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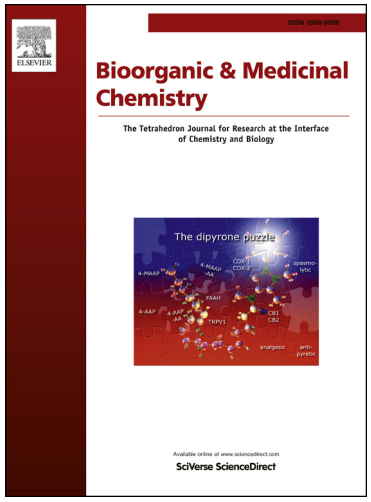

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Antimalarial activity of prodrugs of *N*-branched acyclic nucleoside phosphonate inhibitors of 6-oxopurine phosphoribosyltransferases

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Abstract

Acyclic nucleoside phosphonates (ANPs) that contain a 6-oxopurine base are good inhibitors of the human and *Plasmodium falciparum* 6-oxopurine phosphoribosyltransferases (PRTs), key enzymes of the purine salvage pathway. Chemical modifications, based on the crystal structures of several inhibitors in complex with the human PRTase, led to the design of a new class of inhibitors – the aza-ANPs. Because of the negative charges of the phosphonic acid moiety, their ability to cross cell membranes is, however, limited. Thus, phosphoramidate prodrugs of the aza-ANPs were prepared to improve permeability. These prodrugs arrest parasitemia with IC_{50} values in the micromolar range against *Plasmodium falciparum*-infected erythrocyte cultures (both chloroquine-sensitive and chloroquine-resistant *Pf* strains). The prodrugs exhibit low cytotoxicity in several human cell lines. Thus, they fulfill two essential criteria to qualify them as promising antimalarial drug leads.

Introduction

Malaria remains one of the most important infectious diseases in the world today, with ~48% of the human population living in areas of risk.[1] The two principal protozoan parasites that cause human malaria are *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*). The frontline defence against malaria has been chemotherapeutic drugs (*i.e.* chloroquine, quinine, artemisinin-combination therapies - ACTs). However, because of developing resistance to first-line treatment drugs (*i.e.* ACTs) in Southeast Asia, there is an urgent need to discover new targets and drugs that are capable of combating this disease.[2,3]

Hypoxanthine-guanine-(xanthine) phosphoribosyltransferase [HG(X)PRT] is a recognized target for drugs aimed at malaria.[4,5] This is because HG(X)PRT is a key enzyme of the purine salvage pathway where it synthesises the nucleoside monophosphates required for DNA/RNA production. Mammals are able to synthesize the purine ring *de novo* and, hence, do not have an absolute reliance on the salvage pathway. However, parasites of the genus *Plasmodium* depend entirely on the transport of preformed bases from the host cell and, thus, rely on the activity of HG(X)PRT for both survival and replication.[6] This enzyme catalyses the formation of the 6-oxopurine nucleoside monophosphates (IMP, GMP or XMP) from a purine base (hypoxanthine, guanine or xanthine, respectively) and 5-phospho-α-D-ribosyl-1-pyrophosphate (PRib-PP) (**Fig. 1A**).[7]

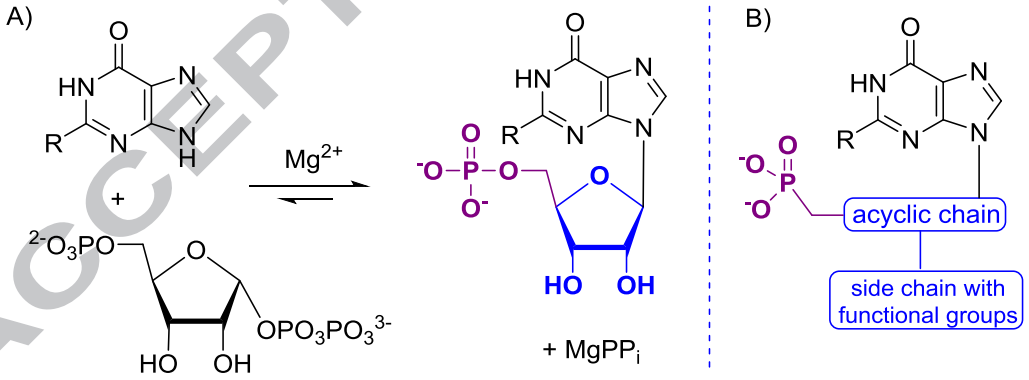


Fig. 1. A) The reaction catalysed by HG(X)PRT. R = -H (hypoxanthine); -NH_2 (guanine); -OH (xanthine). B) General structure of ANP-based inhibitors – analogues of nucleoside monophosphates.

Acyclic nucleoside phosphonates (ANPs) represent a group of compounds with remarkable biological activities and some of them are currently in use as antiviral agents that

act by inhibiting viral DNA polymerases or reverse transcriptases.[8] The absence of a glycosidic bond and the phosphonate moiety replacing phosphate in these nucleotide analogues explains their resistance to enzymatic degradation. We proposed that replacement of the pyrimidine or 6-amino purine base in the structure of antiviral ANPs, by either guanine or hypoxanthine, results in potent and selective inhibition of *Pf* HGXPRT.[9] We have since investigated how the inhibitory potency and selectivity *versus* the human enzyme is affected by acyclic chain length or branching of phosphonoethoxyethyl chain (**Fig. 1B**).[10-12] Since highly conserved regions of amino acid residues are involved in the catalysis, cocrystal structures[9] of ANPs interacting with the active site of human HGPRT provides an excellent model to design suitable new inhibitors. Based on these insights, we have been able to synthesize new compounds that can, with a high degree of complementarity, precisely fill the active site of PRTases.[13] By positioning a nitrogen atom at the branching point in the acyclic moiety, the route to chemical synthesis was simplified. Thus, series of aza-ANPs were synthesized.[14,15]

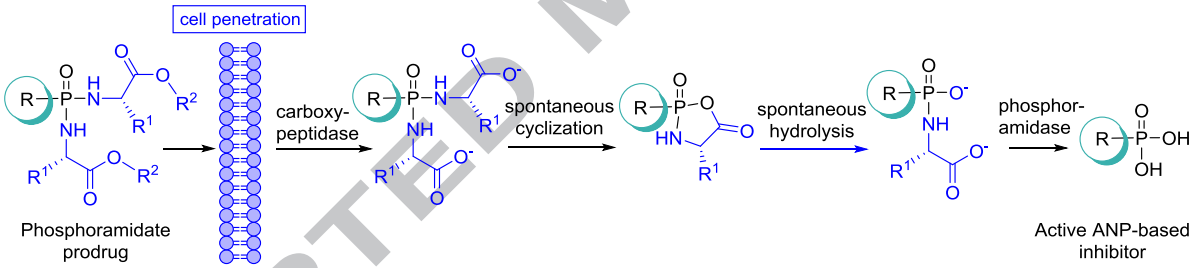
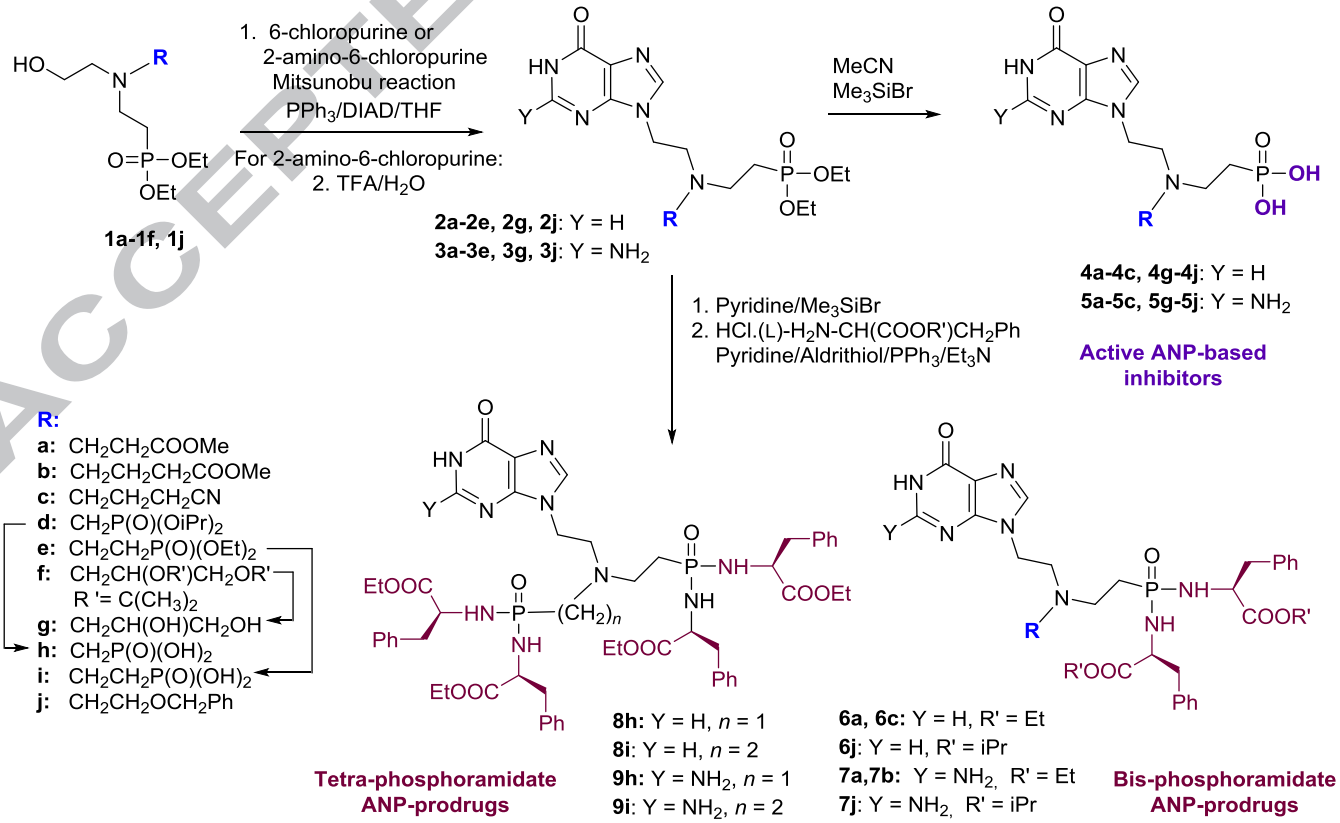


