hybrid **8** was prepared using the classical 'click' conditions, and involved the reaction of the β-isomer of **23** with AZT in the presence of 5 mol % of $CuSO_4{\cdot}H_2O$ and 10 mol % of sodium ascorbate in DCM/H_2O (1:1) at 25 °C.[25] The synthesis of the AZT–tetraoxane hybrid was performed using AZT amine **19**. The amine **19** was converted to the corresponding silylated tetraoxane–AZT hybrid **25** by reaction with ethyl chloroformate in the presence of dry triethylamine and tetraoxane carboxylic acid **24** in dry dichloromethane using the protocols described by Opsenica et al. and O'Neill et al.[26,27] This was followed by desilylation using K_2CO_3/ethanol to yield **9** (Scheme 5).[23]



Scheme 3. Reagents and conditions: (i) compound **16**, dry methanol reflux; (ii) **2a**, K_2CO_3, C_2H_5OH/H_2O, 81%; (ii) **2d**, K_2CO_3, C_2H_5OH/H_2O, 89%; (ii) **4d**, K_2CO_3, C_2H_5OH/H_2O, 68%.



Scheme 4. Reagents and conditions: (i) compound **5**, dry DMF, NaH, N_2, 26%; (i) **6**, dry DMF, NaH, N_2, 80%.



Scheme 5. Reagents and conditions: (i) $BF_3{}^*O(CH_2CH_3)_2$, −10 °C, 63%; (ii) *tert*-butyldiphenylsilylchloride, imidazole, dry dichloromethane, N_2 atm, 88%; (iii) zinc–ammonium chloride, C_2H_5OH/H_2O, reflux, 81%; (iv) ethyl chloroformate, **24**, triethylamine, 0 °C, 57%; (v) K_2CO_3, C_2H_5OH/H_2O, reflux, 62%; (vi) $CuSO_4{\cdot}5H_2O$, sodium ascorbate, DCM/H_2O (1:1), 25 °C, 24 h, 45%.

3. Biological evaluation

3.1. Compound screening for cytotoxicity, antiplasmodial and anti-HIV activity

The series of hybrid molecules generated was evaluated for their inhibitory activity against pseudotyped HIV-1, *P. falciparum* 3D7 and *P. falciparum* Dd2. Target compounds and relevant intermediates were pre-screened at three concentrations (50, 5 and 1 μM) to establish their activity and cytotoxicity (Table 1). Compounds toxic to HeLa cells at 5 μM were removed from the study. Compounds with no activity against HIV and *P. falciparum* at 50 μM (i.e., with IC_{50} >50 μM for both) were not further screened since the exact IC_{50} could not be determined. Compounds with acceptable activities were further evaluated for their cytotoxicity on HeLa cells. Compounds with IC_{50} >15 μM for anti-HIV activity and IC_{50} >10 μM for antiplasmodial activity were classified as not effective in our study. The selectivity index (SI; Table 2) was also used as a cut-off parameter to prioritize the most potent and selective compounds. Therefore a compound with a SI <100 in both assays was considered not effective (NE; Table 1).

Seventeen (17) compounds exhibited strong to moderate inhibition against HIV-1 (VSV-G) and *P. falciparum* (Table 1). The integrase inhibitor raltegravir and the reverse transcriptase inhibitor AZT were used as positive controls in the HIV assay while CQ and DHA were used as positive controls in Malstat assays. Eleven (11) compounds had IC_{50} values in the nanomolar range for both strains of *P. falciparum*. Among these, compounds **3d**, **1a** and **1d** showed significant reduction in the growth of HeLa cells. Nonetheless, **3d** showed high activity against pseudotyped HIV-1 (IC_{50} = 0.9 μM; SI = 31.8) while **1a** showed no effect and **1d** exhibited moderate activity on pseudotyped HIV (IC_{50} = 7.92; SI = 4.6). Both compounds **7** and **8** were not toxic to HeLa cells at 100 μM. Interestingly, hybrid **7** was fourfold more active than **8** against pseudotyped HIV-1 which, speculatively, may be indicative of the importance of the azido group on AZT or the detrimental effect of the triazole ring system on anti-HIV activity. Additionally, compound **7** displayed antiplasmodial activity comparable to reference antimalarial drugs CQ and DHA and was considered our priority since moderate activity against HIV was also observed (IC_{50} = 2.86 μM; SI >35). For further characterization, we decided to focus only on the compound **7** with the most optimal profile.

Compounds **7** and **3d** appeared to be the most active compounds, but **3d** was discontinued due to higher cytotoxicity (CC_{50} = 28.6 μM) and weaker antiplasmodial activity compared to compound **7**. Since DHA is reported to have an additional effect on young and mature gametocytes,[28] gametocytocidal activity of compound **7** was also evaluated. It was apparent that the activity of compound **7** on developing gametocytes is moderate and comparable to that of DHA, but weaker than primaquine, a known gametocytocidal drug used as a control (Fig. 2).

Since HIV-1 (VSV-G) enters cells through the endocytic pathway and can be inhibited by compounds that block endosomal acidification,[29] the activity of compound **7** was further evaluated on a full replicative wild type NL43 HIV-1. Compound **7** was 72× and 600× less potent than AZT against pseudotyped viruses and wild type NL43 HIV-1, respectively (Table 3 and Fig. 3). Viral infection was measured in the presence of drugs tested at six different concentrations and compared to AZT, non-infected TZM cells and non treated-infected TZM cells were used as 100% activity and 0% activity controls, respectively.

3.2. In vivo antimalarial efficacy studies

Hybrid **7** was evaluated in vivo using the mouse model described by Peters.[30] *Plasmodium berghei N* strain infected mice were treated for 4 days and the parasitemia was monitored on days 2, 4 and 7 after the beginning of the treatment. The parasitemia (%) was calculated for each mouse in different groups and the mean parasitemia and standard deviations calculated (Table 4).

Treatment with compound **7** (in pheroid formulation) did not reduce parasitemia at the administered concentration 7 days after the beginning of the treatment. These data suggest that the compound **7** does not suppress parasite growth in vivo after oral administration. Also, compound **7** appeared not to be toxic to mice as mice in the compound **7** treated group became distressed at the same time as mice from the untreated control. For a drug to be effective, enough of the active form must reach the target to elicit the desired effect. We therefore sought to understand why the molecule was not active in vivo after formulation. Drug metabolism and pharmacokinetic studies were thus carried out.

Table 1
Estimation of IC_{50} (concentration resulting in 50% inhibition of cell growth or viral replication) obtained after inhibition of *P. falciparum* sensitive (3D7) strain, resistant (Dd2) strain, pseudotyped HIV-1 and HeLa cells with serial dilution of hybrid molecules. IC_{50}s and standard deviations were calculated using the software GrapPad prism.

Compounds	IC_{50} (μM)			
	P. falciparum 3D7 IC_{50} ± SEM	*P. falciparum* Dd2 IC_{50} ± SEM	HIV-1 (VSV-G) IC_{50} ± SEM	HeLa cells IC_{50} ± SEM
1a, ***n* = 2**	0.37 ± 0.15	0.34 ± 0.20	NE	23.78 ± 1.70
1d, ***n* = 5**	0.58 ± 0.17	0.08 ± 0.02	7.92 ± 2.34	36.13 ± 1.43
2a, ***n* = 2**	3.53 ± 0.56	31.60 ± 14.71	NE	>100
2d, ***n* = 5**	5.18 ± 0.42	8.02 ± 2.43	NE	>100
3d, ***n* = 4**	0.38 ± 0.22	0.08 ± 0.02	0.90 ± 0.11	28.65 ± 5.09
4d, *n* = 4	1.94 ± 0.49	10.57 ± 4.95	NE	>100
5	0.46 ± 0.01	0.65 ± 0.14	7.15 ± 2.57	>100
6	0.16 ± 0.06	0.22 ± 0.06	1.76 ± 0.46	>100
7	0.03 ± 0.01	0.01 ± 0.00	2.86 ± 0.39	>100
8	0.27 ± 0.06	0.10 ± 0.03	11.10 ± 0.46	>100
9	0.22 ± 0.17	0.10 ± 0.08	NE	23.38 ± 4.97
11	2.20 ± 0.14	1.72 ± 0.04	NE	>100
14	1.42 ± 0.28	1.46 ± 0.30	NE	>100
16, *n* = 2	0.03 ± 0.02	0.23 ± 0.04	NE	63.44 ± 6.28
16, *n* = 5	0.13 ± 0.10	0.23 ± 0.12	NE	56.64 ± 17.88
21	11.12 ± 2.77	12.10 ± 1.43	NE	63.97 ± 11.07
25	0.27 ± 0.09	0.19 ± 0.08	NE	>100
AZT	NE	—	0.04 ± 0.02	>100
DHA	0.03 ± 0.01	0.01 ± 0.01	NE	>100
Tetraoxane	0.32 ± 0.28	0.12 ± 0.03	NE	>100
CQ	0.03 ± 0.01	0.71 ± 1.19	12.48 ± 2.45	>100
Raltegravir	NE	—	0.01 ± 0.00	>100

