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Prodrugs of Reverse Fosmidomycin Analogues

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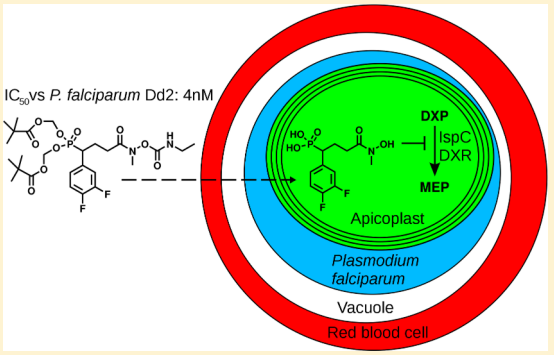
Supporting Information

ABSTRACT: Fosmidomycin inhibits IspC (Dxr, 1-deoxy-D-xylulose 5-phosphate reductoisomerase), a key enzyme in nonmevalonate isoprenoid biosynthesis that is essential in *Plasmodium falciparum*. The drug has been used successfully to treat malaria patients in clinical studies, thus validating IspC as an antimalarial target. However, improvement of the drug's pharmacodynamics and pharmacokinetics is desirable. Here, we show that the conversion of the phosphonate moiety into acyloxymethyl and alkoxycarbonyloxymethyl groups can increase the *in vitro* activity against asexual blood stages of *P. falciparum* by more than 1 order of magnitude. We also synthesized double prodrugs by additional esterification of the hydroxamate moiety. Prodrugs with modified hydroxamate moieties are subject to bioactivation *in vitro*. All prodrugs demonstrated improved antiplasmodial *in vitro* activity. Selected prodrugs and parent compounds were also tested for their cytotoxicity toward HeLa cells and *in vivo* in a *Plasmodium berghei* malaria model as well as in the SCID mouse *P. falciparum* model.



INTRODUCTION

Despite substantial international efforts, malaria remains a major source of global morbidity and mortality. Published estimates for the number of fatal cases in the year 2013 range from 367 000 to 755 000, and the number of nonfatal malaria episodes are orders of magnitude higher.[1] Optimistic reports about a progressive decrease in malaria mortality may need to be reviewed in light of the apparent uncertainty of the numbers quoted above. Even more importantly, progressing attrition of the antimalarial drug arsenal by the emergence and spread of drug-resistant *Plasmodium* parasites and of insecticide-resistant vectors is an acute danger. Development of resistance against antimalarials and insecticides arises as a natural consequence of selection pressure on parasites and mosquitoes.[2] Resistance affects all antimalarial drug classes; recently, resistance to drugs of the artemisinin family, our main line of defense, has been confirmed in several Southeast Asian countries.

In light of the drug resistance problems, a protective vaccine would be a preferable approach. However, the time frame for the development and deployment of a vaccine with a reasonably high level of protection for extended periods cannot be predicted.[3]

There is universal consensus about the urgent need for a continuous influx of novel antimalarials, and, indeed, the malaria drug pipeline of new preclinical and clinical drug candidates is somewhat stronger than it was 5 years ago.[4] However, there is still much scope for improvement. In fact, no major novel antimalarial has been implemented since the introduction of atovaquone in 1992.[5] The total number of druggable targets among the approximately 5300 gene products of *Plasmodium falciparum*,[6] the clinically most important *Plasmodium* species, is not known; in fact, it may be smaller than initially expected. In any case, it would appear sensible to work with any known clinically validated targets that are likely to be exempt from cross-resistance with currently used antimalarial drugs. The proteins of the nonmevalonate isoprenoid biosynthesis pathway, and, most notably, IspC protein catalyzing the first committed step of that pathway,

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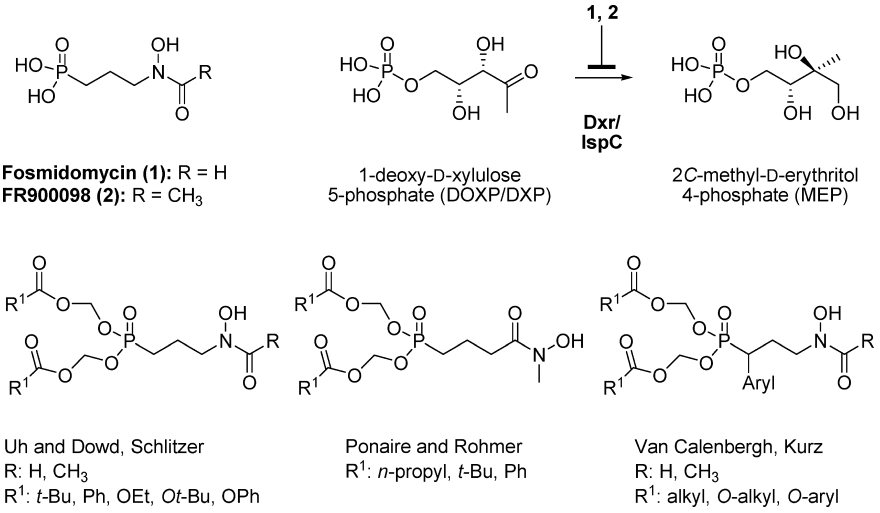
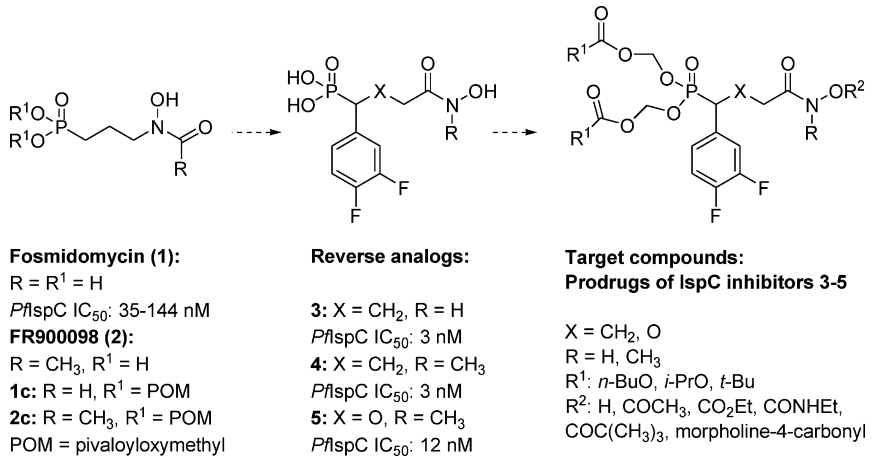
Scheme 1. IspC Inhibitors and Prodrugs with Antibacterial and Antiplasmodial Activity



Scheme 2. Prodrugs of Reverse Fosmidomycin Derivatives 3−5



fulfill these requirements.[7] Specifically, fosmidomycin, an IspC inhibitor originally found as an antibacterial product of *Streptomyces lavendulae*, has been validated as a well-tolerated, safe, and efficacious combination partner in antimalarial treatment regimes in several clinical trials conducted in Africa and South Asia.[8] Moreover, since humans generate isoprenoids via the mevalonate pathway, antimalarials directed at IspC should be exempt from target-related toxicity.

Fosmidomycin inhibits IspC from *P. falciparum* with an IC_{50} in the range of 21−160 nM[9,10] and is active against the blood stage of the parasite with an IC_{50} in the single-digit micromolar range (literature reports varying from 0.4 to 3.7 μM).[9a,11,12] Unfortunately, its pharmacokinetic properties are less than ideal, with only moderate oral bioavailability and a short plasma half-life.[9]

Fosmidomycin acts as a slow, tight-binding substrate-analogue inhibitor whose hydroxamate motif complexes a catalytically essential divalent metal ion at the active site of IspC.[10] Enzyme inhibition has been significantly enhanced by the introduction of aromatic substituents at fosmidomycin's α-position.[11] Other reported structural modifications included the modification of the *N*-acyl group,[12] the modification and inversion of the hydroxamate motif,[13] and the modification of the aliphatic linker that connects the phosphonate motif (mimicking the phosphate moiety of the IspC substrate DXP, 1-deoxy-D-xylulose 5-phosphate) to the hydroxamate motif that interacts with the essential divalent cation of the enzyme.[11c,13b,14] Fosmidomycin prodrugs have been obtained by conversion of the phosphonate moiety into phosphonate esters. (Scheme 1;[15] for review, see Jackson and Dowd 2012[16]).