Fig. 2. Proposed mechanism of phosphoramidate prodrug cleavage.[17]

The presence of the phosphonate group in ANPs is responsible for their highly polar character and deprotonation at physiological pH. Much of the recent commercial success of the antiviral or cytostatic ANPs can be attributed to the development of a range of prodrug groups that are attached to the active compound to mask the charge of the phosphonate moiety. Thus, these groups facilitate transport across cell membranes and improve the pharmacological properties of the ANPs.[16,17] In agreement with the literature, we have observed that the preparation of various types of prodrugs of the parent ANP-based inhibitors can significantly improve the activity in cell-based antimalarial assays.[13,15] As a prodrug of choice, we have selected compounds with a phosphoramidate linkage[18] having two identical amino acid esters attached, to facilitate entry into the cells. Due to the presence of equivalent substituents attached to the phosphorus atom, there are no difficulties associated with prodrug

chirality. In addition, only non-toxic amino acid molecules are released after prodrug cleavage (**Fig. 2**).[17] Several ANP-prodrugs of this type are now in clinical trials as antiviral or anticancer therapeutics.[19] These compounds are able to cross the cellular membranes efficiently and the P-N bond is then cleaved inside the cells. Their relatively high stability in plasma and low cytotoxicity towards mammalian cells make them prime candidates for the development of chemotherapeutics against malaria parasites.

In this paper, we report on the preparation of the phosphoramidate prodrugs of the series of aza-ANP-based PRTase inhibitors. In addition, synthesis of six new aza-ANPs (**4g, 4h, 4j** and **5g, 5h, 5j**) complementing the previously prepared[14,15] inhibitors of *Pf, Pv* and human HG(X)PRT is described, and their inhibitory activity (*Pf*HGXPRT and human HGPRT) is reported to extend our published data. According our recent findings, phenylalanine prodrugs are more stable in plasma and their higher lipophilicity enable better penetration to the cells compared to alanine used in antivirals. The pheylalanine prodrugs are used continuously in the whole SAR-study to allow us comparison among the various types of ANP-based inhibitors. All prepared phosphonate and bisphosphonate prodrugs (**6a, 6c, 6j**, **7a, 7b, 7j**, **8h, 8i** and **9h, 9i**) in this current study carry L-phenylalanine isopropyl- or ethylester moieties to mask the polar phosphonate groups. Their antimalarial activity was evaluated in erythrocyte cell cultures infected with either chloroquine-sensitive (D6) or chloroquine-



Scheme 1: Synthesis of ANP-based inhibitors of *Pf* and human HG(X)PRT and their phosphoramidate prodrugs.

resistant (W2) strains of *Pf*. Their cytotoxicity in mammalian cells was determined as this is a crucial element in determining the potential of such compounds as drug leads.

Chemistry

The synthesis of aza-ANP phosphoramidate prodrugs **6-9** (**Scheme 1**) was based on a recently published method.[20] Starting diethyl esters of phosphonates **2a-2c, 2e, 3a-3c** and **3e** were prepared as we described previously.[14] The series was complemented with new bisphosphonates containing a phosphonomethyl group (**2d** and **3d**), compounds with racemic dihydroxypropyl moiety mimicking the sugar scaffold (**2g** and **3g**) and derivatives bearing an aryl group (**2j** and **3j**) that could enable interaction with a tyrosine side chain that is found in a large mobile loop that is thought to close over the enzyme's active site during catalysis.[21] For the synthesis of these new derivatives, a similar procedure[14] was applied (**Scheme 1**): 2-(2-hydroxyethylamino)ethylphosphonate[14] was *N*-alkylated by diisopropyl bromomethylphosphonate, 4-chloromethyl-2,2-dimethyl-1,3-dioxolane or ((2-bromoethoxy)methyl)benzene, respectively, using K_2CO_3 as a base. Thus formed *N*-branched hydroxyderivatives **1d, 1f** and **1j** were attached to the N^9-position of 6-chloropurine or 2-amino-6-chloropurine *via* Mitsunobu reaction. In the case of alkylation of 2-amino-6-chloropurine, the Mitsunobu reaction had to be followed by heating in water/tetrahydrofuran to decompose the triphenylphosphoranylidene intermediate rising from the presence of the free amino group.[22] The resulting 6-chloropurine derivatives were transformed to hypoxanthine aza-ANP esters **2d, 2g** and **2j** by nucleophilic aromatic substitution in acidic conditions (75% aqueous trifluoroacetic acid). The chlorine atom of 2-amino-6-chloropurine derivatives was displaced with hydroxyl by the same method to form guanine aza-ANP esters **3d, 3g** and **3j**. As expected, the isopropylidene protecting group was simultaneously cleaved from dihydroxyderivatives **2g** and **3g** under these acidic conditions.

The ester groups of the new derivatives **2d, 2g, 2j,** and **3d, 3g, 3j** were cleaved under standard conditions using Me_3SiBr/acetonitrile followed by hydrolysis (**Scheme 1**) to form aza-ANP-based inhibitors **4g, 4h, 4j** and **5g, 5h, 5j**. The synthesis of the free phosphonic acids **4a-4c, 4i** and **5a-5c, 5i** was described in our previous paper.[14]

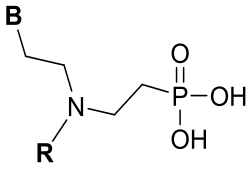
The diethylesters of phosphonates (**2a, 2c, 2j** and **3a, 3b, 3j**) and tetraesters of bisphosphonates (**2d, 2e** and **3d, 3e**) were used for the direct preparation[20] of the

phosphoramidate prodrugs **6-9** suitable for antimalarial testing in the cell-based assays. The selection of this particular type of phosphonate prodrug was based on our previous experience which led to an increase of membrane permeability and bioavailability for the antiviral ANPs.[16,18,23,24] In the first step of the one-pot reaction sequence, the cleavage of the phosphonate esters **2** and **3** with Me_3SiBr in dry pyridine under argon atmosphere forms *in situ* the corresponding trimethylsilyl esters. In the second step, the reaction of these intermediates with ethyl (or isopropyl for **6j** and **7j**) (L)-phenylalanine in the presence of 2,2′-dithiodipyridine (Aldrithiol) and triphenylphosphine yielded the corresponding target bis-amidates **6a, 6c, 6j** and **7a, 7b, 7j** and tetra-amidates **8h, 8i** and **9h, 9i** (**Scheme 1**). To prepare absolutely pure prodrugs for cell-based assays, the complex reaction mixtures were purified by column chromatography on silica gel and the crude products were further purified by preparative HPLC on C18 reverse phase.

Inhibitory effect of the three new aza-ANPs against human HGPRT and *Pf*HGXPRT and evaluation of ten new prodrugs for antimalarial activity and cytotoxicity.