Table 2
Selectivity index values (SI = $^{*}CC_{50}/IC_{50}$) of active hybrid molecules

Compounds	Selectivity index (CC_{50}/IC_{50})		
	P. falciparum 3D7	*P. falciparum* Dd2	Pseudo HIV-1
1a, *n* = 2	64.3	69.9	ND
1d, *n* = 5	62.3	451.6	4.6
2a, *n* = 2	>28.3	>3.2	ND
2d, *n* = 5	>19.3	>12.5	ND
3d, *n* = 4	75.4	358.1	31.8
4d, *n* = 4	>51.5	>9.5	ND
5	>217.4	>153.8	>14
6	>625	>454.5	>56.8
7	>3333.3	>10,000	>35
8	>370.4	>1000	>9
9	106.3	233.8	ND
11	>45.5	>58.1	ND
14	>70.4	>68.5	ND
16, *n* = 2	2114.7	275.8	ND
16, *n* = 5	>435.7	>246.3	ND
21	5.75	5.3	ND
25	>370.4	>526.3	ND
AZT	ND	—	>2500
Dihydroartemisinin	>3333.3	10,000	ND
Tetraoxane	>312.5	>833.3	ND
CQ	>3333.3	140.8	>8
Raltegravir	ND	—	>10,000

* $CC_{50} = IC_{50}$ on HeLa cells: cytotoxic concentration 50, which is concentration resulting in 50% inhibition of HeLa cells growth. ND: Not determined.

3.3. Evaluation of the metabolic stability and PK properties of compound 7 in vitro and in vivo

Compound **7** was administered to mice in two different formulations: the first group of mice received compound **7** dissolved in a mixture of DMSO and distilled water (1:9, v/v) and the second group received compound **7** formulated into Pheroid™ vesicles. We found no trace of the compound **7** in the plasma from the group

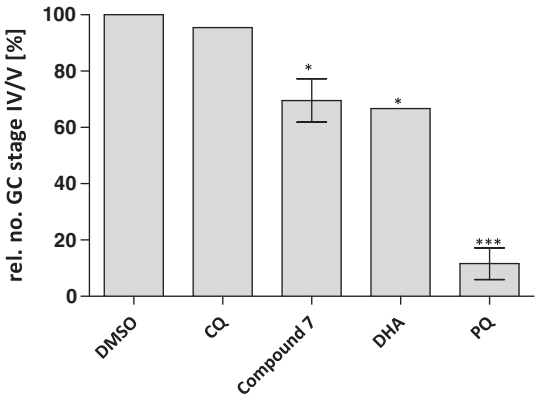


Figure 2. Inhibition of gametocytes maturation. Compounds at IC_{50}s or a 0.5% volume of DMSO were added to stage II gametocyte cultures for 2 days. The numbers of stage IV and V gametocytes were counted after 7 days and correlated to the gametocytemia of the DMSO control (normalized to 100%). The graph represents results of two independent experiments in triplicate for PQ, compound **7**, CQ (mean ± SEM) and a single experiment for DHA (mean ± SD). Statistical analysis was performed using one way ANOVA followed by a *Tukey* test and mean gametocytemia of compounds tested once were compare with mean gametocytemia in DMSO control using t-test (GraphPad Prism 5). Asterisks represent a significant difference between tested compounds and DMSO control (annex), where ***correspond to $P < 0.001$; **correspond to $0.001 < P < 0.01$; *correspond to $0.01 < P < 0.05$ and for $p > 0.05$ the difference in not considered significant and there is no asterisk.

Table 3
IC_{50}s values of compound **7** obtained from the two HIV in vitro assays

	IC_{50} (nM)	
	HIV-1 (VSV-G)	Wild type (NL43)
AZT	40 ± 20	0.8 ± 0.7
7	2860 ± 390	485.5 ± 16.5

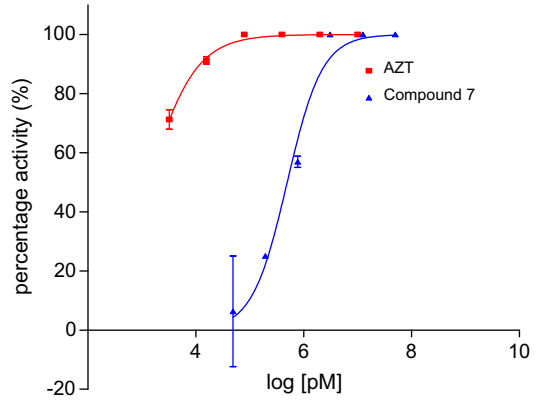


Figure 3. Inhibition of wild type HIV replication. NL43 virus co-culture was maintained in the presence of different concentrations of AZT and compound **7** for 48 h and the supernatant was used to infect TZM cells to quantify the amount of particles formed in the presence of inhibitors. The mean count of wells with 'no drug and no virus' was normalized to 100% activity and the mean count of wells 'no drug and with virus' was normalized to 0% activity. Percentage activities were deducted for treated wells.

of mice treated with compound **7** dissolved in 10% DMSO (Fig. 4A), but after being formulated into Pheroid™ vesicles, a maximal concentration of approximately 40 ng/ml was measured in mice plasma (Fig. 4B). This suggests that absorption of the compound **7** is enhanced by Pheroid™ formulation, but the peak-like shape indicate a rapid clearance of the parent drug (Fig. 4B). The 2 drugs were

quantified in the same plasma samples collected from two groups of mice; AZT was detected at a lower concentration when compound **7** was diluted in 10% DMSO (Fig. 5A) and in a much higher concentration when administered in pheroids (Fig. 5B). DHA was not detected after administration of the compound **7** when dissolved in 10% DMSO or pheroids formulation (Fig. 5C,D). AZT was present in the blood of both groups of mice but not DHA (Fig. 5).

Compound **7**, like other artemisinin derivatives appeared to have poor bioavailability properties. Despite the apparent improved bioavailability observed when formulated in Pheroid™ vesicles, the concentration of **7** in mice plasma remained low to suppress parasite growth in infected mice. The maximal concentration in the blood was estimated to be 40 ng/ml, which is the equivalent of 75 nM; the IC_{50} in vitro was 40 nM, which theoretically is enough to eliminate approximately 50% of the parasites. However, compound **7** did not reduce parasitemia in vivo as mice in the group treated with this compound showed the same parasitemia levels as the untreated group. Data from in vitro metabolism studies clearly showed that compound **7** was a very unstable drug and potentially cleaved by CYP 450 enzymes. In vitro and in vivo studies correlated well and showed that compound **7** releases its precursor drug AZT but not DHA. While AZT was detectable in the mice plasma (in vivo) or in the buffer (in vitro), DHA was not detected in the same plasma or buffer. In vitro studies showed that the presence of AZT in buffer was detected after 15 min incubation while more than 90% of compound **7** was already metabolized (Fig. 6). This further supports the rapid clearance already observed with in vivo studies.

Table 4
In vivo activity of the compound **7** against the erythrocytic forms of *P. berghei N*

Compounds	Dose (mg/kg per day)	Parasitemia day 2 (%)	Parasitemia day 4 (%)	Parasitemia day 7 (%)
7	20	4.24 ± 0.6	11.43 ± 2.6	35.2 ± 3.6
Chloroquine	10	<1%	<1%	<1%
Untreated control	0	3.2 ± 1.3	9.9 ± 1.8	33.1 ± 3.4

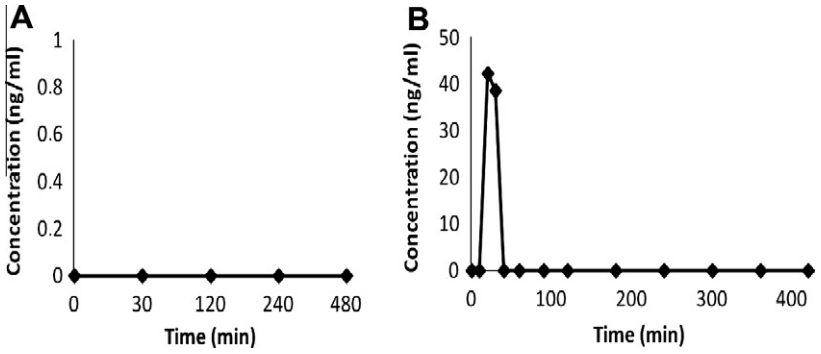


Figure 4. Plasma concentration–time profiles of the compound **7**. (A) Plasma concentration of the compound **7** in mice treated by oral administration of 20 mg/kg dissolve in 10% DMSO; (B) plasma concentration in mice treated by oral administration of 20 mg/kg dissolve in pheroids formulation.