This article reports on a systematic comparison of phosphonate and phosphonate-hydroxamate double prodrugs of reverse fosmidomycin derivatives using two chloroquine-sensitive and one multidrug-resistant *P. falciparum* strains of different geographic origin (3D7, D10, and Dd2). Representative prodrugs and parent compounds were also screened for their inhibition of IspC and their cytotoxicity toward HeLa cells as well as *in vivo* in a *Plasmodium berghei* malaria model and in the SCID mouse *P. falciparum* model.

RESULTS

The reverse fosmidomycin derivative **3**, its *N*-methyl derivate **4**, and the *N*-methyl-substituted oxa-analogue **5** have been shown earlier to inhibit *P. falciparum* IspC (*Pf*IspC) with IC_{50} values in the low nanomolar range (cf. Table 1 and Scheme 2).[17] However, *in vitro* growth inhibition of asexual blood stages of *P. falciparum* by these compounds was weaker. With the aim to improve penetration of the numerous biomembranes that the inhibitors must pass before reaching their target protein inside the apicoplast (Figure 2B), we decided to combine the

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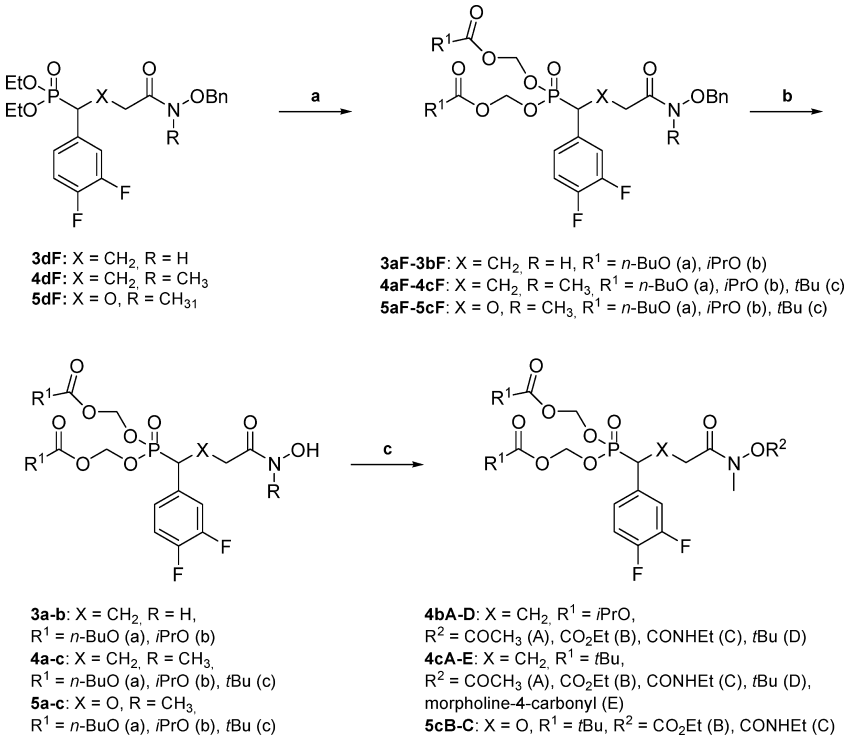
Scheme 3. Synthesis of Target Compounds[a]



[a]Indices **a**–**c** and **A**–**E** indicate phosphonate- and hydroxamate-prodrug functionalities of target compounds, respectively. Reagents and conditions: (a) (1) TMSBr, CH_2Cl_2, rt 24 h; (2) THF/H_2O, RT, 1 h; (3) *n*-butyl chloromethyl carbonate (**3aF**–**5aF**), isopropyl chloromethyl carbonate (**3bF**–**5bF**), or chloromethyl pivalate (**4cF**, **5cF**), TEA, DMF, 80 °C, 6 h; (b) H_2, Pd–C, MeOH, 1 atm, rt, 2 h; (c) acetyl chloride (**4bA**, **4cA**), ethyl chloroformate (**4bB**, **4cB**, **5cB**), ethyl isocyanate (**4bC**, **4cC**, **5cC**), or pivaloylchloride (**4bD**, **4cD**), TEA, CH_2Cl_2, rt, 1 h or CDI, CH_2Cl_2, 4 °C, 1 h; morpholine, CDI, DCM, rt, 12 h (**4cE**).

modification of the phosphonate group and the hydroxamate motif (Scheme 2).

For the modification of the phosphonate motif, we used acyloxymethyl- and alkoxycarbonyloxymethyl ester groups. Numerical estimates predicted that the designed modifications should increase the lipophilicity (clogP) by approximately 4 to 6 orders of magnitude to values that would be in line with Lipinski's rule of five (Table 1).

As starting materials, we selected phosphonic esters **3dF**–**5dF** (Scheme 3). Their dealkylation with bromotrimethylsilane provided the corresponding crude phosphonic acids (structures not shown). Subsequent conversion into acyloxymethyl and alkoxycarbonyloxymethyl esters **3aF**–**3bF**, **4aF**–**4cF**, and **5aF**–**5cF** was accomplished without prior purification by treatment with chloromethyl pivalate and alkyl chloromethyl carbonates in the presence of triethylamine. After column chromatography, all intermediates except **4bF** were obtained in good purity. Catalytic hydrogenation of *O*-benzyl-protected precursors afforded the corresponding hydroxamic acids **3a**,**b**, **4a**–**c**, and **5a**–**c**. With the exception of compound **4c**, all alkoxycarbonyloxyalkyl ester prodrugs were obtained in good purity by column chromatography. Reactions of compounds **4b** and **4c** with acetyl chloride, ethyl chloroformiate, ethyl isocyanate, and pivaloyl chloride yielded bis-prodrugs **4bA**–**4bD** and **4cA**–**4cD**; treatment of crude **4c** with CDI (1,1′-carbonyldiimidazole) and morpholine afforded prodrug **4cE**. The conversion of *β*-oxa-isosteric prodrug **5c** into bis-prodrugs **5cB** and **5cC** has been described elsewhere.[17c] The structures of all novel compounds were confirmed by IR and ^1H and ^{13}C NMR spectra. Their purity was determined by HPLC and elemental analysis, respectively.

Biological Studies. The study compounds were assayed for inhibition of *Pf*IspC as described earlier.[17c] None of the prodrugs (e.g., **3a**,**b**, **4a**,**b**) caused detectable enzyme inhibition. This is in line with expectations based on X-ray structure data, since all prodrugs appear too bulky to be able to be accommodated at the IspC active site. Moreover, the double prodrugs (e.g., **4bB**–**D**) are unable to chelate the essential divalent metal ion of IspC. The calculated logP values of the prodrugs are within the limits of Lipinski's rule of five. Hence, we expected the prodrugs to be cell-permeable (Table 1).

The study compounds were also assayed for growth inhibition of asexual blood stage parasites of three *P. falciparum* strains kept in continuous culture (Table 1). Specifically, we used the chloroquine-sensitive 3D7 and D10 strains as well as the multiresistant Dd2 strain. Parasite proliferation was monitored by an enzyme-linked immunoassay (ELISA) for histidine-rich protein 2 (HRP2).