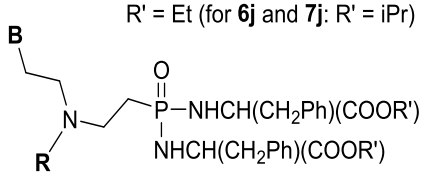
To complement previously published series, six new aza-ANPs (**4g, 4h, 4j** and **5g, 5h, 5j**) were synthesized with either guanine or hypoxanthine as the base. All contain a phosphonate group connected to the N^9 atom of the purine base by five-atom linker (**Scheme 1**). This phoshonate group has been found to occupy the 5′-phosphate binding pocket in the active site in the 6-oxopurine PRTases.[9,13,15] The three structural types of aza-ANPs differ in the substituent that is covalently attached to the nitrogen atom in the linker (Table 1).

Table 1: Comparison of K_i values of aza-ANPs for human HGPRT and *Pf*HGXPRT. Values are means ± SD.

	K_i (μM)					
	B = Guanine			**B** = Hypoxanthine		
R		human	*Pf*		human	*Pf*
$-CH_2CH(OH)CH_2OH$	**5g**	0.6±0.2	0.6±0.2	**4g**	3.8±1.0	5.0±2.0
$-CH_2P(O)(OH)_2$	**5h**	2.0±0.8	1.0±0.6	**4h**	10.0±3.0	6.0±2.0
$-CH_2CH_2OCH_2Ph$	**5j**	1.9±0.4	2.4±0.3	**4j**	13.0±2.0	4.3±0.4

All six of these ANPs inhibit both human HGPRT and *Pf*HGXPRT. Compound **5g** exhibited the lowest K_i value (*i.e.* 0.6 μM) for *Pf*HGXPRT, and thus, this scaffold has good potential for further development of highly potent inhibitors of this enzyme. For both enzymes, ANPs with guanine as the base had lower K_i values compared with those ANPs where hypoxanthine is the base. These ANPs exhibited similar affinity for both enzymes, suggesting that the interactions between the inhibitor and the active site residues could be identical for the two enzymes. The range of K_i values for *Pf*HGXPRT for previously published inhibitors **4a-c**, **4i** and **5a-c**, **5i** was 0.1-6 μM.[14]

Table 2: *In vitro* antimalarial activity and cytotoxicity of the aza-ANP prodrugs.

R' = Et (for **6j** and **7j**: R' = iPr)  **R**		IC_{50} (μM) D6[a]	IC_{50} (μM) W2[b]	CC_{50} (μM) A549[c]	CC_{50} (μM) C32[d]	CC_{50} (μM) C32-TG[e]	SI*	Free aza-ANP inhibitor
B = Hypoxanthine								
CH_2CH_2COOMe	**6a**	3.5±1.0	2.9±1.1	70±9	>300	212±3	>44	**4a**
$CH_2CH_2CH_2CN$	**6c**	4.2±1.4	5.5±1.6	30±8	102±22	89±12	15	**4c**
$CH_2CH_2OCH_2Ph$	**6j**	1.5±0.5	2.8±1.2	23±1	13±2	18±4	8	**4j**
$CH_2P(O)(NHZ)_2$ Z = $CH(CH_2Ph)(COOR')$	**8h**	4.7±1.1	1.6±0.2	41±5	23±0	25±0	9	**4h**
$CH_2CH_2P(O)(NHZ)_2$ Z = $CH(CH_2Ph)(COOR')$	**8i**	3.1±0.3	2.7±0.3	79±10	48±2	48±2	20	**4i**
B = Guanine								
CH_2CH_2COOMe	**7a**	11.8±1.6	5.6±1.8	>300	>300	>300	>34	**5a**
$CH_2CH_2CH_2COOMe$	**7b**	8.6±1.5	5.7±3.9	≥232	234±11	≥246	>33	**5b**
$CH_2CH_2OCH_2Ph$	**7j**	1.6±0.2	2.9±1.0	20±4	17±4	23±1	9	**5j**

$CH_2P(O)(NHZ)_2$ Z = $CH(CH_2Ph)(COOR')$	**9h**	10.4±1.4	4.6±0.2	>300	65±24	56±1	>8	**5h**
$CH_2CH_2P(O)(NHZ)_2$ Z = $CH(CH_2Ph)(COOR')$	**9i**	6.3±0.1	7.4±2.1	144±83	51±1	53±5	12	**5i**

[a]Chloroquine-sensitive (D6) or [b]chloroquine-resistant (W2) *Plasmodium falciparum* cultured in erythrocyte cell cultures. [*]Estimated selectivity index = average CC_{50} for A549, C32 and C32-TG divided by the average IC_{50} for *Pf* in erythrocyte cell cultures. [c]Human lung carcinoma cells [d]Human melanoma cells [e]Thioguanine-resistant mutant, expressing no functional human HGPRT due to exon-2 deletion.[25] IC_{50} and CC_{50} values are means ± SD and means ± SEM, respectively.

The antimalarial activities and cytotoxicity data for ten new prodrugs of the aza-ANPs are given in Table 2. The potential cytotoxicity of the prodrugs was determined in three human tumor cell lines, *i.e.* human lung carcinoma A549 cells; human C32 melanoma cells; and C32-TG, an HGPRT-deficient mutant form of the C32 cells. In general, the 50% cytotoxic concentrations (CC_{50}) were very similar in the three cell lines. All ten aza-ANP prodrugs exhibited *in vitro* antimalarial activity in the micromolar range in erythrocyte cell cultures infected with either the chloroquine-sensitive (D6) or chloroquine-resistant (W2) strains of *Pf*. In all cases, except for the compounds with benzyl substituent **6j** and **7j**, the aza-ANPs with the lowest IC_{50} values were those with hypoxanthine as the base. However, the prodrugs with the lowest cytotoxicity were those with guanine as the base. As a result of these preferences, compounds **6a**, **7a** and **7b** have the best selectivity index ratios suggesting that they could be the optimal compounds for further development as antimalarials amongst this group of prodrugs. The latter two compounds showed no or marginal cytotoxicity at concentrations of 230-300 μM, yet produced activity against the malaria parasite in the concentration range 6-12 μM.

The lowest IC_{50} values were for compounds **6j** and **7j** (approx. 1.5 μM for D6 and 2.8 μM for W2), but these prodrugs also have the lowest selectivity indexes (SI: 8 and 9). For these prodrugs, both the IC_{50} values and CC_{50} values were the same irrespective of the purine base. This prodrug carries an isopropyl moiety instead of the ethyl group used in all the other prodrugs examined here. Thus, this data could reflect that this type of prodrug has greater cell permeability into the pathogen and/or that this group is more easily hydrolyzed by the enzymes inside the pathogen or less easily hydrolyzed by the mammalian host cell. On the

other hand these elusive properties could be caused also by the distinctive character of the aryl substituent in the side chain of **6j** and **7j**.

Conclusions

New aza-ANPs have been synthesized all of which inhibit human HGPRT and *Pf*HG(X)PRT activity. Phosphoramidate prodrugs of these and previously published aza-ANP-based inhibitors are able to arrest the growth of *Pf* in cell culture and also possess low cytotoxicity in human cells. Three prodrugs have been found from these studies that have selectivity indexes of >33. The findings also suggest that replacing of the ethyl group by *e.g.* isopropyl group in the prodrug design may influence the biological activity. The results show that subtle differences in the chemical structure of parent ANPs and their prodrugs can have a significant effect on their *in vitro* antimalarial activity and cytotoxicity.

Experimental Section

Synthesis and Analytical Chemistry. Unless otherwise stated, solvents were evaporated at 40 °C/2 kPa, and the compounds were dried over P_2O_5 at 2 kPa. NMR spectra were recorded on Bruker Avance 500 (^{1}H at 500 MHz, ^{13}C at 125.8 MHz) and Bruker Avance 400 (^{1}H at 400 MHz, ^{13}C at 100.6 MHz) spectrometers with TMS as internal standard or referenced to the residual solvent signal. Mass spectra were measured on UPLC-MS (Waters SQD-2). The purity of the tested compounds was determined by HPLC (H_2O-CH_3CN, linear gradient) and combustion analysis (C, H, N) and was higher than 95%. The basic chemicals were obtained from commercial sources or prepared according to the published procedures. Preparative HPLC purifications were performed on columns packed with 7 μm C18 reversed phase resin (Waters Delta 600 chromatograph column), 17 × 250 mm; in ca. 200 mg batches of mixtures using gradient MeOH/H_2O as eluent.