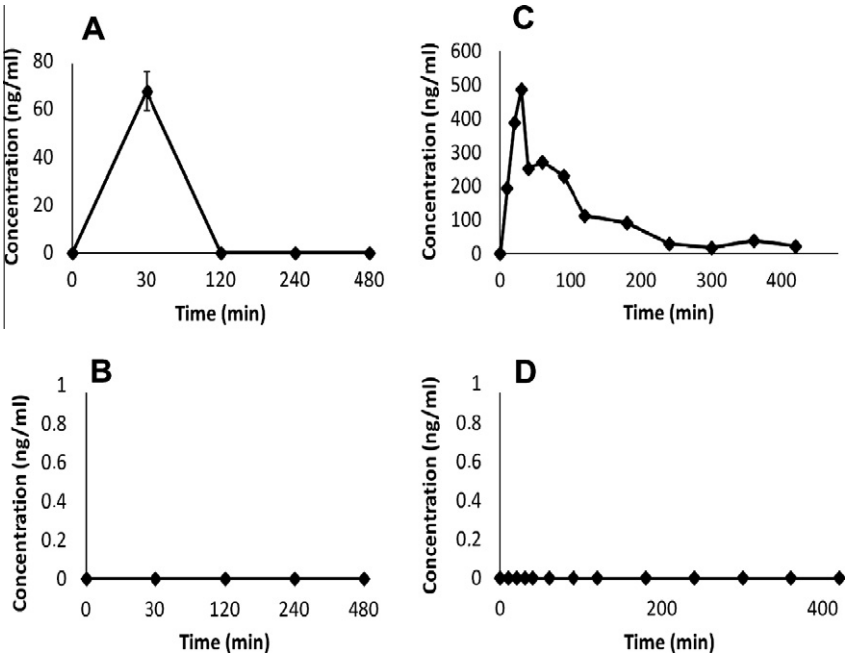


Figure 5. Plasma concentration–time profiles of AZT and DHA. (A) Plasma concentration of AZT in mice treated by oral administration of 20 mg/kg dissolve in 10% DMSO; (B) plasma concentration of DHA in mice treated by oral administration of 20 mg/kg dissolve in 10% DMSO; (C) plasma concentration of AZT in mice treated by oral administration of 20 mg/kg dissolve in Pheroids) and (D) plasma concentration of DHA in mice treated by oral administration of 20 mg/kg dissolve in Pheroids.

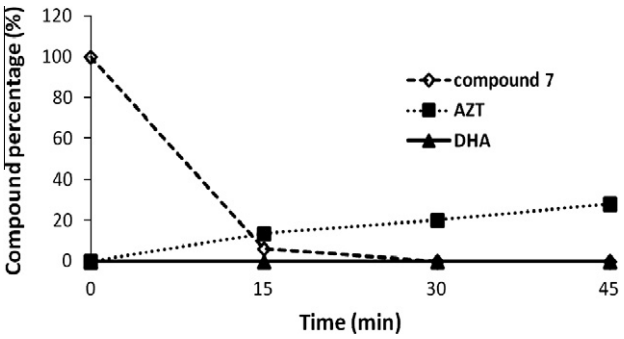


Figure 6. Metabolism of compound 7 in mice liver microsomes.

4. Discussion and conclusion

The interaction between HIV and malaria infection is bidirectional and synergistic,[31] and currently there are no specific recommended treatment regimens for malaria and HIV/AIDS co-infection. In this study, we investigated hybrid molecules for their ability to target both *P. falciparum* and HIV. We anticipated that these molecules could be used to treat malaria infected individuals while offering protection against HIV. This strategy could be particularly beneficial for intermittent preventive treatment in HIV infected pregnant women. Hybrid molecules synthesized in this study showed strong to moderate potency in vitro against both, HIV and *P. falciparum*, but most were not investigated further because of their cytotoxicity. Compound **7**, the most potent hybrid in our in vitro assays has poor aqueous solubility and displayed poor bioavailability in vivo. However, our preliminary data are encouraging as the antiplasmodial activity of compound **7** was comparable to that of DHA in vitro. DHA is practically insoluble in water just like compound **7** and because of its poor aqueous solubility, DHA has only been formulated as oral and rectal tablet preparations.[32] A combination of DHA with piperaquine is currently an option for the treatment of uncomplicated malaria. These data suggest that compound **7** could be developed further and requires appropriate formulation for its delivery. If successfully developed as a drug, compound **7** could have an advantage over DHA, since it might confer some additional protection against HIV.

Compound **7** did not suppress parasite growth in vivo when administered orally in pheroid vesicles. Taking into account that compound **7** was administered in pheroid vesicles to improve absorption, moderate antimalarial activity was at least expected. Since unacceptable PK properties is one of the main reasons for clinical failure of drug candidates,[33] it was therefore necessary to evaluate other factors contributing to the poor PK profile of compound **7**, and to better understand the poor in vivo activity.

Our attempt to study PK properties was to use the data as a guide in determining whether or not compound **7** was likely to be improved. The hybridization of AZT and DHA obviously leads to the generation of a higher molecular weight (MW) compound which might explain the predicted poor solubility of compound **7**, as the trend towards increased MW is likely to worsen both aqueous solubility and intestinal permeability.[34] The reduction in MW is a useful approach in drug development for increasing solubility and might also improve metabolic stability. In our current context we envisaged that the two moieties would remain linked and thus preclude the need to reduce the MW. Compound **7** displayed poor solubility and prompted us to dissolve in DMSO for in vitro assays and to prepare a formulation for in vivo studies since the latter can be used to increase the absorption of the molecule. However, the observed poor solubility is not surprising, considering the point of attachment of AZT to DHA through the primary hydroxyl group of AZT, resulting in compound **7** not having a free hydroxyl group, which would contribute to improved solubility.

Despite the apparent improved bioavailability observed when formulated in pheroid vesicles, the concentration of compound **7** in mice plasma remained low to suppress parasite growth in infected mice and rapid clearance was observed. This suggested poor stability and a short half life in vivo. The O-dealkylation of the oxygen linking AZT and DHA in compound **7** appeared to be the main reason for the poor metabolic stability of compound **7**. In vitro and in vivo studies correlated well and showed that compound **7** releases AZT, but not DHA.

DHA is reported to be metabolized extensively to hydroxylated derivatives by rat liver microsomes.[35] However, a study carried out in vivo and in vitro using human microsome preparations revealed that DHA is mainly glucuronidated.[36] AZT is also known to be metabolized by glucuronide conjugation to a major inactive metabolite, 3-azido-3-deoxy-5-O-beta-D-glucopyranuronosylthymidine.[37] Compound **7** was synthesized by linking AZT and DHA at their respective glucuronidation sites,[37,38] on this basis, Compound **7** cannot be conjugated via glucuronidation unless it is first hydroxylated. The DHA moiety released as the result of a cleavage product might have undergone some hydroxylations prior to cleavage, and could not be detected in mouse plasma in vivo or in the buffer in vitro, as the method was strictly designed to detect DHA and not hydroxylated products.

We have demonstrated the use of hybrid molecules to inhibit two non-related organisms and our data showed that appropriately designed hybrid molecules could have applications in the treatment of HIV/malaria co-infections. It could be advantageous to exploit compounds targeting aspartic proteases as recent studies have shown that clinically approved HIV Protease Inhibitors (PIs) were able to inhibit malaria parasite growth and to augment the antimalarial action of artemisinin in vitro.

5. Experimental data

Chemicals and reagents were purchased from either Sigma–Aldrich or Merck, South Africa. Chromatography solvents were purchased from Kimix Chemicals or Protea Chemicals, South Africa, Chemically Pure (CP grade) solvents and distilled before use.