Several prodrugs under study, phosphonate prodrugs (**3a**, **4a**, **5a**) and double prodrugs (**4bA**, **4cC**), inhibit the multiplication of *P. falciparum* blood stages with IC_{50} values in the single-digit nanomolar range (IC_{50} values vs *Pf*Dd2: 4–9 nM, Table 1). Among them, the most active prodrugs are phosphonates **4a** and **4b** and double prodrug **4cC** (Table 1). Their inhibitory efficacy exceeds that of the parent free phosphonic acid **4** by about 1 order of magnitude. Notably, the antiparasite IC_{50} values of prodrugs **3a**, **4a**, **4b**, and **4cC** are similar to the IC_{50} values for IspC inhibition by their respective mother compounds, **3** and **4**. To the best of our knowledge, **4a**, **4b**,

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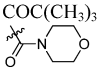
Table 1. Inhibition of *Pf*IspC, *in Vitro* Antiplasmodial Activity, and Calculated logP

Entry	R	R[1]	X	R[2]	*Pf*IspC[a] IC_{50} [μM]	*Pf*Dd2[b,c] IC_{50} [μM]	*Pf*3D7[b,c] IC_{50} [μM]	*Pf*D10[b,c] IC_{50} [μM]	logP[d]
1					0.16 ± 0.02[f]	0.81[f]	0.88[f]	1.3	-2.5 ± 0.6
2					0.015 ± 0.001[e]	0.16	0.16	0.35	-2.6 ± 0.6
1c		*t*-Bu			n. d.	0.49	0.48	1.8	1.4 ± 0.6
2c		*t*-Bu			n. d.	0.028	0.041	0.098	1.6 ± 0.6
3	H		CH_2	H	0.0028 ± 0.0004[f]	0.65	0.075	0.21	-1.5 ± 0.7
4	CH_3		CH_2	H	0.0034 ± 0.0013[f]	0.04	0.12	0.083	-0.8 ± 0.7
5	CH_3		O	H	0.012 ± 0.001[g]	0.13[g]	0.54[g]	0.52	-0.2 ± 0.8
3a	H	*n*-BuO	CH_2	H	>1000	0.009	0.039	0.10	3.2 ± 0.7
4a	CH_3	*n*-BuO	CH_2	H	>1000	0.005	0.013	n. d.	3.9 ± 0.8
5a	CH_3	*n*-BuO	O	H	n. d.	0.009	0.014	0.031	5.2 ± 0.9
3b	H	*i*-PrO	CH_2	H	>1000	0.029	0.049	n. d.	1.8 ± 0.7
4b	CH_3	*i*-PrO	CH_2	H	887 ± 240	0.007	0.008	n. d.	2.5 ± 0.8
5b	CH_3	*i*-PrO	O	H	n. d.	0.029	0.035	0.031	3.7 ± 0.9
5c	CH_3	*t*-Bu	O	H	n. d.	0.022[g]	0.013[g]	0.086	4.5 ± 0.8
4bA	CH_3	*i*-PrO	CH_2	$COCH_3$	n. d.	0.008	0.013	0.061	2.7 ± 0.8
4bB	CH_3	*i*-PrO	CH_2	$CO_2C_2H_5$	>1000	0.018	0.028	0.13	3.7 ± 0.8
4bC	CH_3	*i*-PrO	CH_2	$CONHC_2H_5$	>1000	0.012	0.014	0.072	3.1 ± 0.8
4bD	CH_3	*i*-PrO	CH_2	$COC(CH_3)_3$	>1000	0.034	0.043	0.22	3.9 ± 0.8
4cA	CH_3	*t*-Bu	CH_2	$COCH_3$	n. d.	0.044	0.114	0.29	3.5 ± 0.8
4cB	CH_3	*t*-Bu	CH_2	$CO_2C_2H_5$	n. d.	0.018	0.083	0.22	4.4 ± 0.8
4cC	CH_3	*t*-Bu	CH_2	$CONHC_2H_5$	n. d.	0.004	0.045	0.058	3.8 ± 0.8
4cD	CH_3	*t*-Bu	CH_2	$COC(CH_3)_3$	n. d.	0.070	0.20	0.46	4.7 ± 0.8
4cE	CH_3	*t*-Bu	CH_2		>1000	>10	>10	>10	2.5 ± 0.8
5cB	CH_3	*t*-Bu	O	$CO_2C_2H_5$	n. d.	0.019[g]	0.023[g]	0.123	5.7 ± 0.8
5cC	CH_3	*t*-Bu	O	$CONHC_2H_5$	n. d.	0.022[g]	0.031[g]	0.128	5.1 ± 0.8

[a]Values (mean ± SD calculated from nine or more data points) were determined using nonlinear regression analysis as described earlier.[18] [b]Values are the mean of at least two independent experiments conducted in duplicate, each using 12 serial dilutions. [c]n.d., not determined. [d]logP values were calculated using ACD/ChemSketch freeware, version 12.01. [e]Data from Behrendt et al.[17b] [f]Data from Behrendt et al.[17a] [g]Data from Brücher et al.[17c]

and **4cC** are the most potent fosmidomycin derivatives with regard to inhibition of *P. falciparum* blood stage proliferation. A more detailed comparison of the biological activities of the study compounds is described below.

Figure 1 compares the impact of different phosphonate and hydroxamate prodrug functionalities applied to three α-substituted mother compounds with regard to their activity against three different *P. falciparum* strains. As phosphonate prodrug functionalities, we used *n*-butyloxycarbonyloxymethyl group **a**, isopropyloxycarbonyloxymethyl group **b**, and pivaloyloxymethyl group **c**, which have been used earlier for antiviral prodrug strategies and, to some extent, in earlier work on IspC inhibitors. The ordinate of each graph represents the specific activity gain resulting from the modification of the respective parent compound (defined as the difference of the $\log_{10}$ of the IC_{50} values of parent compound and prodrug derivative). For each data point, the abscissa value represents the IC_{50} value of the respective prodrug when applied to the respective strain.

The three-way comparison affords the following conclusions: (i) Whereas the overall sensitivity of strains 3D7 and Dd2 against the study compounds are similar, the D10 strain appears to be somewhat less sensitive. (ii) With few exceptions, *n*-butyloxycarbonyloxymethyl group **a** showed the largest activity enhancement of the prodrugs studied. (iii) The impact of the prodrug functionality differs substantially among the tested *P. falciparum* strains. Enhancements up to 50-fold are observed with the 3D7 laboratory strain. On the other hand, the maximum observed enhancement with the D10 strain was by an approximate factor of 17. (iv) Parent compounds differ with regard to the activity enhancement caused by conversion into prodrugs. Notably, parent compound **5** showed the highest respective enhancement by conversion into prodrugs. Moreover, the impact of the respective prodrug functionality is larger with the 3D7 strain than with the Dd2 strain. (v) The modification of the hydroxamic acid motif introduces an additional level of complexity; simple esters, carbonates, and carbamates have been synthesized and tested. Specifically, results obtained with acetyl, pivaloyl, ethoxycarbonyl, and ethylaminocarbonyl residues are summarized in Table 1 and Figures 1 and 2A (in Figure 1, the double prodrugs are marked by colored rings around the data points). In parallel to Figure 1, the abscissa of Figure 2A represents the IC_{50} value of double-derivatized prodrugs when assayed with one of the three *P. falciparum* strains under study. The ordinate shows the difference of the $\log_{10}$ (IC_{50}) values of the phosphonate-modified (single-modified) and the double-modified compounds.

With few exceptions (e.g., **4cC**, the most active compound under study), the additional modification of the hydroxamic acid group led to reduced antiplasmodial activity. Only the morpholine derivative had no detectable antiparasite activity.