Synthesis of hydroxyderivatives 1 - General Procedure

To the mixture of diethyl 2-(2-hydroxyethylamino)ethylphosphonate[14] (2 g, 8.9 mmol), K_2CO_3 (1.2 g, 8.9 mmol) and KI (0.05 g) in dry acetonitrile (30 ml) a corresponding halogenoderivative (10 mmol) was added. The reaction mixture was stirred at 70 °C for 3 days. The solvent was then removed by evaporatation. H_2O and $CHCl_3$ were added, the organic layer separated, washed with brine and dried over anhydrous $MgSO_4$. After filtration, solvent was evaporated and the residue was purified by column chromatography on silica gel ($CHCl_3$-MeOH), the product was obtained as oil.

[N-(Diethyl)phosphonoethyl-N-(diisopropyl)phosphonomethyl]-2-aminoethanol (1d): Starting from diisopropyl bromomethylphosphonate, yield 61%. ^{1}H NMR (DMSO-d_6): 4.59 m, 2 H (iPr); 4.44 t, 2 H, J = 5.4 (OH); 3.97 m, 4 H (Et); 3.45 dd, 2 H, J = 11.6 and 5.9 (H-1'); 2.86 d, 2 H, J = 9.9 (H-5'); 2.84 m, 2 H (H-3'); 2.63 t, 2 H, J(2',1') = 6.0 (H-2'); 1.92 m, 2 H (H-4'); 1.23 m, 18 H (iPr and Et). ^{13}C NMR (DMSO-d_6): 69.54 and 69.47 (iPr); 60.77 and 60.72 (Et); 59.11 (C-1'); 56.29 (C-2'); 49.78 d, J(P,C) = 160.9 (C-5'); 48.44(C-3'); 23.70 m, 4 C (iPr); 22.03 d, J(P,C) = 134.1 (C-4'); 16.17 and 16.11 (Et). MS (ESI): m/z = 404 $[M+H]^+$.

Diethyl 2-(((2,2-dimethyl-1,3-dioxolan-4-yl)methyl)(2-hydroxyethyl)amino)ethylphosphonate (1f): Starting from racemic 4-chloromethyl-2,2-dimethyl-1,3-dioxolane, yield 45%. ^{1}H NMR (DMSO-d_6): 4.37 t, 2 H, J = 5.2 (OH); 4.11 m, 1 H (H-6'); 3.98 m, 4 H (Et); 3.53 m, 1 H, (H-7'a); 3.41 t, 2 H, J(1',2') = 5.4 (H-1'); 3.39 m, 1 H, (H-7'b); 2.74 m, 2 H (H-3'); 2.56 t, 2 H, J(2',1') = 6.4 (H-2'); 2.48 m, 2 H (H-5'); 1.89 m, 2 H (H-4'); 1.24 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 74.21 (C-6'); 67.45 (C-7'); 60.73 and 60.67 (Et); 59.05 (C-1'); 56.23 and 55.81 (C-3' and C-2'); 47.78 (C-5'); 26.65 and 25.41 (Me); 22.16 d, J(P,C) = 134.5 (C-4'); 16.17 and 16.11 (Et). MS (ESI): m/z = 340 $[M+H]^+$.

Diethyl (2-((2-(benzyloxy)ethyl)(2-hydroxyethyl)amino)ethyl)phosphonate (1j): Starting from ((2-bromoethoxy)methyl)benzene, yield 52%. ^{1}H NMR (DMSO-d_6): 7.32 m, 5 H (Ar); 4.46 s, 2 H (CH_2Ph); 4.36 t, 1 H, J = 5.4 (OH); 3.95 m, 4 H (Et); 4.48 t, 2 H, J = 6.0 (CH_2O); 3.42 m, 2 H (CH_2OH); 2.74 m, 2 H (CH_2N); 2.66 t, 2 H, J = 5.9 (CH_2N); 2.53 t, 2 H, J = 6.2 (CH_2N); 1.89 m, 2 H (CH_2P); 1.20 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 138.44, 128.06, 2 C, 127.25, 127.19, 2 C (Ar); 71.93 (CH_2Ph); 68.35 (CH_2O); 60.69, 2 C (Et); 59.13 (CH_2OH); 55.71, 52.70 and 47.57 (CH_2N); 22.42 d, J(P,C) = 134.5 (CH_2P); 16.12, 2 C (Et). MS (ESI): m/z = 360 $[M+H]^+$.

Derivatives 1a-1c and **1e:** Prepared according the literature.[14]

Synthesis of N^9-substituted hypoxanthines 2 and guanines 3 - General Procedure

To a solution of triphenylphosphine (0.9 g, 3.44 mmol) in dry THF (25 ml) cooled to –30 °C under argon atmosphere diisopropylazadicarboxylate (DIAD, 0.6 ml, 3.3 mmol) was added slowly. The mixture was stirred for 30 minutes and this preformed complex was added to 6-chloropurine (0.34 g, 2.2 mmol) or 2-amino-6-chloropurine (0.37 g, 2.2 mmol), dry THF (25 ml) and corresponding hydroxyderivative **1** (1.9 mmol) at –30 °C under argon. The resulting mixture was slowly warmed to room temperature and stirred for 3 days. (In the case of 2-amino-6-chloropurine derivatives, water (10 ml) was then added and the mixture was heated at 80 °C for additional 30 h.) Solvent was evaporated and the crude mixture was purified by

chromatography on silica gel (MeOH-$CHCl_3$) and intermediates were characterized. Thus obtained 6-chloropurine or 2-amino-6-chloropurine intermediate was dissolved in trifluoroacetic acid (aqueous, 75%, 20 ml) and stirred overnight. The solvent was evaporated and the residue codistilled with water (3x) and ethanol. After chromatography on silica gel (MeOH-$CHCl_3$) the pure hypoxanthine or guanine derivative was obtained as colorless foam.

9-[{N-(Diethyl)phosphonoethyl-N-(diisopropyl)phosphonomethyl}-2-aminoethyl]hypoxanthine (2d): Starting from **1d** and 6-chloropurine, yield 53% in two steps. ^{1}H NMR (DMSO-d_6): 12.28 s, 1 H (NH); 8.13 s, 1 H and 8.03 s, 1 H (H-2 and H-8); 4.50 m, 2 H (iPr); 4.19 t, 2 H, J(1',2') = 5.8 (H-1'); 3.93 m, 4 H (Et); 2.96 t, 2 H, J(2',1') = 5.8 (H-2'); 2.90 d, 2 H, J = 10.1 (H-5'); 2.79 m, 2 H (H-3'); 2.74 m, 2 H (H-4'); 1.19 m, 18 H (iPr and Et). ^{13}C NMR (DMSO-d_6): 156.55 (C-6); 148.23 (C-4); 145.16 (C-2); 140.67 (C-8); 123.66 (C-5); 69.62 and 69.55 (iPr); 60.85 and 60.79 (Et); 53.36 (C-2'); 49.37 d, J(P,C) = 159.9 (C-5'); 48.02 (C-3'); 41.24 (C-1'); 23.68 m, 4 C (iPr); 21.89 d, J(P,C) = 134.1 (C-4'); 16.15 and 16.09 (Et). MS (ESI): m/z = 522 $[M+H]^+$.

Diethyl 2-((2,3-dihydroxypropyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethylphosphonate (2g): Starting from **1f** and 6-chloropurine, isopropylidene protecting group was cleaved under acidic conditions as expected, yield 50% in two steps. ^{1}H NMR (DMSO-d_6): 12.32 s, 1 H (NH); 8.12 s, 1 H and 8.03 s, 1 H (H-2 and H-8); 4.17 m, 2 H, (H-1'); 3.94 m, 4 H (Et); 3.41 m, 2 H (H-6'); 3.20 m, 2 H, (H-7'); 2.85 m, 2 H (H-2'); 2.69 m, 2 H (H-3'); 2.55 m, 1 H and 2.39 m, 1 H (H-5'); 1.76 m, 2 H (H-4'); 1.20 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 156.58 (C-6); 148.24 (C-4); 145.24 (C-2); 140.71 (C-8); 123.71 (C-5); 69.38 (C-6'); 63.78 (C-7'); 60.88 and 60.82 (Et); 56.29 and 53.22 (C-3' and C-2'); 47.54 (C-5'); 39.14 (C-1'); 20.90 d, J(P,C) = 124.6 (C-4'); 16.17 and 16.11(Et). HRMS calcd. for $C_{16}H_{29}N_5O_6P$: 418.18500; found: 418.18498. MS (ESI): m/z = 418 $[M+H]^+$.