5.1. Procedures of hybrid molecules synthesis

5.1.1. Procedure for the synthesis of phenyl 2-((*tert*-butyldiphenylsilyloxy) methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-ylcarbamate 20

To a well stirred solution of **19** (200 mg, 0.417 mmol, 1 equiv) in dry DCM was added (36.2 mg, 0.458 mmol, 1.1 equiv) of dry pyridine. The resulting solution was stirred for 5 min followed by the addition of (72.0 mg, 0.458 mmol,1.1 equiv) of phenyl chloroformate. The resulting reaction mixture was then refluxed for 16 h and the progress of the reaction was monitored by TLC. On completion, the reaction mixture was diluted with DCM (200 ml) and washed with 1 M sodium bicarbonate solution, water and brine in succession. The organic layer was dried over anhydrous magnesium sulphate and the solvent was removed under reduced pressure. The concentrated reaction mixture was subjected to further reaction without purification. δ_H (400 MHz, $CDCl_3$) 9.01 (s, 1H, NH, exchangeable with D_2O), 8.59 (d, 1H, *J* 8.09, NH, exchangeable with D_2O), 7.46 (m, 11H, ArH), 7.14 (m, 5H, ArH), 6.57 (m, 1H), 4.93 (m, 1H), 4.01 (m, 3H), 2.37 (m, 2H), 1.50 (d, 3H, *J* 1.12), 1.08 (s, 9H); δ_C (100.6 MHz) 163.4, 161.0, 156.6, 151.5, 135.8, 135.2, 132.8, 133.6, 133.5, 130.5, 130.3, 128.9, 128, 127.9, 125.3, 121.4, 112.8, 86.5, 84.6, 64.1, 50.1, 37.5, 27.1, 13.7, 12.1; Mass 599.34. Elemental analysis for; $C_{33}H_{37}N_3O_6Si$ Calculated: C, 66.09; H, 6.22; N, 7.01; Found: C, 65.95; H, 6.19; N, 7.10.

5.1.2. Procedure for the synthesis of ethyl-2-(2-((*tert*-butyldiphenylsilyloxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-ylamino)-2-oxoacetate 21

To the well stirred solution of **19** (200 mg, 0.417 mmol, 1 equiv) in dry DCM, was added (62.8 mg, 0.459 mmol, 1.1 equiv) of ethyl 2-chloro-2-oxoacetate. The reaction mixture was stirred at room temperature for 6 h. Upon completion, as evidenced by TLC, a saturated solution of sodium bicarbonate was added and the solution was stirred vigorously until it turned slightly alkaline in nature. The reaction mixture was then extracted with DCM and the organic layer was dried over anhydrous magnesium sulphate. The solvent was removed under reduced pressure resulting in the isolation of crystalline white solid. The structure was assigned on the basis of spectral data and analytical evidences. δ_H (400 MHz, $CDCl_3$) 8.98 (s, 1H, NH, exchangeable with D_2O), 8.57 (d, 1H, *J* 8.09, NH, exchangeable with D_2O), 7.65 (m, 4H), 6.54 (m, 1H), 7.39 (m, 7H), 4.88 (m, 1H), 4.38 (q, 2H, *J* 7.15), 3.98 (m, 3H), 2.35 (m, 2H), 1.51 (d, 3H, *J* 1.12 Hz), 1.39(t, 3H, *J* 7.14), 1.09(s, 9H); δ_C (100.6 MHz) 163.2, 161.2, 156.6, 151.4, 135.6, 135.2, 133.4, 133.3, 132.3, 130.1, 130, 128, 127.9, 112.3, 86.2, 84.4, 64.5, 63.9, 50.3, 37.8, 27, 19.5, 13.9, 11.9. Mass 579.25. Elemental analysis for $C_{30}H_{37}N_3O_7Si$ Calculated: C, 62.15; H, 6.43; N, 7.25. Found: C, 62.05; H, 6.32; N, 7.19.

5.1.3. 1-(2-((*tert*-Butyldiphenylsilyloxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)tetrahydrofuran-3-yl)-3-(2-(7-chloroquinolin-4-ylamino)ethyl)urea 1a

To a solution of **20** (200 mg, 0.333 mmol, 1 equiv) was added (81.4 mg, 0.367 mmol, 1.1 equiv) of 4,7-dichloroquinoline-based amines in anhydrous methanol (MeOH). The resulting reaction mixture was refluxed for 12 h. The progress of the reaction was monitored by TLC. The solvent was removed under reduced pressure on the completion of the reaction and the residue was column chromatographed using 10% MeOH/DCM mixture, resulting in the isolation of the corresponding amides in good yields. The structure of the amides was assigned on the basis of spectral data and analytical evidences. δ_H (300 MHz, DMSO-d_6) 11.28 (br s, 1H, NH, exchangeable with D_2O), 8.40 (d, 1H, *J* 5.54), 8.19 (d, 1H, *J* 9.11), 7.78 (d, 1H, *J* 2.21), 7.62–7.67 (m, 5H), 7.36–7.44 (m, 7H), 6.62 (br s, 1H, NH, exchangeable with D_2O), 6.59 (br s, 1H, NH, exchangeable with D_2O), 6.57 (d, 1H, *J* 5.62), 6.23 (t, 1H, *J* 6.65, NH, exchangeable with D_2O), 6.15 (m, 1H), 4.4 (m, 1H), 3.86 (m, 3H), 3.23 (m, 4H), 2.21 (m, 2H), 1.53(d, 3H, *J* 0.82), 1.0(s, 9H); δ_C (75.4 MHz- DMSO-d_6) 163.5, 161.2, 158.1, 151, 150.6, 150.2, 135.4, 135.0, 134.8, 132.5, 129.8, 127.7, 126.7, 124.2, 123.8, 109.5, 117.1, 98.5, 84.6, 83.2, 63.9, 49.5, 43.6, 38.0, 37.3, 26.6, 18.8, 11.7; Mass calculated: 727.32, Found: 727.41; Elemantal analysis for; $C_{38}H_{43}ClN_6O_5Si$ Calculated: C, 62.75; H, 5.96; N, 11.5. Found: C, 59.85; H, 6.19; N, 11.1.

5.1.4. 1-(2-((*tert*-Butyldiphenylsilyloxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(*2H*)-yl)tetrahydrofuran-3-yl)-3-(5-(7-chloroquinolin-4-ylamino)pentyl)urea 1d

δ_H (400 MHz, DMSO-d_6) 11.25 (s, 1H, NH, exchangeable with D_2O), 8.33 (d, 1H, *J* 5.45), 8.21 (dd, 1H, *J* 5.23, *J′* 9.05), 7.74 (d, 1H, *J* 2.14); 7.60 (m, 5H), 7.38 (m, 7H), 6.43 (t, 1H, *J* 5.21), 6.24 (d, 1H, *J* 7.51, NH, exchangeable with D_2O), 6.15 (t, 1H, *J* 6.44, NH, exchangeable with D_2O), 5.82 (t, 1H, *J* 5.75, NH, exchangeable with D_2O), 4.32 (m, 1H), 3.82 (m, 3H), 3.29 (m, 2H), 3.22 (m, 2H), 2.18–2.27 (m, 2H), 1.65 (m, 4H), 1.47 (s, 3H), 1.37 (m, 2H), 0.96 (s, 9H,); δ_C (75.4 MHz) 163.5, 157.4, 151.7, 150.2, 150.0, 148.9, 135.4, 134.9, 134.8, 132.5, 129.7, 127.7, 127.3, 123.9, 123.8, 117.3, 109.5, 98.5, 84.6, 83.2, 63.9, 49.4, 42.3, 37.4, 29.7, 27.5, 26.5, 24.2, 23.9, 18.7, 11.7; Mass calculated: 769.40 Observed: 769.45; Elemantal analysis for; $C_{41}H_{49}ClN_6O_5Si$ Calculated: C, 64.00; H, 6.69; N, 10.92. Found: C, 62.34; H, 6.44; N, 11.11.