We also determined the cytotoxicity and selectivity of parent compounds (**4**, **5**) and prodrugs (**4a**, **5a**, **4bA**–**4bC**, **4cA**–**4cD**, **5cC**) for the parasite versus mammalian cells (Table 2). Parent compounds **4** and **5** and all prodrugs tested displayed no cytotoxicity toward mammalian cells and are characterized by excellent selectivity indices (SIs, Table 2).

DISCUSSION

Phosphonates play an important role in pharmacology as stable mimics of naturally occurring phosphates, most notably as

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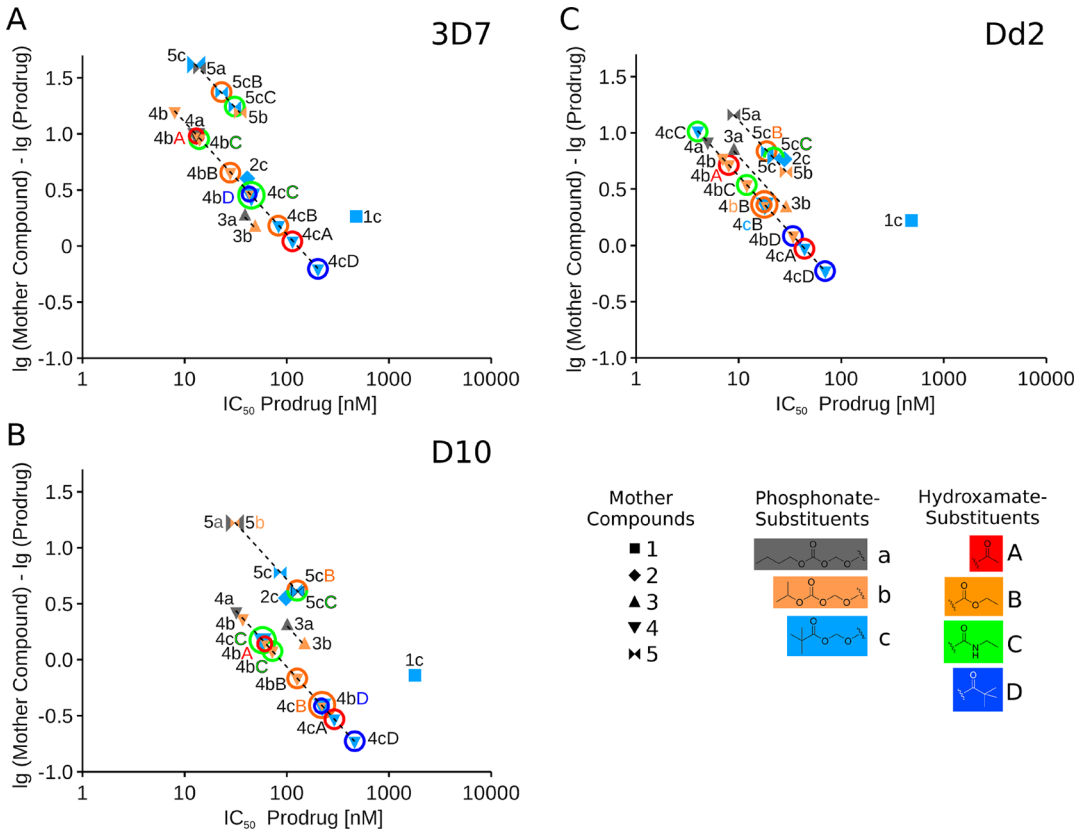

Figure 1. Inhibitory effect of prodrug minilibraries versus three *P. falciparum* strains. Minilibraries derived from a given parent compound are connected by dashed lines. Double prodrugs feature colored rings.

Table 2. Cytotoxicity and Selectivity Indices

entry	R	R^1	R^2	HeLa[a] IC_{50} [μM]	selectivity index[b]
4	CH_3	H	H	>1000	>2500
5	CH_3	H	H	>1000	>7692
4a	CH3	*n*-BuO	H	190	38 000
5a	CH_3	*n*-BuO	H	190	21 111
4bA	CH_3	*i*-PrO	$COCH_3$	188	23 500
4bB	CH_3	*i*-PrO	$CO_2C_2H_5$	205	11 389
4bC	CH_3	*i*-PrO	$CONHC_2H_5$	168	14 000
4cA	CH_3	*t*-Bu	$COCH_3$	175	3977
4cB	CH_3	*t*-Bu	$CO_2C_2H_5$	157	8722
4cC	CH_3	*t*-Bu	$CONHC_2H_5$	219	54 750
4cD	CH_3	*t*-Bu	$COC(CH_3)_3$	69	986
5cC	CH_3	*t*-Bu	$CONHC_2H_5$	158	7182

[a]Cytotoxicity test with HeLa cells. Values are the mean of two duplicate determinations. [b]Selectivity indices were calculated as the HeLa cell IC_{50}/*Pf*Dd2 IC_{50}; larger values indicate greater parasite activity.

antiviral drugs. However, the highly polar phosphonate motif frequently causes difficulties in uptake. Extensive literature has accumulated about the masking of the phosphonate group by prodrug groups that are designed to be enzymatically removed at the desired site of drug action (for review, see refs 19a and 19b).

Modification of the phosphonate motif of fosmidomycin and the analogous natural product FR900098 has been addressed repeatedly over the past decade[15,20] (for review, see also Jackson and Dowd 2012[16]). Initial work on substituted phenylphosphonate esters of fosmidomycin and FR900098 was followed by work with more complex groups such as the isopropyloxycarbonyloxymethyl group.

The most potent prodrug candidates reported in this article exceed the efficacy of authentic fosmidomycin by almost 2 orders of magnitude, as judged by their *in vitro* IC_{50} values against the asexual blood stage of *P. falciparum*. Moreover, efficacy enhancement of reverse analogues **3**–**5** by prodrug derivatization is about 1 order of magnitude higher than in the case of fosmidomycin and FR900098. The additional derivatization of the hydroxamic acid motif yielded IC_{50} values between 4 and 70 nM vs strain Dd2. In some cases, the antiplasmodial activity of the double prodrugs is reduced; however, the most active prodrug under study is double-prodrug **4cC** (IC_{50} value vs *Pf*Dd2: 4 nM). The introduction of a morpholine-containing carbamate resulted in complete loss of antiplasmodial activity (**4cE**).

The prodrugs under study are too bulky to access the active site of IspC, and the compounds with modifications of the hydroxamic acid motif would be unable to complex the divalent metal of the enzyme. Not surprisingly, single and double prodrugs fail to inhibit isolated IspC protein. However, the inhibitory activity in the parasite assay shows that several hydroxamate prodrug functionalities can be hydrolyzed in viable *P. falciparum* asexual blood stage cultures. Possibly, their hydrolytic cleavage may be a limiting factor that is responsible for the observed reduction of antiplasmodial activity caused by several modifiers of the hydroxamate motif.