Diethyl (2-((2-(benzyloxy)ethyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethyl)phosphonate (2j): Starting from **1j**, yield 52%. ^{1}H NMR (DMSO-d_6): 12.27 s, 1 H (NH); 8.08 s, 1 H and 8.01 s, 1 H (H-2 and H-8); 7.29 m, 5 H (Ar); 4.37 s, 2 H (CH_2Ph); 4.16 m, 2 H, (H-1'); 3.92 m, 4 H (Et); 3.38 m, 2 H (H-6'); 2.85 m, 2 H, (H-2'); 2.67 m, 4 H (H-3' and H-5); 1.77 m, 2 H (H-4'); 1.18 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 156.53 (C-6); 148.23 (C-4); 145.19 (C-2); 140.67 (C-8); 135.91, 128.08, 2 C, 127.38, 127.30, 2 C (Ar); 123.69 (C-5); 71.99 (C-Ph); 69.17 (C-6'); 60.82, 2 C (Et); 52.11, 52.09 and 47.43 (C-5', C-3' and C-2'); 40.81 (C-1'); 20.89 d, J(P,C) = 128.4 (C-4'); 16.13, 2 C (Et). MS (ESI): m/z = 478 $[M+H]^+$.

Hypoxanthine derivatives 2a-2c and 2e: Prepared according the literature.[14]

9-[{N-(Diethyl)phosphonoethyl-N-(diisopropyl)phosphonomethyl}-2-aminoethyl]guanine

(3d): Starting from **1d** and 2-amino-6-chloropurine, yield 75% in two steps. ^{1}H NMR (DMSO-d_6): 10.61 s, 1 H (NH); 7.73 s, 1 H (H-8); 6.48 s, 2 H (NH_2); 4.53 m, 2 H (iPr); 3.99 t, 2 H, J(1',2') = 6.0 (H-1'); 3.93 m, 4 H (Et); 2.90 d, 2 H, J = 10.3 (H-5'); 2.88 m, 2 H (H-2'); 2.79 m, 2 H (H-3'); 1.74 m, 2 H (H-4'); 1.20 m, 18 H (iPr and Et). ^{13}C NMR (DMSO-d_6): 156.72 (C-6); 153.39 (C-2); 150.99 (C-4); 137.85 (C-8); 116.29 (C-5); 69.64 and 69.57 (iPr); 60.88 and 60.82 (Et); 53.39 (C-2'); 49.41 d, J(P,C) = 159.9 (C-5'); 48.15 (C-3'); 40.79 (C-1'); 23.69 (iPr); 22.10 d, J(P,C) = 134.3 (C-4'); 16.16 and 16.10 (Et). MS (ESI): m/z = 537 $[M+H]^+$.

Diethyl 2-((2,3-dihydroxypropyl)(2-(guanin-9-yl)ethyl)amino)ethylphosphonate (3g): Starting from **1f** and 2-amino-6-chloropurine, isopropylidene protecting group was cleaved under acidic conditions as expected, yield 56% in two steps. ^{1}H NMR (DMSO-d_6): 11.08 s, 1 H (NH); 8.02 s, 1 H (H-8); 6.75 s, 2 H (NH_2); 4.40 t, 2 H, J(1',2') = 5.1 (H-1'); 4.03 m, 4 H (Et); 3.89 m, 1 H (H-6'); 3.64 m, 2 H, (H-7'); 3.43 m, 3 H (H-2' and H-5'a); 3.34 m, 2 H (H-3'); 3.21 m, 1 H (H-5'b); 2.32 m, 2 H (H-4'); 1.25 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 155.96 (C-6); 153.99 (C-2); 150.55 (C-4); 137.33 (C-8); 114.72 (C-5); 66.02 (C-6'); 63.46 (C-7'); 61.69 and 61.63 (Et); 55.17 and 51.53 (C-3' and C-2'); 48.22 (C-5'); 38.29 (C-1'); 21.16 d, J(P,C) = 134.0 (C-4'); 16.12 and 16.06(Et). MS (ESI): m/z = 433 $[M+H]^+$.

Diethyl (2-((2-(benzyloxy)ethyl)(2-(guanin-9-yl)ethyl)amino)ethyl)phosphonate (3j): Starting from **1j**, yield 71%. ^{1}H NMR (DMSO-d_6): 10.56 s, 1 H (NH); 7.68 s, 1 H (H-8); 7.29 m, 5 H (Ar); 6.43 s, 2 H (NH_2); 4.39 s, 2 H (CH_2Ph); 3.98 m, 2 H, (H-1'); 3.92 m, 4 H (Et); 3.42 m, 2 H (H-6'); 2.83 m, 2 H, (H-2'); 2.73 m, 4 H (H-3' and H-5'); 1.81 m, 2 H (H-4'); 1.18 m, 6 H (Et). ^{13}C NMR (DMSO-d_6): 156.67 (C-6); 153.34 (C-2); 150.95 (C-4); 138.22 (Ar); 137.89 (C-8); 128.09, 2 C, 127.33, 2 C, 127.26 (Ar); 116.24 (C-5); 72.02 (C-Ph); 72.00 (C-6'); 60.83, 2 C (Et); 52.51, 52.17 and 47.43 (C-5', C-3' and C-2'); 39.81 (C-1'); 22.36 d, J(P,C) = 138.7 (C-4'); 16.13, 2 C (Et). MS (ESI): m/z = 493 $[M+H]^+$.

Guanine derivatives 3a-3c and 3e: Prepared according the literature.[14]

Synthesis of the free phosphonic acids – General Procedure

A mixture of the corresponding diester **2** or **3** (1 mmol), acetonitrile (20 ml) and $BrSiMe_3$ (1 ml) was stirred for 2 days at room temperature. After evaporation and codistillation with acetonitrile, the residue was treated with aqueous methanol (2 : 1, 30 ml) for 1 h and evaporated. The residue was purified by preparative HPLC (water-methanol).

2-((2,3-Dihydroxypropyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethylphosphonic acid (4g): Starting from **2g**, yield 67%. ^{1}H NMR (DMSO-d_6): 12.32 s, 1 H (NH); 8.15 s, 1 H and 8.04 s,

1 H (H-2 and H-8); 4.29 t, 2 H, *J*(1',2') = 6.2 (H-1'); 3.53 m, 1 H (H-6'); 3.23 m, 2 H, (H-7'); 3.03 t, 2 H, *J*(2',1') = 6.2 (H-2'); 2.89 m, 2 H (H-3'); 2.71 m, 1 H and 2.56 m, 1 H (H-5'); 1.64 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 156.55 (C-6); 148.22 (C-4); 145.33 (C-2); 140.67 (C-8); 123.70 (C-5); 68.45 (C-6'); 63.80 (C-7'); 56.28 and 52.91 (C-3' and C-2'); 48.95 (C-5'); 40.73 (C-1'); 24.54 d, *J*(P,C) = 131.0 (C-4'). HRMS calcd. for $C_{12}H_{19}N_5O_6P$: 360.10784; found: 360.10761. MS (ESI): *m/z* = 360 $[M-H]^-$.

9-[(N-Phosphonoethyl-N-phosphonomethyl)-2-aminoethyl]hypoxanthine (4h): Starting from **2d**, yield 55%. ^{1}H NMR (DMSO-d_6): 12.47 s, 1 H (NH); 8.30 s, 1 H and 8.10 s, 1 H (H-2 and H-8); 4.51 t, 2 H, *J*(1',2') = 6.0 (H-1'); 3.53 t, 2 H, *J*(2',1') = 6.0 (H-2'); 3.35 d, 2 H, *J* = 12.8, (H-5'); 3.50 m, 2 H (H-3'); 1.96 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 156.15 (C-6); 148.15 (C-4); 145.98 (C-2); 140.33 (C-8); 122.99 (C-5); 53.04 and 50.23 (C-2' and C-3'); 48.94 d, *J*(P,C) = 146.4 (C-5'); 23.41 d, *J*(P,C) = 133.6 (C-4'). HRMS calcd. for $C_{10}H_{16}N_5O_7P_2$: 380.05304; found: 380.05285. MS (ESI): *m/z* = 380 $[M-H]^-$.

(2-((2-(Benzyloxy)ethyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethyl)phosphonate (4j): Starting from **2j**, yield 65%. ^{1}H NMR (DMSO-d_6): 12.31 s, 1 H (NH); 8.08 s, 1 H and 8.01 s, 1 H (H-2 and H-8); 7.28 m, 5 H (Ar); 4.39 s, 2 H (CH_2Ph); 4.25 t, 2 H, *J*(1',2') = 6.1 (H-1'); 3.44 t, 2 H, *J*(6',5') = 5.4 (H-6'); 3.01 t, 2 H, *J*(2',1') = 6.1 (H-2'); 2.88 m, 2 H (H-3'); 2.83 t, 2 H, *J*(5',6') = 5.4 (H-5'); 1.65 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 156.54 (C-6); 148.24 (C-4); 145.27 (C-2); 140.60 (C-8); 138.11, 128.10, 2 C, 127.33, 2 C and 127.27 (Ar); 123.69 (C-5); 71.99 (C-Ph); 67.33 (C-6'); 52.30, 52.13 and 48.57 (C-5', C-3' and C-2'); 40.87 (C-1'); 24.58 d, *J*(P,C) = 131.9 (C-4'). MS (ESI): *m/z* = 420 $[M-H]^-$.