5.1.5. N1-(2-((*tert*-butyldiphenylsilyloxy)methyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl)-N2-(5-(7-chloroquinolin-4-ylamino)pentyl)oxalamide 3d

To a solution of **21** (200 mg, 0.345 mmol, 1 equiv) was added of (94.9 mg, 0.380 mmol, 1.1 equiv) 4,7-dicholoquniloine-based amines in anhydrous MeOH. The resulting reaction mixture was then refluxed for 12 h. The progress of the reaction was monitored by TLC. On the completion of the reaction, the solvent was removed under reduced pressure to yield the corresponding keto-amides in good to excellent yields. δ_H (400 MHz, DMSO-d_6) 11.30 (br s, 1H, NH, exchangeable with D_2O), 9.14 (d, 1H, *J* 8.67, NH, exchangeable with D_2O), 8.77 (t, 1H, *J* 6.23, NH, exchangeable with D_2O), 8.37 (d, 1H, *J* 5.34), 8.26 (dd, 1H, *J* 3.02, 9.09), 7.76 (d, 1H, *J* 2.22), 7.60–7.62 (m, 4H), 7.36–7.45 (m, 8H), 6.45 (d, 1H, *J* 5.50), 6.25 (t, 1H, *J* 6.72), 4.62 (m, 1H), 3.99 (m, 1H), 3.81 (m, 2H), 3.23 (m, 2H), 3.13 (m, 2H), 2.30 (m, 2H), 1.69 (m, 2H), 1.61 (m, 2H), 1.53 (s, 3H), 1.38 (m, 2H), 0.98 (s, 9H); δ_C (100.6 MHz, DMSO-d_6) 164.5 156.7, 151.9, 151.2, 150.9, 148.8, 136.7, 135.6, 135.5, 133.5, 133.1, 130.6, 128.5, 127.1, 125.0, 124.6, 115.7, 110.5, 99.2, 84.2, 83.6, 63.8, 48.8, 42.8, 36.8, 28.9, 27.9, 27.1, 24.4, 21.0, 19.4, 12.3; Mass calculated 797.41. Found: 797.41; Elemental analysis for; $C_{42}H_{49}ClN_6O_6$ Si Calculated: C, 63.26; H, 6.19; N, 10.54. Found: C, 60.17; H, 6.25; N, 9.98.

5.1.6. 1-[2-(7-Chloro-quinolin-4-ylamino)-ethyl]-3-[2-hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-2pyrimidin-1-yl)-tetrahydro-furan-3-yl]-urea 2a

To a mixture of **1a** (318 mg, 0.415 mmol, 1 equiv) in ethanol (2 ml) and water (0.5 ml) was added potassium carbonate (1.38 g, 10 mmol, 24 equiv). The reaction mixture was refluxed at 90 °C for 3 days. The completion of the reaction mixture was checked using TLC with anisaldehyde spray in 10% MeOH/DCM solvent system. The reaction mixture was concentrated and purification was done using column chromatography with 15% MeOH: DCM solvent system. white solid; mp 178 °C; δ_H (400 MHz, CD_3OD) 8.36 (1H, d, *J* 5.6), 8.0(1H, *J* 9.0, d), 7.86 (1H, q, *J* 1.2), 7.8 (1H, d, *J* 2.1), 7.41(dd, 1H, *J* 2.1, *J′* 9.0), 6.58 (d, 1H, *J* 5.6), 6.17 (dd, 1H, *J* 5.6, *J′* 12.2), 4.33 (dd, 1H, *J* 6.46, 7.5), 3.48 (m, 4H,), 3.77 (3H, m), 2.23 (1H, m, 2H), 1.89 (3H, d, *J* 1.13), δ_C (75.4 MHz; CD_3OD) 159.8, 151, 136.7, 135, 132.9, 130.1, 126.2, 124.7, 123.4, 122.8, 117.3, 110, 98.1, 85.5, 84.4, 60.9, 49.5, 43.7, 38.43, 37.9, 11; Calculated Mass 488.15, LCMS single peak, 3.65 min, *m*/*z* 489.2(M+1). Elemental analysis for; $C_{22}H_{25}ClN_6O_5$ Calculated: C, 54.04; H, 5.15; N, 17.19. Found: C, 54.15; H, 5.05; N, 17.13.

5.1.7. 1-[5-(7-Chloro-quinolin-4-ylamino)-pentyl-3-[2-hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-urea 2d

To a mixture of **1d** (318 mg, 0.415 mmol, 1 equiv) in ethanol (2 ml) and water (0.5 ml) was added potassium carbonate (1.15 g, 8.4 mmol, 20 equiv). The reaction mixture was refluxed for 7 days. The completion of the reaction mixture was checked using TLC with anisaldehyde spray in 10% MeOH/DCM solvent system. The reaction mixture was concentrated and purification was done using column chromatography with 15% MeOH/DCM solvent system. Yellow solid; mp 152 °C; δ_H (400 MHz, CD_3OD) 8.36 (1H *J* 6.3, d), 8.24 (*J* 9.0, d, 1H), 7.85 (1H, q, *J* 1.19), 7.8 (1H, d, *J* 2.0), 7.53 (1H, dd, *J* 2.0, *J′* 9.0), 6.67 (1H, d, *J* 6.42), 6.16 (dd, *J* 5.5, *J′* 12.12), 4.33 (d, *J* 6.6 H), 3.75 (m, 3H), 3.49 (t, *J* 7.13), 3.15 (t, *J* 6.44,), 2.27 (2H, m), 1.88 (d, 3H), 1.82 (quintet, 2H, *J* 6.9, CH_2), 1.51 (4H, m, CH_2CH_2); δ_C (75.4 MHz, CD_3OD) 165, 159.28, 153.6, 150.9, 146.9, 143.84, 137, 136.6, 125.8, 123.6, 122.8, 116.5, 110, 98.2, 85.61, 84.4, 61.0, 49.5, 42.9, 39.3, 38.0, 29.6, 27.5, 23.8, 11.

Calculated mass 530, LCMS single peak, 3.65 min. Calculated mass 531.2(M+1). LCMS single peak, 3.65 min, *m*/*e*, 531.3(M+1). Elemental analysis for; $C_{25}H_{31}ClN_6O_5$ Calculated: C, 56.55; H, 5.88; N, 15.83. Found: C, 56.43; H, 5.85; N, 15.89.

5.1.8. *N*-[5-(7-chloro-quinolin-4-ylamino)-pentyl]-*N*-[2-hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-oxalamide 4d

To a mixture of **3d** (318 mg, 0.415 mmol, 1 equiv) in ethanol (2 ml) and water (0.5 ml) was added potassium carbonate (1.38 g, 10 mmol, 24 equiv). The reaction mixture was refluxed at 90 °C for 3 days. The completion of the reaction mixture was checked using T.L.C with anisaldehyde spray in 10% MeOH/DCM solvent system. The reaction mixture was concentrated and purification was done using column chromatography with 13% MeOH: DCM solvent system. white solid; mp 257 °C; δ_H (400 MHz, CD_3OD) 11.2 (br s, NH), 8.69 (*J* 6.11, t, 1H, NH), 8.35 (d, *J* 5.42, 1H), 8.23 (d, *J* 9.0, H_e), 7.72(d, 1H, *J* 2.2), 7.71 (q, 1H, *J* 1.2),7.39 (1H,dd, *J* 2.2, *J*′ 8.96), 7.21 (t, 1H, *J* 5.0, NH), 6.42 (d, 1H, *J* 5.47), 6.17 (t, 1H, *J* 6.22), 4.97 (t, 1H, *J* 5.0, OH), 4.39 (m, 1H), 3.58(1H, m), 3.51 (1H, m), 3.22 (2H, m), 3.12 (2H, m), 1.75 (3H, d, *J* 1.0), 1.61 (2H, quintet, *J* 7.5), 1.5 (2H, quintet, *J* 6.84), 2.22 (2H, m), 1.34 (2H, q, *J* 6.88), 3.85(1H, m); δ_C (75.4 MHz, CD_3OD) 164.2, 160.6, 160.1, 152.3, 150.8, 150.5, 149.5, 136.8, 133.8, 127.9, 124.5, 124.4, 117.9, 109.8, 99.1, 84.4, 84, 61.5, 49.2, 42.8, 37.1, 28.9, 27.9, 12.6; Calculated mass 559.2(M+1), LCMS single peak, 4.23, min, *m*/*e*, 559.5(M+1). Elemental analysis for; $C_{26}H_{31}ClN_6O_6$ Calculated: C, 55.86; H, 5.59; N, 15.03. Found: C, 56.83; H, 5.65; N, 15.14.