How Are the Phosphonate Prodrugs Bioactivated in the Cell Culture Assay? The first-generation prodrugs of FR900098 were structurally simple aryl phosphonates.[21] Obviously, their hydrolysis would have to involve hydrolytic

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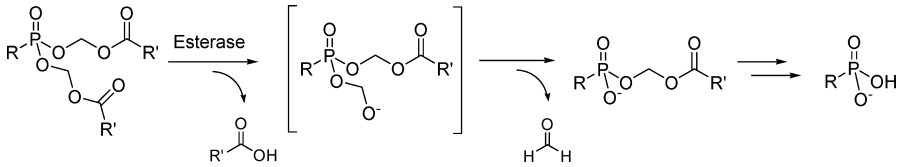
Scheme 4. Hypothetical Sequence of Reactions for Bioactivation of Phosphonate Prodrugs



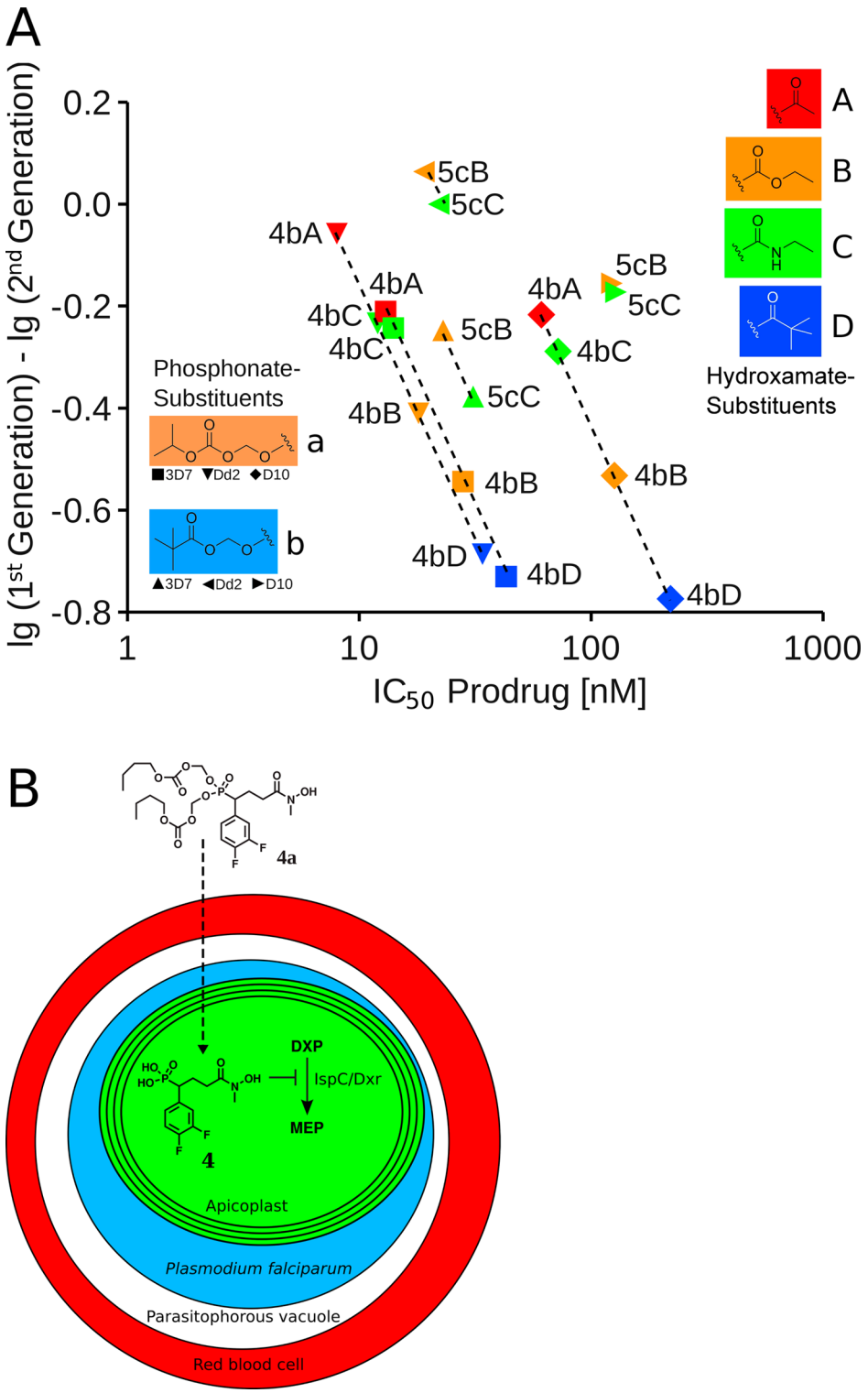


Figure 2. (A) Impact of hydroxamate modification on inhibitory activity. Double prodrugs derived from a given phosphonate prodrug (**4b**, **5b**) are connected by dashed lines. (B) Passage of an IspC inhibitor across membrane barriers in *Plasmodium*-infected red blood cells.

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cleavage of oxygen phosphorus bonds by phosphatases or similar hydrolases. Interestingly, ordinary alkyl phosphonates are not active against *P. falciparum*, most likely due to a lack of bioactivation. However, alkyl phosphonates have been reported to be active against *Mycobacterium tuberculosis in vitro*.[20]

The more complex acyloxyalkyl and alkoxycarbonyloxyalkyl esters offer additional opportunities for cleavage by esterase type enzymes (Scheme 4).[19] The concept of phosphonate drug modification, especially the alkoxycarbonyloxymethyl and pivaloyloxyalkyl derivatives, have resulted in the success of antiviral drugs Tenofovir disoproxil fumarate[22] and Adefovir dipivoxil,[23] whose pharmacological properties have been investigated in considerable detail.

In the present study, the best results were obtained with the *n*-butyloxycarbonyloxymethyl group. This may indicate a preferred cleavage of the alkoxycarbonyloxymethyl motif, where the *n*-butyl residue would be preferable to the isopropyl residue for steric reasons. The enzymatic step could be followed by spontaneous release of carbon dioxide, followed by formaldehyde.

How Are the Hydroxamic Acid Prodrugs Bioactivated? It appears obvious that the hyxdroxamate prodrugs would have to be activated by esterase-like enzymes of the host or parasite cells, although details are not known. Because of the presence of different phosphonate ester groups and the relatively small number of double prodrugs tested, it is difficult to derive comprehensive structure− activity relationships.

However, the data show that *O*-acetyl hydroxamates (**4bA**, **4cA**) are more active than the quite bulky *O*-pivaloyl hydroxamates (**4dD**, **4cD**) and that *N*-ethyl substituted carbamates (**4bC**, **4cC**, **5cC**) are stronger *P. falciparum* growth inhibitors than ethyl carbonates (**4bB**, **4cB**, **5cB**). One exception is the bulky morpholine based carbamate **4cE**, which is inactive *in vitro* toward all *P. falciparum* strains used in this study. While the structure−metabolism relationships of various carbamate prodrugs has recently been reviewed by Testa and co-workers,[24] no comparable studies have yet been published on carbamate prodrugs of hydroxamic acids. However, it appears that steric restrictions are responsible for the lack of *in vitro* activity of **4cE**. It remains unknown whether the hydrolysis of the dual-modified prodrugs begins at the hydroxamate or phosphonate motif. Compounds with exclusive modification of the hydroxamate motif would be useful for further studies but have not yet been obtained.

Where Are the Phosphonate Prodrugs Bioactivated? By comparison with antiviral drugs and prodrugs, the antimalarial activity involves a much more complex situation at the level of cellular topology. Specifically, oral antiviral drugs must be capable of gastrointestinal absorption and of permeation into the nuclear and/or cytoplasmic compartments of target cells, the site of viral replication and assembly. By contrast, in order to attack IspC, which is located in the apicoplast compartment of the erythrocyte stage of *Plasmodium*, an oral drug must be capable of gastrointestinal absorption, passage through the erythrocyte membrane (whose properties are known to be modified by the parasite),[25] and passage through up to six additional membranes.[26] More specifically, in the *in vitro* parasite assay, inhibitors (i.e., mother compounds, prodrugs, and/or the products resulting from partial or complete hydrolysis or unmodified fosmidomycin type compounds) must pass through the erythrocyte membrane, the parasitophorous vacuole membrane (PVM), the *Plasmodium* cell membrane, and the four membrane layers of the apicoplast. Partial or terminal prodrug hydrolysis could be proceeding in any or all of the following compartments: (i) the erythrocyte cytoplasm, (ii) the parasitophorous vacuole, (iii) the parasite cytoplasm, and/or (iv) inside the apicoplast (Figure 2B).