Phosphonic acids 4a-4c and 4i: Prepared previously.[14]

2-((2,3-Dihydroxypropyl)(2-(guanin-9-yl)ethyl)amino)ethylphosphonic acid (5g): Starting from **3g**, yield 46%. ^{1}H NMR (DMSO-d_6): 10.76 s, 1 H (NH); 7.75 s, 1 H (H-8); 6.62 s, 2 H (NH_2); 4.24 m, 2 H, (H-1'); 3.76 m, 1 H (H-6'); 3.30 m, 6 H, (H-7', H-2' and H-3'); 3.05 m, 1 H (H-5'a); 2.35 m, 1 H (H-5'b); 1.86 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 156.66 (C-6); 153.57 (C-2); 150.89 (C-4); 137.55 (C-8); 116.25 (C-5); 67.11 (C-6'); 63.66 (C-7'); 55.85 and 52.40 (C-3' and C-2'); 49.07 (C-5'); 23.60 d, *J*(P,C) = 131.6 (C-4'). HRMS calcd. for $C_{12}H_{20}N_6O_6P$: 375.11874; found: 375.11830. MS (ESI): *m/z* = 375 $[M-H]^-$.

9-((N-Phosphonoethyl-N-phosphonomethyl)-2-aminoethyl)guanine (5h): Starting from **3d**, yield 71%. ^{1}H NMR (DMSO-d_6): 11.53 (NH); 9.04 s, 1 H (H-8); 7.30 (NH_2); 4.51 t, 2 H, *J*(1',2') = 6.0 (H-1'); 3.63 t, 2 H, *J*(2',1') = 6.0 (H-2'); 3.50 m, 4 H (H-3' and H-5'); 2.03 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 155.13 (C-6); 153.72 (C-2); 149.66 (C-4); 137.63 (C-8); 115.98 (C-5); 53.91 and 50.01 (C-2' and C-3'); 48.64 d, *J*(P,C) = 146.2 (C-5'); 40.34 (C-1');

23.06 d, *J*(P,C) = 132.45 (C-4'). HRMS calcd. for $C_{10}H_{17}N_6O_7P_2$: 395.06394; found: 395.06342. MS (ESI): *m/z* = 395 $[M-H]^-$.

(2-((2-(Benzyloxy)ethyl)(2-(guanin-9-yl)ethyl)amino)ethyl)phosphonate (5j): Starting from **3j**, yield 51%. ^{1}H NMR (DMSO-d_6): 10.62 s, 1 H (NH); 7.68 s, 1 H (H-8); 7.30 m, 5 H (Ar); 6.54 s, 2 H (NH_2); 4.41 s, 2 H (CH_2Ph); 4.08 t, 2 H, *J*(1',2') = 5.9 (H-1'); 3.50 t, 2 H, *J*(6',5') = 5.3 (H-6'); 3.02 t, 2 H, *J*(2',1') = 5.9 (H-2'); 2.97 m, 4 H (H-3'); 2.90 t, 2 H, *J*(5',6') = 5.3 (H-5'); 1.72 m, 2 H (H-4'). ^{13}C NMR (DMSO-d_6): 156.68 (C-6); 153.46 (C-2); 150.96 (C-4); 138.04 (Ar); 137.67 (C-8); 128.13, 2 C, 127.59, 127.31, 2 C (Ar); 116.24 (C-5); 72.05 (C-Ph); 66.94 (C-6'); 52.37, 52.18 and 48.68 (C-5', C-3' and C-2'); 39.80 (C-1'); 24.47 d, *J*(P,C) = 130.4 (C-4'). MS (ESI): *m/z* = 435 $[M-H]^-$.

Phosphonic acids 5a-5c and 5i: Prepared previously.[14]

Synthesis of bisphosphoramidate prodrugs of phosphonic acids 6 and 7 - General procedure

A mixture of corresponding diester **2** or **3** (0.5 mmol), dry pyridine (8 ml) and $BrSiMe_3$ (0.4 ml) was stirred overnight at room temperature under argon. After evaporation and codistillation with pyridine under argon atmosphere, the residue was dissolved in dry pyridine (5 ml) and ethyl (L)-phenylalamine hydrochloride (0.5 g, 2.1 mmol) and triethylamine (1 ml) were added. The mixture was heated to 70 °C under argon atmosphere and then solution of Aldrithiol (0.66 g, 3 mmol) and triphenylphosphine (0.8 g, 3 mmol) in dry pyridine (5 ml) was added. The reaction mixture was heated at 60 °C for 2 days, the solvent was evaporated and the residue was purified by column chromatography on silica gel and the crude product further purified by preparative HPLC. The phosphoramidate prodrug was obtained as foam.

(2S,2'S)-Diethyl 2,2'-{[2-((3-methoxy-3-oxopropyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (6a): Starting from **2a**, yield 85%. ^{1}H NMR (DMSO-d_6): 12.26 s, 1 H (NH); 8.02 s, 1 H and 7.99 s, 1 H (H-8 and H-2); 7.20 m, 10 H (Ar); 4.51 t, 1 H, *J* = 11.2 (NH); 4.19 t, 2 H, *J* = 10.6 (NH); 4.09 t, 2 H, *J*(1',2') = 6.2 (H-2'); 4.02 q, 4 H, *J* = 7.1 (Et); 4.01 m, 1 H and 3.87 m, 1 H (CH); 3.53 s , 3 H (Me); 2.87 m, 3 H and 2.75 m, 1 H (CH_2Ph); 2.56 t, 2 H, *J*(5',6') = 7.0 (H-5'); 2.62 t, 2 H, *J*(2',1') = 6.2 (H-2'); 2.43 m, m, 2 H (H-3'); 2.24 t, 2 H, *J*(6',5') = 7.1 (H-6'); 1.33 m, 2 H (H-4'); 1.11 t, 3 H and 1.06 t, 3 H, *J* = 7.1 (Et). ^{13}C NMR (DMSO-d_6): 173.09, 172.92 and 172.16 (CO); 156.56 (C-6); 148.17 (C-4); 145.17 (C-2); 140.48 (C-8); 137.21, 137.11, 129.30, 2 C, 129.24, 2 C, 127.98, 2 C, 127.94, 2 C, 126.35 and 126.26 (Ar); 123.69 (C-5); 60.21 and 60.11 (Et); 53.94 and 53.82 (NHCH); 51.90 (C-2'); 51.08 (Me); 47.89 (C-5');

46.76 (C-3'); 41.16 (C-1'); 31.51 (C-7'); 25.92 (C-6'); 21.52, *J*(P,C) = 136.3 (C-4'); 13.83 and 13.78 (Et). HRMS calcd. for $C_{35}H_{47}N_7O_8P$: 724.32182; found: 724.32161. MS (ESI): *m/z* = 724 $[M+H]^+$.

(2S,2'S)-Diethyl 2,2'-{[2-((3-cyanopropyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (6c): Starting from **2c**, yield 58%. ^{1}H NMR (DMSO-d_6): 12.30 s, 1 H (NH); 8.04 s, 1 H and 8.02 s, 1 H (H-8 and H-2); 7.21 m, 10 H (Ar); 4.52 t, 1 H, *J* = 11.6 (NH); 4.20 t, 2 H, *J* = 11.6 (NH); 4.11 t, 2 H, *J*(1',2') = 6.2 (H-2'); 4.03 q, 4 H, *J* = 7.1 (Et); 3.98 m, 1 H and 3.89 m, 1 H (CH); 2.86 m, 3 H and 2.75 m, 1 H (CH_2Ph); 2.63 t, 2 H, *J*(2',1') = 6.2 (H-2'); 2.48 m, 1 H and 2. 39 m, 1 H (H-3'); 2.30 t, 2 H, *J*(5',6') = 6.7 (H-5'); 2.18 t, 2 H, *J*(7',6') = 7.2 (H-7'); 1.45 m, 2 H (H-6'); 1.36 m, 2 H (H-4'); 1.12 t, 3 H and 1.07 t, 3 H, *J* = 7.1 (Et). ^{13}C NMR (DMSO-d_6): 173.04 and 172.85 (CO); 156.49 (C-6); 147.38 (C-4); 145.20 (C-2); 140.44 (C-8); 137.08, 2 C, 129.27, 2 C, 129.22, 2 C, 127.96, 2 C, 127.93, 2 C, 126.33 and 126.24 (Ar); 120.29 (C-5); 118.24 (CN); 60.20 and 60.11 (Et); 53.87 and 53.81 (NHCH); 51.88 (C-2'); 50.98 (C-5'); 46.63 (C-3'); 42.07 (C-1'); 41.18 (CH_2Ph); 22.56 (C-7'); 13.80 and 13.77 (Et); 13.50 (C-6'). HRMS calcd. for $C_{35}H_{46}N_8O_6P$: 705.32724; found: 705.32730. MS (ESI): *m/z* = 705 $[M+H]^+$.