5.1.9. 1-(4-Azido-5-hydroxymethyl-tetrahydro-furan-2-yl)-3-[3-(7-chloro-quinolin-4-ylamino)-benzyl]-5-methyl-1*H*-pyrimidine-2,4-dione 6

Sodium hydride (7.99 mg, 0.33 mmol, 1 equiv) was added at 0 °C to AZT (89 mg, 0.33 mmol, 1 equiv) in dry DMF under N_2 atmosphere. The solution was stirred 25 min at 0 °C followed by stirring at room temperature for 20 min. The (**15**) quinoline derivative (100.66 mg, 0.33 mmol, 1 equiv) in dry DMF was added dropwise at room temperature to the above mentioned solution. The solution was stirred for 12 h and monitored with TLC using anisaldehyde spray. Extraction was done using water and ethyl acetated solvent mixture. The compound was purified with 5% MeOH/DCM solvent system. Yellow solid; mp 238 °C; yield 80%; δ_H (400 MHz, DMSO-d_6) 9.0 (br s, 1H), 8.44 (d, 1H, *J* 5.28, H^b), 8.38 (d, 1H, *J* 9.0), 7.88 (d, 1H, *J* 2.1), 7.55 (dd, *J* 2.1, *J*′ 9.0), 7.79(d, 1H, *J* 1.0), 7.36 (m, 1H), 7.24 (m, 2H), 7.0 (d, *J* 7.65), 6.91 (d, *J* 5.34), 6.16 (t, *J* 6.31), 5.2 (OH, br s), 5.01 (2H, m), 4.39 (dt, *J* 5.44, *J*′ 12.0), 3.84 (dd, *J* 3.7, *J*″ 8.67), 3.64 (2H, m), 2.42(1H,ddd, *J* 6.91, *J*′ 13.64, *J*″ 20.43), 2.31 (1H,ddd, *J* 6.54, *J*′ 13.73, *J*″ 19.44), 1.85 (br s, 3H); δ_C (75.45 MHz, DMSO-d_6) 163.1, 152.3, 150.9, 150.1, 148.2, 140.7, 138.9, 135.5, 134.3, 129.7, 128.15, 125.4, 124.9, 123.8, 121.9, 121.5, 118.9, 109.1, 102.4, 84.9, 84.6, 61.1, 60.4, 44.1, 36.8, 13.34. Calculated Mass 534.16 (M+1). LCMS single peak, 5.65 min, *m*/*e*, 534.4 (M+1).

5.1.10. Compound/intermediate 23

Boron trifluoride diethyl etherate complex (5 drops) was added slowly to a solution of dihydroartemisinin (1.00 g, 3.54 mmol, 1 equiv) and propargyl alcohol (794 mg, 14.2 mmol, 4 equiv) in 10 mL of dry DCM at 0 °C under a nitrogen atmosphere. The resulting mixture was allowed to warm to 25 °C and stirred at this temperature for 10 h. The product mixture, after dilution with DCM, was washed successively with 5% aqueous $NaHCO_3$, water and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. Purification of the crude product using column chromatography (0–20% EtOAc/Hex) afforded the β-isomer as colourless needles. mp 116 °C; yield 80%; δ_H (400 MHz, $CDCl_3$) 5.39 (1H, s, H 12), 4.95 (1H, d, *J* 3.3, H 10), 4.29 (2H, d, *J* 2.4), 2.65 (1H, m), 2.40–2.30 (2H, m), 2.05–1.17 (10H, m), 1.42 (3H, s), 0.93 (3H, d, *J* 6.0), 0.91 (3H, d, *J* 7.2); δ_C (75 MHz, $CDCl_3$) 104.1, 100.6, 88.0, 81.0, 79.7, 73.9, 54.9, 52.5, 44.3, 37.4, 36.4, 34.6, 30.6, 26.1, 24.7, 24.4, 20.3, 12.7; HRMS(ESI) found *m*/*z* 323.1207 $[M+H]^+$ for $C_{18}H_{26}O_5$ requires 322.17802; Anal. Calcd for $C_{18}H_{26}O_5$: C, 67.06; H, 8.13. Found: C, 66.70; H, 8.10.

5.1.11. Hybrid 8

Copper(II) sulphate pentahydrate (0.0232 mmol, 5 mol %) and sodium ascorbate (0.0696 mmol, 15 mol %) was added to a mixture of AZT (0.464 mmol, 1.0 equiv) and the acetylene **23** (0.510 mmol, 1.1 equiv) in 3 mL of DCM/H_2O (1:1). The resulting mixture was stirred for 24 h at 25 °C. Upon completion, the product mixture was diluted with water and extracted with EtOAc. The combined organic layer was washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product so obtained was subjected to column chromatography (0.5–10% MeOH/DCM) to give the β-isomer as a light green foam (199.9 mg, 45%); mp 122 °C; δ_H (400 MHz, $CDCl_3$) 9.27 (1H, s), 7.68 (1H, s), 7.46 (1H, s), 6.24 (1H, br s), 5.46 (1H, s), 4.93 (1H, br s), 4.91 (1H, d, *J* 12.8), 4.71 (1H, d, *J* 12.8), 4.43 (1H, br s), 4.03 (1H, br d, *J* 11.2), 3.81 (1H, br d, *J* 11.6), 3.57 (1H, br s, OH), 3.00 (2H, br s), 2.65 (1H, m), 2.40–1.16 (11H, m), 1.93 (3H, s), 1.44 (3H, s), 0.94 (3H, d, *J* 5.6), 0.90 (3H, d, *J* 7.2); δ_C (100 MHz, $CDCl_3$) 163.8, 150.5, 145.6, 137.9, 122.8, 111.3, 104.2, 102.1, 88.8, 88.0, 85.4, 81.1, 61.8, 61.6, 59.3, 52.5, 44.4, 37.5, 37.4, 36.4, 34.6, 30.9, 26.1, 24.7, 24.5, 20.3, 12.3, 12.4; HRMS(ESI) found *m*/*z* 590.2811 $[M+H]^+$ for $C_{28}H_{39}N_5O_9$ requires 589.27478; Anal. Calcd for $C_{28}H_{39}N_5O_9$: C, 57.03; H, 6.67; N, 11.88. Found: C, 56.78; H, 6.61; N, 11.49.

5.1.12. 1-(4-Azido-5-hydroxymethyl-tetrahydro-furan-2-yl)-3-[2-(7-chloro-quinolin-4-ylamino)-ethyl]-5-methyl-1*H*-pyrimidine-2,4-dione 5

Sodium hydride (12 mg, 0.5 mmol, 1 equiv) was added at 0 °C to AZT (133 mg, 0.5 mmol, 1 equiv) in dry DMF under N_2 atmosphere. The solution was stirred for 25 min at 0 °C followed by stirring at room temperature for 20 min. The (**12**) quinoline derivative (142 mg, 0.5 mmol, 0.5 equiv) in dry DMF was added drop wise at room temperature to the above mentioned solution. The solution was stirred for 12 h and monitored by TLC using anisaldehyde spray. Extraction was done using water and ethyl acetated solvent mixture. The compound was purified with 5% MeOH/DCM solvent system. White solid; mp 157–159 °C; Yield 26%; δ_H (400 MHz, DMSO-d_6) 8.41(1H, d, *J* 5.41, H_b), 8.11 (1H, *J* 9.1, d), 7.77(1H, d, *J* 2.2), 7.75 (1H, Q, *J* 1.19), 7.48(t, *J* 5.6, NH), 7.43 (dd, 1H, *J* 2.2, *J*′ 8.8), 6.69(1H, d, *J* 5.46), 6.0 (1H, t, *J* 6.3), 5.21 (1H, br s, OH), 4.35 (1H, dt, *J* 5.56, *J*′ 6.9), 4.0 (2H, m,), 3.82 (1H, m), 3.66 (1H, m), 3.60 (1H, m), 3.48 (2H, m), 2.25(1H, ddd, *J* 5.86, *J*′ 6.59, *J*″ 13.7), 2.23(1H, ddd, *J* 5.8, *J*′ 7, *J*″ 13.5), 1.82 (3H, d, *J* 1.0); δ_C (75.45 MHz, DMSO-d_6) 163.4, 152.3, 151, 150.6, 149.6, 135.3, 133.8, 128, 124.6, 124.3, 117.9, 109, 85, 84.7, 61.1, 60.2, 36.9, 13.3. Calculated mass 472.15 (M+1), LCMS, single peak, 4.94 min, *m*/*e*, 472.4(M+1).