Since the activity ratio of prodrugs and their mother compounds is different for the three *P. falciparum* strains used, it appears unlikely that the compounds undergo complete hydrolysis in the erythrocyte cytoplasm. Hence, at least a significant fraction of the prodrugs must be able to access the parasite without prior hydrolysis and must then be hydrolyzed at some point inside the parasite to yield the actual inhibitor. Notably, however, it remains unknown whether hydrolysis proceeds in the parasite cytoplasm and/or inside the apicoplast.

***In Vivo* Antimalarial Studies with Prodrugs 4bC and 4cC and Its Parent Compound 4 in Two Mouse Models of Malaria.** On the basis of their promising antiplasmodial *in vitro* properties and excellent selectivity indices, compounds **4** and **4cC** were selected and tested for their *in vivo* activity in *P. berghei*-infected NMRI mice (Table 3).[27] A standard Peters test using daily oral or intraperitoneal doses of 50 mg/kg body weight was conducted for 4 days. To preliminarily assess their efficacy, pharmacokinetic properties, and compound stability *in vivo*, both compounds were administered via the oral and intraperitoneal routes. However, regardless of the method of application, neither mother compound **4** nor prodrug **4cC** displayed pronounced efficacy in this *in vivo* model, evidenced by activities of 56.9−68.4% and MSD of 4.0−6.7 days, which is comparable to the MSD of control mice (~6.0 days) (Table 3).

Table 3. *In Vivo* Studies Using *Plasmodium berghei*-Infected Mice[a,28]

	parasitized erythrocytes [%][b]	activity [%]	survival [days]
control	35	0	4.0[c]
4 (p.o.)	15	57	6.0
4 (i.p.)	13	62	6.7
4cC (p.o.)	15	58	6.3
4cC (i.p.)	11	68	6.3

[a]A standard Peters test using daily oral or intraperitoneal doses of 50 mg/kg body weight was conducted for 4 days. Briefly, compounds were dissolved or suspended in 70:30 Tween 80/ethanol and diluted 10× with water. Experimental groups (n = 3 mice) were administered 4× by the oral (p. o.) or intraperitoneal (i.p.) route (once per day; 4, 24, 48, and 72 h postinfection). [b]Blood was collected on day 4 (96 h after infection). Parasitemia reduction (activity) and mean survival time in days (MSD) for multidose regimens are reported. Activity was calculated as the difference between the mean percent parasitaemia for the control and treated groups expressed as a percent relative to the control group. [c]Mice were euthanized on day 4 postinfection in order to prevent death otherwise occurring on day 6. For comparison, clinically used antimalarial drugs such as artesunate and mefloquine, administered in this *P. berghei* model at four daily oral doses of 30 mg/kg, showed a reduction in parasitemia of 99.0 and 99.9% with a mean survival time of 10 and 29 days.[29]

While parent compound **4** and prodrug **4cC** showed high potency in the cell culture assays against sensitive and multidrug-resistant strains of *P. falciparum*, the outcome of our initial *in vivo* studies was less promising (Table 3). Both compounds displayed <70% *in vivo* efficacy with a mean survival time of 6.3 to 6.7 days, which is comparable with the mean survival time of untreated, infected control mice (6 days, Table 3). There are several explanations for the weak *in vivo*

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activity of **4** and **4cC** in the *P. berghei* mouse model such as chemical instability, metabolic instability, insufficient bioactivation of **4cC**, and structural differences between the targets *Pf*IspC and *Pb*IspC. It should be noted, however, that species differences cannot be addressed *in vitro* since studies on IspC protein of *P. berghei* are not available.

To exclude differences between both *Plasmodium* species (and both target enzymes), the *in vivo* antimalarial activity of parent compound **4** and prodrug **4bC** was also studied in the SCID mouse *P. falciparum* model. This recently established model uses SCID mice engrafted with human erythrocytes, offering the possibility to investigate the actual target parasite *P. falciparum in vivo*. Parasitemia reduction (activity) is reported in Table 4 and is in accordance with the *P. berghei* model; neither mother compound **4** nor prodrug **4bC** displayed significant *in vivo* activity in the SCID mouse *P. falciparum* model.[30,31]

Table 4. *In Vivo* Studies Using the *P. falciparum* Mouse Model[a]

	parasitized erythrocytes [%][b]	activity [%]
control (n = 5 mice)	12	0
4bC (p.o.) (n = 2 mice)	14	0
4 (i.p.) (n = 2 mice)	14	0

[a]A standard Peters test using daily oral or intraperitoneal doses of 50 mg/kg body weight was conducted for 4 days. Compounds were dissolved or suspended in 70:30 Tween 80/ethanol and diluted 10× with water. [b]Blood was collected on day 4 after the first treatment. All mice were euthanized after blood collection in order to prevent death otherwise occurring 3−5 days later. Experimental and control groups (n = 2 and 5 mice, respectively) were treated 4× by the oral (p.o.) or intraperitoneal (i.p.) route (once per day 3, 4, 5, and 6 days postinfection). For comparison, clinically used antimalarial drugs such as artesunate and chloroquine, administered in this *P. falciparum* model, showed a reduction in parasitemia of 90% at four daily oral doses of 13 and 5 mg/kg.[30]

Since both prodrugs **4cC** and **4bC** are not cytotoxic to mammalian cells and highly active against different strains of *P. falciparum in vitro*, we assume that the lack of significant *in vivo* antiplasmodial activity in both mouse models of malaria (regardless the way of administration) may be attributed to the metabolic and/or chemical instability of the parent compound **4**, which was inactive in both animal models. One possible explanation is the hydrolysis of the essential hydroxamic acid pharmacophore of **4** into the corresponding carboxylic acid that is known to be inactive toward *Pf*IspC and *Pf*K1 strain.[17b]

CONCLUSIONS

The *P. falciparum in vitro* proliferation assay is a valid tool for the *in vitro* characterization of preclinical antimalarial drug candidates. In this study, we used chloroquine-sensitive and multidrug-resistant *P. falciparum* strains of different geographic origin (3D7, D10, and Dd2). We showed that the *in vitro* efficacy of reverse fosmidomycin derivatives can be enhanced by more than an order of magnitude by esterification of the hydrophilic phosphonate motif. The most active phosphonate prodrugs are *n*-butyloxycarbonyloxymethyl prodrugs with IC_{50} values in the single-digit nanomolar range. We also modified the hydroxamate pharmacophore by esterification. With the exception of the morpholine-based carbamate group, all hydroxamate prodrugs were activated in the experimental system under study. In light of the present, initial study, the development of double prodrugs failed to provide a significant activity gain in all cases. Only a few double prodrugs were highly active with IC_{50} values in the single-digit nanomolar range; among them, the most active prodrug under study was **4cC** (IC_{50} value vs *Pf*Dd2: 4 nM). Additional experiments demonstrated that parent compounds **4** and **5** and the most active prodrugs showed no cytotoxicity against mammalian cells and are characterized by excellent selectivity indices. To preliminarily assess their activity *in vivo*, prodrug **4cC** and its parent compound **4** were administered via the oral and intraperitoneal routes in the *P. berghei* malaria model. However, neither mother compound **4** nor prodrug **4cC** demonstrated pronounced *in vivo* activity in the *P. berghei*-infected mouse model. To exclude species differences and thereby structural differences of the drug targets *Pf*IspC and *Pb*IspC, the *in vivo* activity of parent compound **4** and a structurally related prodrug **4bC** was also tested in the SCID mouse *P. falciparum* model. Again, neither mother compound **4** nor prodrug **4bC** displayed *in vivo* efficacy.