(2S,2'S)-Diisopropyl 2,2'-{[2-((2-(benzyloxy)ethyl)(2-(hypoxanthin-9-yl)ethyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (6j): Starting from **2j**, yield 76%. ^{1}H NMR (DMSO-d_6): 12.27 s, 1 H (NH); 8.02 s, 1 H and 7.99 s, 1 H (H-8 and H-2); 7.20 m, 15 H (Ar); 4.80 m, 2 H (iPr); 4.47 t, 1 H, *J* = 11.5 (NH); 4.36 s, 2 H (CH_2Ph); 4.17 t, 2 H, *J* = 11.5 (NH); 4.11 t, 2 H, *J*(1',2') = 6.2 (H-1'); 3.97 m, 1 H and 3.84 m, 1 H (CH); 3.30 t, 2 H, *J*(6',5') = 5.9 (H-6'); 2.85 m, 3 H and 2.74 m, 1 H (CH_2Ph); 2.72 t, 2 H, *J*(2',1') = 6.2 (H-2'); 2.54 t, 2 H, *J*(5',6') = 5.9 (H-5'); 2.44 m, 2 H (H-3'); 1.38 m, 2 H (H-4'); 1.10 m, 12 H (iPr). ^{13}C NMR (DMSO-d_6): 172.49, 2 C (CO); 156.54 (C-6); 148.17 (C-4); 145.14 (C-2); 140.51 (C-8); 138.29, 137.10, 2 C, 129.27, 2 C, 129.32, 2 C, 129.28, 2 C, 128.07, 2 C, 127.96, 2 C, 127.91, 2 C, 127.23, 2 C, 126.34 and 126.25 (Ar); 123.66 (C-5); 71.95 (C-Ph); 68.13 (C-6'); 67.71 and 67.55 (iPr); 54.02 and 53.84 (NHCH); 52.73 (C-2'); 52.08 (C-5'); 47.77 (C-3'); 41.27 (C-1'); 40.00, 2 C (CH_2Ph); 25.91, *J*(P,C) = 109.7 (C-4'); 21.29 m, 4 C (iPr). MS (ESI): *m/z* = 800 $[M+H]^+$.

(2S,2'S)-Diethyl 2,2'-{[2-((2-(guanin-9-yl)ethyl)(3-methoxy-3-oxopropyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (7a): Starting from **3a**, yield 40%. ^{1}H NMR (DMSO-d_6): 10.55 s, 1 H (NH); 7.59 s, 1 H (H-8); 7.20 m, 10 H (Ar); 6.46 s, 2 H (NH_2); 4.54 t, 1 H, *J* = 11.6 (NH); 4.18 t, 1 H, *J* = 11.8 (NH); 4.02 q, 4 H, *J* = 7.3 (Et); 3.97 m, 1 H (NHCH); 3.87 m, 3 H (NHCH and H-1'); 3.55 s, 3 H (Me); 2.87 m, 3

H and 2.71 m, 1 H (CH_2Ph); 2.56 m, 2 H (H-2' and H-5'); 2.45 m, 2 H (H-3'); 2.28 t, 2 H, *J*(6',5') = 6.9 (H-6'); 1.37m, 2 H (H-4'); 1.11 t, 3 H and 1.06 t, 3 H (Et). ^{13}C NMR (DMSO-d_6): 173.08, 172. 90 and 172.24 (CO); 156.71 (C-6); 153.32 (C-2); 150.95 (C-4); 137.60 (C-8); 137.21, 137.11, 129.29, 2 C, 129.24, 2 C, 127.99, 2 C, 127.94, 2 C, 126.36 and 126.27 (Ar); 116.29 (C-5); 60.21 and 60.11 (Et); 53.98 and 53.83 (NHCH); 51.90 (C-2'); 51.12 (Me); 47.91 (C-5'); 46.64 (C-3'); 40.53 (C-1'); 31.54 (C-6'); 13.83 and 13.77 (Et). HRMS calcd. for $C_{35}H_{48}N_8O_8P$: 739.33272; found: 739.33281. MS (ESI): *m/z* = 739 $[M+H]^+$.

(2S,2'S)-Diethyl 2,2'-{[2-((2-(guanin-9-yl)ethyl)(4-methoxy-4-oxobutyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (7b): Starting from **3b**, yield 41%. ^{1}H NMR (DMSO-d_6): 10.53 s, 1 H (NH); 7.61 s, 1 H (H-8); 7.21 m, 10 H (Ar); 6.42 s, 2 H (NH_2); 4.53 m, 1 H (NH); 4.20 m, 1 H (NH); 4.02 q, 4 H, *J* = 7.1 (Et); 3.97 m and 3.87 m, 4 H (H-1'and NHCH); 3.56 s, 3 H (Me); 3.88 m, 2 H (NHCH); 2.86 m, 3 H and 2.75 m, 1 H (CH_2Ph); 2.54 m, 2 H (H-2'); 2.40 m, 2 H (H-5'); 2.23 m, 2 H (H-3'); 2.11 m, 2 H (H-7'); 1.44 m, 2 H (H-6'); 1.35 m, 2 H (H-4'); 1.12 t, 3 H and 1.06 t, 3 H, *J* = 7.1 (Et). ^{13}C NMR (DMSO-d_6): 173.38 and 173.36 (CO); 156.98 (C-6); 153.65 (C-2); 151.23 (C-4); 137.88 (C-8); 137.48, 137.39, 129.59, 2 C, 129.55, 2 C, 128.30, 2 C, 128.25, 2 C, 126.68 and 126.59 (Ar); 116.65 (C-5); 60.52 and 60.42 (Et); 54.27 and 54.14 (NHCH); 51.81 (C-2'); 51.38 (Me); 47.01 (C-5'); 44.10 (C-3'); 30.91 (C-7'); 24.36 d, *J*(P,C) = 124.0 (C-4'); 22.26 (C-6'). 14.13 and 14.07 (Et). HRMS calcd. for $C_{36}H_{50}N_8O_8P$: 753.34837; found: 753.34843. MS (ESI): *m/z* = 753 $[M+H]^+$.

(2S,2'S)-Diethyl 2,2'-{[2-((2-(benzyloxy)ethyl)(2-(guanin-9-yl)ethyl)amino)ethyl]phosphoryl}bis(azanediyl)bis(3-phenylpropanoate) (7j): Starting from **3j**, yield 61%. ^{1}H NMR (DMSO-d_6): 10.54 s, 1 H (NH); 7.62 s, 1 H (H-8); 7.20 m, 15 H (Ar); 6.43 s, 2 H (NH_2); 4.78 m, 2 H (iPr); 4.50 t, 1 H, *J* = 11.5 (NH); 4.37 s, 2 H (CH_2Ph); 4.19 t, 2 H, *J* = 11.5 (NH); 3.90 t, 2 H, *J*(1',2') = 6.2 (H-1'); 3.87 m, 2 H (CH); 3.33 t, 2 H, *J*(6',5') = 6.0 (H-6'); 2.85 m, 3 H and 2.72 m, 1 H (CH_2Ph); 2.64 t, 2 H, *J*(2',1') = 6.2 (H-2'); 2.53 t, 2 H, *J*(5',6') = 6.0 (H-5'); 2.45 m, 2 H (H-3'); 1.42 m, 2 H (H-4'); 1.08 m, 12 H (iPr). ^{13}C NMR (DMSO-d_6): 172.50, 2 C (CO); 156.74 (C-6); 153.33 (C-2); 150.97 (C-4); 138.33 (Ar); 137.64 (C-8); 137.08, 2 C, 129.32, 2 C, 129.28, 2 C, 128.08, 2 C, 127.96, 2 C, 127.91, 2 C, 127.28, 2 C, 127.22, 2 C; 126.35 and 126.26 (Ar); 116.29 (C-5); 71.99 (C-Ph); 68.18 (C-6'); 67.72 and 67.56 (iPr); 54.06 and 53.84 (NHCH); 52.71 (C-2'); 52.14 (C-5'); 47.74 (C-3'); 40.66 (C-1'); 40.00, 2 C (CH_2Ph); 25.90, *J*(P,C) = 111.3 (C-4'); 21.28 m, 4 C (iPr). MS (ESI): *m/z* = 815 $[M+H]^+$.