5.1.13. *N*-[2-(*tert*-Butyl-diphenyl-silanyloxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-2-(1,2,4,5-tetraoxa-3-bicyclo[3.3.1]nonane-spiro[5.5]undec-9-yl)-acetamide 25

A solution of tetraoxane (80 mg, 0.24 mmol), Et_3N (33 μl, 0.24 mmol) and $ClCO_2Et$ (22.52 μl, 0.24 mmol) in dry DCM (10 ml) was stirred for 90 min at 0 °C. Then the amine (223 mg, 0.46 mmol) was added and after 30 min of stirring, the reaction mixture was warmed to room temperature. After an additional 90 min it was diluted with water and the layers were separated,

the organic layer was with water dried with $MgSO_4$ and evaporated to dryness. The purification was done using 90% ethylacetate and hexane and TLC was checked using anisaldehyde stain. mp 98–100 °C; δ_H (400 MHz, CD_3OD) 10.18 (br s, 1H, NH), 7.58 (1H, Q, *J* 1.2, H_6), 7.40 (6H, m); 7.68 (m, 4H), 6.43 (dd, 1H, *J* 5.9, *J*′ 8.93), 4.61 (1H, t), 4.07 (m, 3H), 2.12 (2H, d, *J* 6.5), 2.27 (2H, m), 1.95 (m, 5H), 1.85 (2H, br s), 1.64 (14H, m), 1.55 (3H, d, *J*1.1, C_5-Me), 1.26 (2H, m, adamantyliden), 1.10 (s, 9H, $(CH_3)_3$; δ_C (75.45 MHz, CD_3OD) 172.6, 164.2, 150.9, 135.7, 135.3133.5, 132.6, 130.4, 130.0, 128.4, 127.7, 111.6, 110.5, 107.9, 87.4, 87.37, 85.4, 84.9, 68.3, 65.2, 51.5, 42.6, 37.1, 34.1, 33.8, 33.3, 33.2, 32.9, 27.2, 19.6, 12.5.

5.1.14. 1-(4-Azido-5-(3*R*,5a*S*,6*R*,8a*S*,9*R*,12*S*,12a*R*)-decahydro-3,6,9-trimethyl-3,12-epoxy-12*H*-pyrano[4,3-*j*]-1,2-benzodioxepin-10-oxymethyl-tetrahydro-furan-2-yl)-5-methyl-1*H*-pyrimidine-2,4-dione 7

To a solution of dihydroartemisnin 0.284 g (1 mmol) and 0.267 g (1 mmol AZT) in 150 ml of dry DCM was added 5 drops of $BF_3{\cdot}Et_3O$ at −10 °C. The mixture was stirred at room temp until reaction completed. The solution was worked up using 5% $NaHCO_3$ solution, and purified by column chromatography which resulted in a diastereomeric mixture of α and β isomer (1:15) with a combined yield of 63%. White solid; mp 85–87 °C; δ_H (300 MHz, $CDCl_3$) NMR for β isomer 8.5 (1H, br, s, D_2O exchangeable NH), 7.17 (1H, q, *J* 1.1), 6.13 (1H, t, *J* 6.6), 5.4 (1H, s), 4.80 (1H, d, *J* 3.5), 4.09 (3H, m), 3.64 (1H, dd, *J* 2.5, *J*′ 9.9), 2.71 (1H, m), 2.42 (2H, m), 1.92 (d, 3H, *J* 1.0), 1.43 (br s, 3H), 0.96 (3H, br s), 0.94 (3H, d, *J* 1.5); δ_C (75.45 MHz, $CDCl_3$)163.3, 149.9, 134.6, 111.3, 104.3, 102.1, 87.9, 84.8, 82.6, 80.7, 67.7, 60.8, 52.4, 44.1, 37.55, 37.51, 36.34, 34.4, 30.7, 26, 24.6, 22, 20.2, 13.1, 12.7; FAB MS 534$(M+1)^+$; Elemantal analysis for; $C_{25}H_{35}N_5O_8$ Calculated: C, 56.27; H, 6.61; N, 13.13. Obtained: C, 56.22; H, 6.40; N, 12.96. Calculated Mass 533, LCMS, single peak, 8.78 min, *m*/*z*, 556(M+23), 572 (M+39).

5.1.15. *N*-[2-Hydroxymethyl-5-(5-methyl-2,4-dioxo-3,4-dihydro-2*H*-pyrimidin-1-yl)-tetrahydro-furan-3-yl]-2-(1,2,4,5-tetraoxa-3-bicyclo[3.3.1]nonane-spiro[5.5]undec-9-yl)-acetamide 9

To a mixture of (**25**) silylated AZT-tetraoxane compound (50 mg, 0.062 mmol, 1 equiv) in ethanol (10 ml) and water (0.5 ml) was added potassium carbonate (171 mg, 1.24 mmol, 20 equiv). The reaction mixture was refluxed at 90 °C for 2 days. The completion of the reaction mixture was checked using TLC with anisaldehyde spray in 10% MeOH/DCM solvent system. The reaction mixture was concentrated and purification was done using column chromatography with 8% MeOH/DCM solvent system. White solid; mp 178 °C; δ_H (400 MHz, CD_3OD) 8.37 (1H, br s, NH), 7.70 (1H, q, *J* 1.2, H_6), 6.14 (t, 1H, *J* 5.3), 5.91 (d, 1H, *J* 7.0, NH), 4.47 (dd, 1H, *J* 7.4, *J*′ 14.9), 3.93 (dd, 1H, *J* 2.2, *J*′ 12.5), 3.75 (2H, m, 2H), 2.34 (2H, m), 2.12 (2H, d, *J* 6.5), 1.91 (3H, d, *J*1.1), 1.85 (2H, br s, 2H), 1.62 (14H, m), 1.92 (m, 5H), 1.28 (2H, m), δ_C (75.45 MHz; CD_3OD) 173.15, 169.1, 163.8, 148.9, 139.4, 135.5, 107.4, 86.7, 84.3, 61.5, 47.6, 43.1, 38.4, 36.7, 33.9, 33.5, 27, 12.5. Calculated mass 562 2 (M+1), LCMS single peak, 7.59 *m*/*z*, 562.5(M+1).

5.2. Antiviral activity of hybrid molecules

5.2.1. Cells

MT4 and TZM cells were cultured in RPMI 1640 containing glutamine (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen) and penicillin/streptomycin. HeLa and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine, 10% FCS and penicillin/streptomycin. The FCS was heat-inactivated at 56 °C before use.

5.2.2. Production of pseudotyped lentiviral particles

Viral particles were generated by co-transfection of HEK293T cells with the transfer vector pWPXL (Addgene, 12257), the packaging plasmid psPAX2 (Addgene, 12260), and the vesicular stomatitis virus glycoprotein (VSV-G) expression plasmid, pMD2.G (Addgene, 12259). These 3 plasmids were used to transfect HEK93T using the PEI (Polyethyleneimine) method as described.[39] At 48 h and 72 h posttransfection, the viral supernatant was harvested by filtration through a 0.45-μm cellulose acetate filter to remove cell debris and viral particles were further concentrated by using Amicon ultrafiltration concentrators with a pore size of 100 kDa (Millipore). The concentrated viral supernatant was used for the subsequent infection of target cells.

5.2.3. Compound screening

The reporter viruses were first used to screen hybrid molecules for anti-HIV properties. 100 μl of culture containing approximately 1.2×10^4 HeLa cells were seeded in a 96-well plate and incubated overnight at 37 °C. The next day, compounds were diluted in DMSO, then in medium. Virus stock was thawed, diluted with DMEM and plated in 96 well plates. Compounds were added to virus suspension and were immediately transferred to HeLa cells by replacing old medium with virus suspension and incubated for 48 h at 37 °C. The final DMSO concentration was 1%. After 48 h incubation, the plate was removed from the incubator and the medium containing virus particles was discarded and replaced with 100 μl ice cold PBS to rinse the cells. The washing step was performed twice and 110 μl lysis buffer (1 mM DTT, 0.1% triton X-100 in 20 mM HEPES, pH 7.4) was added and incubated at −80 °C for 45–60 min to break cells membrane. The plate was then thawed at room temperature with gentle shaking. Plates were subsequently centrifuged at 1500 rpm for 10 min at 4 °C to pellet cell debris and 100 μl of supernatant was then transferred to a black plate for fluorescence measurement. Fluorescence intensity was measured using a spectrofluorometer (*Safire*^{2},Tecan) with the following settings. Measurement mode: from the top, excitation wavelenght/bandwidth: 488 nm/10 nm, emission wavelenght/bandwidth: 509 nm/10 nm, gain (manual): 100, at room temperature.

5.2.4. Infectivity assay using infectious wild type HIV-1 virus

For the most active compound, the anti-HIV activity was evaluated on the replication of a wild type infectious virus in a biosafety level 3 laboratory. MT4 cells are immortalized T-cells used to replicate HIV in vitro.[40] A co-culture is initiated by mixing a viral suspension with an uninfected MT4 cells culture and a fresh co-culture is prepared every 48 h by diluting an existing co-culture with uninfected MT4 cells in a 1:10 ratio (5 ml of co-culture, 5 ml uninfected MT4 culture, 40 ml RPMI medium). A freshly prepared co-culture was assayed in a 48 well plate at a final volume of 500 μl of diluted co-culture per well. After 4 h incubation at 37 °C, 5 μl of inhibitors dissolved in DMSO were added to each well and plates were incubated for 48 h. Fourty eight (48) hours after incubation, 1×10^4 TZM cells were seeded in a 96 well plate and incubated at 37 °C to allow the cells to attach at the bottom and the 48-well plate containing the co-culture was centrifuged at 800 g for 10 min. One hundred (100) μl of MT4 cell-free supernatant from each concentration was used to infect TZM cells in triplicate, which enables to determine the amount of infectious particles that have been produced in the presence of inhibitor.[41,42] After 2 days post infection, cells were fixed with aceton/ethanol (1:1), washed three times with PBS. The infected TZM cells were further incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining solution for 2–4 h. After incubation, the number of blue cells in each well was counted under bright field illumination and the mean number of blue cells in each well and for each concentration was calculated.