EXPERIMENTAL SECTION

Chemistry. *General Procedures.* All solvents and chemicals were used as purchased without further purification. The progress of all reactions was monitored on Merck precoated silica gel plates (with fluorescence indicator UV_{254}) using ethyl acetate/*n*-hexane as solvent system. Column chromatography was performed with Fluka silica gel 60 (230−400 mesh ASTM) with the solvent mixtures specified in the corresponding experiment. Spots were visualized by irradiation with ultraviolet light (254 nm). IR spectra were recorded on a Varian 800 FT-IR Scimitar series. Proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded on a Bruker Avance 500 (500.13 MHz for ^{1}H; 125.76 MHz for ^{13}C) and a Bruker Avance III-600 (600.22 MHz for ^{1}H; 150.93 MHz for ^{13}C) using DMSO-d_6 and $CDCl_3$ as solvents. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak for ^{1}H and ^{13}C and to external tetramethylsilane). Elemental analysis was performed on a PerkinElmer PE 2400 CHN elemental analyzer. If necessary, the purity was determined by HPLC. Analytical high-pressure liquid chromatography (HPLC) was performed in analogy to a previously reported procedure.[1] Instrument: Varian ProStar HPLC System [Varian ProStar 210 (pump), Varian ProStar 320 (UV-detector), and Varian ProStar 410 (autosampler)]; column: Phenomenex Luna C-18(2) 5 μm particle size (250 mm × 4.6 mm), supported by Phenomenex Security Guard Cartridge Kit C18 (4.0 mm × 3.0 mm). The purity of all final compounds determined by HPLC was 95% or higher.

Experimental Data for Compounds. Experimental data are listed below for selected compounds: **5bF**, **4b**, and **4bB**.

General Procedure for the Synthesis of *O*-Bn-Protected Prodrugs 3aF−3bF, 4aF−4cF, and 5aF−5bF. To a solution of protected phosphonohydroxamic acids **3dF−5dF** (1 eq., 3 mmol) in dry dichloromethane (20 mL) was added trimethylsilyl bromide (5 equiv, 15 mmol, 2.0 mL) at 0 °C. After 1 h, the solution was allowed to warm to room temperature and stirred for a further 23 h. The solvent was removed under reduced pressure; the residue was dissolved in THF (20 mL) and treated with water (0.2 mL). After 30 min, the solvent was evaporated, and the residue was dried in vacuo overnight. The residue was dissolved in anhydrous DMF (15 mL) and treated with triethylamine (3 equiv, 9 mmol, 1.25 mL); after stirring for 10 min at room temperature, *n*-butyl chloromethyl carbonate (**3aF−5aF**, 10 equiv, 30 mmol, 5.0 g), isopropyl chloromethyl carbonate (**3bF−5bF**, 10 equiv, 30 mmol, 4.6 g), or chloromethyl pivalate (**4cF**, 10 equiv, 30 mmol, 4.5 g) was added, and the solution was heated at 70 °C for 2 h. The mixture was treated again with triethylamine (1 equiv, 3 mmol, 0.4 mL) and *n*-butyl chloromethyl carbonate (**3aF−5aF**, 1.5 equiv, 4.5 mmol, 0.75 g), isopropyl chloromethyl carbonate (**3bF−5bF**, 1.5 equiv, 4.5 mmol, 0.69 g), or chloromethyl pivalate (**4cF**, 1.5 equiv, 4.5 mmol, 0.68 g) and stirred for a further 2 h at 70 °C. The

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procedure of adding triethylamine and alkylating reagent was repeated once again. After two more hours at 70 °C, the reaction mixture was allowed to cool to room temperature and stirred overnight. The solution was diluted with diethyl ether (120 mL) and washed with water (60 mL), saturated aqueous solution of $NaHCO_3$ (2 × 60 mL), and once again with water (60 mL). The organic layer was dried over $MgSO_4$ and filtered, and the solvent was evaporated in vacuo. Purification of crude products **3aF−3bF**, **4aF−4cF**, and **5aF−5bF** was accomplished by column chromatography on silica gel with diethyl ether as the eluent.

*((((3,4-Difluorophenyl)(2-(hydroxy(methyl)amino)-2-oxoethoxy)-methyl)phosphoryl)bis(oxy))bis(methylene)diisopropyl Dicarbonate (**5bF**).* Yellow oil (0.39 g, 20%); ^{1}H NMR (500.13 MHz, $CDCl_3$): δ 1.30 (d, J = 6.3 Hz, 6H, $CH(CH_3)_2$), 1.31 (d, J = 6.2 Hz, 6H, $CH(CH_3)_2$), 3.18 (s, 3H, NCH_3), 4.02 (d, J = 16.2 Hz, 1H, OCH_2C=O), 4.35 (dd, J_1 = 16.1 Hz, J_2 = 0.9 Hz, 1H, OCH_2C=O), 4.74 (dd, J_1 = 15.7 Hz, J_2 = 10.9 Hz, 2H, OCH_2Ph), 4.90 (dq, J_1 = 12.1 Hz, J_2 = 6.1 Hz, $CH(CH_3)_2$), 4.97 (d, $^2J_{H,P}$ = 14.0 Hz, 1H, PCH), 5.62 (dd, J_1 = 12.4 Hz, J_2 = 5.3 Hz, 1H, $POCH_2$), 5.68 (dd, J_1 = 11.6 Hz, J_2 = 5.5 Hz, 1H, $POCH_2$), 5.68 (d, J = 11.7 Hz, 2H, $POCH_2$), 7.10−7.18 (m, 2H, $C_6H_3F_2$), 7.22−7.36 (m, 6H, C_6H_5, $C_6H_3F_2$) ppm; ^{13}C NMR (125.76 MHz, $CDCl_3$): δ 21.61 ($CH(CH_3)_2$), 33.44 (NCH_3), 66.85 (d, $^3J_{C,P}$ = 12.3 Hz, OCH_2C=O), 73.25 ($CH(CH_3)_2$), 76.25 (OCH_2Ph), 76.62 (d, $^1J_{C,P}$ = 156.6 Hz, PCH), 84.67 (d, $^2J_{C,P}$ = 6.5 Hz, $POCH_2$), 84.80 (d, $^2J_{C,P}$ = 6.5 Hz, $POCH_2$), 117.40 (m), 124.57 (m), 128.75, 129.23, 129.44, 129.94 (m), 133.91, 150.32 (ddd, $^1J_{C,F}$ = 248.2 Hz, $^2J_{C,F}$ = 12.08 Hz, $^5J_{C,P}$ = 2.4 Hz), 150.66 (ddd, $^1J_{C,F}$ = 251.1 Hz, $^2J_{C,F}$ = 12.2 Hz, $^4J_{C,P}$ = 3.4 Hz, $C_6H_3F_2$: C3), 153.03 (C=O, carbonate), 153.06 (C=O, carbonate), 170.51 (C=O, hydroxamate) ppm; IR (NaCl): $\tilde{\nu}$ = 2986 ($C-H_{aliph.}$), 1760 (C=O), 1266 (P=O), 1030 (P−O) cm^{-1}; Anal. Calcd For $C_{27}H_{34}F_2NO_{12}P$: C, 51.19; H, 5.41; N, 2.21. Found: C, 51.17; H, 5.27; N, 2.27.

General Procedure for the Synthesis of Prodrugs 3a,b, 4a−c, and 5a,b. Bn-protected prodrugs **3aF−3bF**, **4aF−4cF**, and **5aF−5bF** (0.4 mmol) were dissolved in methanol (30 mL). Pd−C calalyst (10%, 15% w/w) was added, and the respective mixture was hydrogenated for 2 h at atmospheric pressure. The catalyst was removed by filtration. and the solvent was evaporated in vacuo to yield target compounds **3a,b**, **4a−c**, and **5a,b**.

*(((1-(3,4-Difluorophenyl)-4-(hydroxy(methyl)amino)-4-oxobutyl)-phosphoryl)bis(oxy))bis(methylene)diisopropyl Dicarbonate (**4b**).* Colorless oil (0.21 g, 97%); ^{1}H NMR (500.13 MHz, DMSO-d_6): δ 1.38−1.15 (m, 12H, $CH(CH_3)_2$), 1.87−1.72 (m, 1H, $CHCH_2$), 1.97 (q, J_1 = 14.2 Hz, J_2 = 7.9 Hz, 1H, $CHCH_2$), 2.20 (tdd, J_1 = 19.9 Hz, J_2 = 17.2 Hz, J_3 = 14.2 Hz, J_4 = 8.4 Hz, 2H, CH_2CO), 3.50−3.40 (m, 1H, PCH), 4.79 (ddq, J_1 = 30.9 Hz, J_2 = 12.1 Hz, J_3 = 6.1 Hz, 2H, OCH), 5.45 (d, J = 12.7 Hz, 2H, OCH_2O), 5.81−5.50 (m, 2H, OCH_2O), 7.21−6.98 (m, 1H), 7.31 (dd, J_1 = 11.9 Hz, J_2 = 7.7 Hz, 1H), 7.41 (q, J = 9.1 Hz, 1H), 9.70 (s, 1H, NH) ppm; ^{13}C NMR (125.76 MHz, DMSO-d_6): δ 21.10 ($CH(CH_3)_2$), 21.14 ($CH(CH_3)_2$), 21.18 (2C, $CH(CH_3)_2$), 23.76 (CH_2CH_2), 29.10 (d, $^2J_{C,P}$ = 16.8 Hz, $CHCH_2$), 35.53 (NCH_3), 41.54 (d, $^1J_{C,F}$ = 137.0 Hz, PCH), 72.62 ($CH(CH_3)_2$), 72.76 ($CH(CH_3)_2$), 83.90 (d, $^2J_{C,P}$ = 6.5 Hz, $POCH_2$), 83.99 (d, $^2J_{C,P}$ = 6.8 Hz, $POCH_2$), 117.44 (d, $^2J_{C,F}$ = 17.0 Hz), 117.97 (dd, $^2J_{C,F}$ = 17.6 Hz, $^3J_{C,F}$ = 6.7 Hz), 126.01, 132.32, 148.62 (dd, $^1J_{C,F}$ = 244.9, $^2J_{C,F}$ = 12.0 Hz), 149.09 (dd, $^1J_{C,F}$ = 248.9 Hz, $^2J_{C,F}$ = 12.6 Hz), 152.42 (C=O, carbonate), 152.51 (C=O, carbonate), 171.57 (C=O, hydroxamate) ppm; IR (NaCl): $\tilde{\nu}$ = 3217 (O−H), 3061 ($C-H_{aromat.}$), 2988 ($C-H_{aliph.}$), 1762 (C=O, carbonate) 1647 (C=O, hydroxamate), 1272 (P=O), 1032 (P−O) cm^{-1}; Anal. Calcd for $C_{21}H_{30}F_2NO_{11}P$: C, 46.59; H, 5.58; N, 2.59. Found: C, 46.67; H, 5.61; N, 2.43.

General Procedure for the Synthesis of Double Ester Prodrugs 4bA−D, 4cA−D, and 5cB−C. Prodrugs **4b**, **4c**, and **5c** (1 equiv, 0.3 mmol) were dissolved in dry dichloromethane (5 mL) and treated with triethylamine (1 equiv, 0.3 mmol, 0.05 mL). At 0 °C, solutions of acetyl chloride (**4bA**, **4cA**, 1.1 equiv, 0.33 mmol, 0.026 g), ethyl chloroformiate (**4bB**, **4cB**, **5cB**, 1.1 equiv, 0.33 mmol, 0.013 g), ethyl isocyanate (**4bC**, **4cC**, **5cC**, 1 equiv, 0.3 mmol, 0.021 g), or pivaloyl chloride (**4bD**, **4cD**, 1 equiv, 0.3 mmol, 0.036 g) in dry dichloromethane (2 mL) were added dropwise, respectively. After 10 min, the reaction mixture was allowed to warm to room temperature and stirred for 30 min. The solution was concentrated under reduced pressure, and the residue was dissolved in 20 mL of ethyl acetate. The organic layer was washed with water (2 × 5 mL), dried over $MgSO_4$, and filtered, and the solvent was removed under reduced pressure. Compounds **4bA−C**, **4cA−C**, and **5cB−C** were purified by column chromatography using ethyl acetate as the eluent.

*((((1-(3,4-Difluorophenyl)-4-(((ethoxycarbonyl)oxy)(methyl)-amino)-4-oxobutyl)-phosphoryl)bis(oxy))bis(methylene)diisopropyl Dicarbonate (**4bB**).* Light yellow oil (0.07 g, 38%); ^{1}H NMR (500.13 MHz, DMSO-d_6): δ 1.19−1.31 (m, 15H, CH_3), 1.92−2.05 (m, 1H, CH_2), 2.05−2.15 (m, 1H, CH_2), 2.15−2.29 (m, 2H, CH_2), 3.17 (s, 3H, NCH_3), 3.49 (dd, J = 24.6, 10.1 Hz, 1H, PCH), 4.24 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 4.77 (td, J = 12.5, 6.3 Hz, 1H, OCH), 4.84 (td, J = 12.5, 6.3 Hz, 1H, OCH), 5.42−5.51 (m, 2H, OCH_2O), 5.53−5.65 (m, 2H, OCH_2O), 7.07−7.14 (m, 1H), 7.29 (dd, J_1 = 10.6 Hz, J_2 = 8.8 Hz, 1H,), 7.42 (dd, J_1 = 19.1 Hz, J_2 = 8.7 Hz, 1H) ppm; ^{13}C NMR (125.76 MHz, DMSO-d_6): δ 21.54 (CH_3), 21.58 (CH_3), 21.62 (CH_3), 24.08 (m, CH_2CH_2), 29.20 (d, $^2J_{C,P}$ =17.5 Hz, $CHCH_2$), 35.30 (NCH_3), 35.95 (OCH_2CH_3), 41.67 (d, $^1J_{C,P}$ = 137.8 Hz, PCH), 73.09 (OCH), 73.23 (OCH), 84.38 (d, $^2J_{C,P}$ = 6.6 Hz, OCH_2O), 84.47 (d, $^2J_{C,P}$ = 7.0 Hz, OCH_2O), 117.95 (d, $^2J_{C,F}$ = 15.4 Hz), 118.41 (dd, $^2J_{C,F}$ = 17.6 Hz, $^3J_{C,F}$ = 6.7 Hz), 126.41 (m), 132.49 (m), 149.17 (dd, $^1J_{C,F1}$ = 245.2 Hz, $^2J_{C,F2}$ = 12.4 Hz), 149.57 (dd, $^1J_{C,F1}$ = 244.2 Hz, $^2J_{C,F2}$ = 12.3 Hz), 152.87, 152.95, 153.52 (C=O, carbonate), 175.99 (C=O, hydroxamate) ppm; IR (NaCl): $\tilde{\nu}$ = 2987 ($C-H_{aliph.}$), 1765 (C=O, hydroxamate), 1684 (C=O, carbonate), 1267 (P=O), 1030 (P−O) cm^{-1}; Anal. Calcd for $C_{24}H_{34}F_2NO_{13}P$: C, 46.99; H, 5.59; N, 2.28. Found: C, 47.18; H, 5.59; N, 2.32.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental procedures for syntheses, enzyme assays, and evaluation of biological activity; analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CDI, 1,1′-carbonyldiimidazole; DOXP/DXP, 1-deoxy-D-xylulose 5-phosphate; Dxr/IspC, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; ELISA, enzyme linked immunoassay; HRP2, histidine-rich protein 2; Pd−C, palladium on activated carbon; *Pf*IspC, *P. falciparum* IspC; PVM, parasitophorous vacuole membrane; SD, standard deviation; TEA, triethylamine; TMSBr, trimethylsilyl bromide

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