5.3. Evaluation of the antimalarial activity of hybrid molecules

5.3.1. Malstat assay

Compounds were screened for growth inhibition activity against *P. falciparum* using the Malstat assay as described.[43,44] Synchronized ring stages of *P. falciparum* strains 3D7 and Dd2 were plated in triplicate in 96-well plates (200 μl/well) at a parasitemia of 1% in the presence of the compounds dissolved in dimethyl sulfoxide (DMSO) and the Malstat assay was performed as described by Aminake et al.[45]

5.3.2. Gametocyte toxicity test

P. falciparum NF54 parasites were grown at high parasitemia to favor gametocyte formation as described.[46] Upon the appearance of gametocyte stage II, 1 ml of culture was aliquoted in triplicate in a 24-well plate in the presence of compounds at their respective IC_{50}s. The gametocytes were cultivated for 7 days, and the medium was replaced daily. For the first 48 h of cultivation, the gametocytes were treated with test compounds, and subsequently the medium was compound free. After 7 days, Giemsa-stained blood smears were prepared and the gametocytemia was evaluated by counting the numbers of gametocytes of stage IV and V in a total number of 1000 erythrocytes.

5.4. Evaluation of compounds cytotoxicity

The cytotoxicity of compounds was evaluated using MTT assay. This assay was first described by Mosmann.[47] MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 Diphenyltetrazoliumbromid) is uptaken by living cells and converted by mitochondrial dehydrogenases to a violet formazan that cannot diffuse through cell membranes and crystallizes in vital cells. The method was carried out as described by Aminake et al.[45]

5.5. Compound administration and blood collection

A stock solution of Pheroid™ vesicles containing the compound **7** were prepared at the Unit for Drug Research and Development (North-West University, South Africa) and were dissolved in NO_2–water for the assay. The compound **7** was also dissolved in 10% DMSO, and the two formulations were compared. The compound **7** was administered to two groups of male mice (C57/BL6) at 20 mg/kg by oral gavage and blood was collected from mice by tail bleeding at different time points. The blood collected was transferred to heparanized tubes and immediately centrifuged at 1500*g* for 10 min at 4 °C. The plasma was separated from red blood cells and stored at −80 °C until analysis. The study and all procedures were approved by the Ethics Committee of the University of Cape Town (REC REF: 010/026). The compound **7** dissolved in DMSO–water (1:9) formed a white precipitate and was resuspended prior to administration. The total volume per administration was 200 μl and 20–25 μl of blood were collected at 30, 120, 240, 480 min after drug administration (compound **7** dissolved in DMSO). For mice treated with the compound **7** entrapped in pheroid vesicles, blood was collected from one mouse at a time at 10, 20, 30, 40, 60, 90, 120, 180, 240, 300, and 360, 420 min after drug administration to maximize the number of samples collected and to reduce the gap between times of collection.

5.6. Quantification of plasma level of the compound 7, AZT and dihydroartemisin

5.6.1. Calibration standard

Stock solutions of test compounds (**7**, AZT and DHA) were prepared in acetonitrile at a concentration of 1 mg/ml. Ten (10) microliters of the stock solutions were diluted in human plasma to obtain standard (S) 1 at a concentration of 10 μg/ml. S2 (5 μg/ml), S3 (2.5 μg/ml), S4 (1.25 μg/ml), STD 5 (0.625 μg/ml), S 6 (0.313 μg/ml), S 7 (0.156 μg/ml), S 8 (0.0781 μg/ml) and S 9 (0.0391 μg/ml) were prepared by serial dilution. Standards 4–9 were used to obtain calibration curves (for compound **7**, AZT and DHA) between 39.1 and 1250 ng/ml. The calibration standards were briefly vortexed and used immediately or stored at −20 °C. The calibration standards were analyzed in duplicate in each study sample batch. The accuracies (%Nom) of the calibration standards of compound **7**, AZT and DHA were between 88.2% and 111.1%, and the precision (%CV) were between 1.4% and 13.9% during study sample analysis.

5.6.2. Compounds extraction

To extract drugs from plasma samples collected from mice, 20 μl of standards and plasma samples were thawed on ice and mixed with 80 μl of internal standard solution (100 ng/ml of deoxythymidine in acetonitrile). The samples were vortexed for 1 min, and sonicated for 5 min, then centrifuged at 1500*g* for 5 min at room temperature. The supernatant (approximately 60 μl) was transferred to a clean tube and evaporated under vacuum in a rotor evaporation system at 30 °C for 45 min or until the samples were completely dried. The residue was reconstituted in 100 μl of mobile phase (10 mM ammonium acetate/methanol, 85:15, v/v).

5.7. Chromatography and mass spectrometry

The compound **7**, DHA and AZT were quantified using an Agilent 1200 HPLC (Agilent technologies, USA) connected in tandem to an AB Sciex API 3200 mass spectrometer (AB SCIEX, 110 Marsh Drive, Foster City, California, USA) equipped with a turbo ion spray (ESI) source. The software Analyst 1.5.1 was used to control both, the liquid chromatogram and the mass spectrometer. The Phenomenex Luna PFP (50 × 2.0 mm, 5 μm) column (Phenomenex, USA) was used for compounds separation. The mobile phase consisted of a gradient of methanol and 10 mM ammonium acetate. The system operated at a flow rate of 0.5 ml/min and the volume of injection was 20 μl. The ESI interface was used in positive mode and the turbo ion spray source was heated to 450 °C.

The AB Sciex API 3200 mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at *m*/*z* 534.1 to the product ions at *m*/*z* 126.9 for the compound **7**, the protonated molecular ions at *m*/*z* 268.1 to the product ions at *m*/*z* 127.0 for AZT, the ammonium adduct at *m*/*z* 302.2 to the product ions *m*/*z* 267.1 for DHA, and the the protonated molecular ions at *m*/*z* 227.1 to the product ions at *m*/*z* 127.0 for the internal standard.

5.8. In vivo efficacy studies of the compound 7

To start an infection, a cryotube containing frozen of *P. berghei N* (CQ sensitive) parasites was quickly thawed at room temperature, then mixed with equal volume of PBS and 200 μl of the resulting mixture was injected immediately intra-peritoneally into a naive recipient C57/BL6 male mouse. After 3–4 days, parasites were monitored in peripheral blood by Giemsa staining, and when parasitemia was higher than 10%, blood was obtained by cardiac puncture and diluted in equal volume of PBS. Each animal was inoculated intraperitoneally with 200 μl of this blood suspension. The test drug the compound **7** entrapped in Pheroid vesicles (20 mg/kg), the standard drug chloroquine (10 mg/kg) and PBS control were administered to parasitized mice 30 min after administration of infected erythrocytes on day zero. The same dose was repeated on days 1, 2 and 3 after infection. Blood was collected by tail bleeding and smears were prepared and stained with Giem-

sa. Parasitemia was assessed to qualitatively assess the activity of the compound at the tested dose. A dose resulting in survival times greater than that of infected non treated mice was considered active. Death occurring before day 6 of infected and treated mice was regarded as toxic death.

5.9. In vitro metabolism studies

In vitro metabolism studies were performed to confirm metabolism observations made in vivo. A final concentration of 1 μM of the compound **7** was incubated at 37 °C with mice liver microsomes. The incubation mix consisted of compound **7** (1 μM), 0.2 mg/ml mice microsomal protein, and 100 mM KPO_4 in 200 mM phosphate buffer, pH 7.4. The reaction was initiated by the addition of NADPH (1 mM). The mixture was rotated at 37 °C. Aliquots were collected before initiating the reaction. At varying time points 200 μl of incubation mixture were removed and added to 100 μl ice cold acetonitrile to stop the reaction. The rate of loss of parent compound was determined at 0, 15, 30, and 45 min by LC–MS/MS analysis. The amount of compound in the samples was expressed as a percentage of remaining compounds compared to time point zero (100%).

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