Synthesis of tetraphosphoramidate prodrugs of bisphosphonic acids 8 and 9 - General procedure

A mixture of corresponding tetraester **2d, 2e** or **3d, 3e** (0.5 mmol), dry pyridine (8 ml) and $BrSiMe_3$ (1 ml) was stirred overnight at room temperature under argon. After evaporation and codistillation with pyridine under argon atmosphere, the residue was dissolved in dry pyridine (8 ml) and ethyl (L)-phenylalamine hydrochloride (1.4 g, 10 mmol) and triethylamine (2.5 ml) were added. The mixture was heated to 70 °C under argon atmosphere and then solution of Aldrithiol (1.85 g, 8 mmol) and triphenylphosphine (2.1 g, 8 mmol) in dry pyridine (8 ml) was added. The reaction mixture was heated at 70 °C for 3 days, the solvent was evaporated and the residue was purified by column chromatography on silica gel and the crude product further purified by preparative HPLC. The phosphoramidate prodrug was obtained as foam.

Tetra-(ethyl L-phenylalanine) prodrug of 9-[(N-Phosphonoethyl-N-phosphonomethyl)-2-aminoethyl]hypoxanthine (8h): Starting from **2d**, yield 20%. ^{1}H NMR (DMSO-d_6): 12.27 s, 1 H (NH); 8.08 s, 1 H and 7.99 s, 1 H (H-8 and H-2); 7.19 m, 20 H (Ar); 4.51 m, 1 H, 4.35 m, 1 H and 4.07 m, 2 H (NH); 3.99 m, 14 H (H-1', NHCH and Et); 2.55-2.90 m, 12 H (CH_2Ph, H-2' and H-5'); 2.26 m, 2 H (H-3'); 1.30 m, 2 H (H-4'); 1.06 m, 12 H and (Et). ^{13}C NMR (DMSO-d_6): 172.81 m (CO); 156.52 (C-6); 148.17 (C-4); 145.16 (C-2); 140.32 (C-8); 137.09, 2 C, 137.03, 2 C, 129.27, 4 C, 129.20, 4 C, 127.96, 4 C, 127.92, 4 C, 126.32, 2 C and 126.24, 2 C (Ar); 123.69 (C-5); 60.15 m (Et); 53.78 m (NHCH); 51.37 (C-2'); 49.83, J(P,C) = 162.9 (C-5'); 45.99 (C-3'); 41.06 (C-1'); 13.77 and 13.73 (Et). HRMS calcd. for $C_{54}H_{70}N_9O_{11}P_2$: 1082.46645; found: 1082.46721. MS (ESI): m/z = 1083 $[M+H]^+$.

Tetra-(ethyl L-phenylalanine) prodrug of 9-[(N,N-(bis-2-phosphonoethyl))-2-aminoethyl]hypoxanthine (8i): Starting from **2e**, yield 25%. ^{1}H NMR (DMSO-d_6): 12.28 s, 1 H (NH); 8.00 m, 2 H (H-2 and H-8); 7.19 m, 20 H (Ar); 4.-11-4.52 m, 4 H (NH); 3.75-4.07 m, 14 H (H-1', Et, NHCH); 2.88 m, 6 H and 2.77 m, 2 H (CH_2Ph); 2.52 m, 2 H (H-2'); 2.39 m, 4 H (H-3', H-5'); 1.35 m, 4 H (H-4', H-6'); 1.08 m, 12 H (Et). ^{13}C NMR (DMSO-d_6): 173.42, 173.33, 173.19 and 173.15 C (CO); 156.84 (C-6); 148.44 (C-4); 145.51(C-2); 140.65 (C-8); 137.43, 2 C, 137.33, 2 C 129.56, 4 C, 129.52, 4 C, 128.28, 4 C, 128.26, 4 C, 126.65, 2 C and 126.62, 2 C (Ar); 123.99 (C-5); 60.51 and 60.42, 4 C (Et); 54.22 m, 4 C (NHCH); 51.84 (C-2'); 46.63 and 46.58 (C-3' and C-5'); 41.19 (C-1'); 24.91 d, J(P,C) = 144.03 (C-4' and C-6'); 14.11 and 14.07, 4 C (Et). HRMS calcd. for $C_{55}H_{72}N_9O_{11}P_2$: 1096.48265; found: 1096.48260. MS (ESI): m/z = 1096 $[M+H]^+$.

Tetra-(ethyl L-phenylalanine) prodrug of 9-[(N-Phosphonoethyl-N-phosphonomethyl)-2-aminoethyl]guanine (9h): Starting from **3d**, yield 20%. ^{1}H NMR (DMSO-d_6): 10.55 s, 1 H

(NH); 7.67 s, 1 H (H-8); 7.15 m, 20 H (Ar); 6.44 s, 2 H (NH_2); 4.55 m, 1 H, 4.33 m, 1 H, 4.14 m, 1 H and 4.08 m, 1 H (NH); 3.78-4.03 m, 14 H (H-1', NHCH, Et); 2.81 m, 8 H (CH_2Ph); 2.56 m, 4 H (H-2', H-3'); 2.25 d, 2 H, J = 10.8 (H-5'); 1.36 m, 2 H (H-4'); 1.06 m, 12 H and (Et). ^{13}C NMR (DMSO-d_6): 173.20 m (CO); 157.06 (C-6); 153.69 (C-2); 151.28 (C-4); 137.81 (C-8); 137.22, 2 C, 137.50, 2 C, 129.54, 4 C, 129.66, 4 C, 128.27, 4 C, 128.35, 4 C, 126.53, 2 C and 126.74, 2 C (Ar); 116.61 (C-5); 60.55 m (Et); 54.23 m (NHCH, C-2'); 52.28 d, J = 132.3 (C-5'); 49.14 (C-3'); 40.73 (C-1'); 40.29 m (CH_2Ph); 24.43 d, J = 110.6 (C-4'); 14.06 and 14.12 (Et). HRMS calcd. for $C_{54}H_{71}N_{10}O_{11}P_2$: 1097.47735; found: 1097.47834. MS (ESI): m/z = 1097 $[M+H]^+$.

Tetra-(ethyl L-phenylalanine) prodrug of 9-[(N,N-(bis-2-phosphonoethyl))-2-aminoethyl]guanine (9i): Starting from **3e**, yield 70%. ^1H NMR (DMSO-d_6): 10.56 s, 1 H (NH); 7.19 s, 1 H (H-8); 7.18 m, 20 H (Ar); 6.47 s, 2 H (NH_2); 4.52 t, 1 H, J = 11.6 (NH); 4.21 t, 1 H, J = 11.0 (NH); 3.96 m, 10 H (H-1', Et); 3.79 m, 4 H, (NHCH); 2.84 m, 6 H and 2.77 m, 2 H (CH_2Ph); 2.41 m, 4 H (H-3', H-5'); 1.40 m, 4 H (H-4', H-6'); 1.10 t, 6 H and 1.04 t, 6 H, J = 7.2 (Et). ^{13}C NMR (DMSO-d_6): 173.04, 173.02, 172.89 and 172.85 (CO); 156.72 (C-6); 153.36 (C-2); 150.97 (C-4); 137.38 (C-8); 137.12, 2 C, 137.04, 2 C 129.27, 4 C, 129.22, 4 C, 127.99, 4 C, 127.95, 4 C, 126.36, 2 C and 126.29, 2 C (Ar); 116.30 (C-5); 60.21 and 60.12, 4 C (Et); 53.98 m, 4 C (NHCH); 51.46 (C-2'); 46.26 (C-3' and C-5'); 40.00 (C-1'); 25.64 d, J(P,C) = 113.61 (C-4' and C-6'); 13.80 m, 4 C (Et). HRMS calcd. for $C_{55}H_{73}N_{10}O_{11}P_2$: 1111.49300; found: 1111.49303. MS (ESI): m/z = 1111.5 $[M+H]^+$.

Determination of K_i values

The K_i values were determined using a spectrophotometric assay at 25°C, 0.1 M Tris-HCl, 10 mM $MgCl_2$, pH 7.4 with *P*Rib-*PP* as the variable substrate and guanine as the fixed substrate.[15] The K_i values are $K_{i(app)}$ as they were measured at a single concentration of the second substrate. The concentration of the second substrate (guanine) was saturating: 60 μM. $K_{i(app)}$ was calculated using the equation $K_{m(app)} = K_m(1+ [I]/K_{i(app)})$.

Evaluation of *in vitro* antimalarial activity of aza-ANP prodrugs

P. falciparum D6 (Sierra-Leone) laboratory line, sensitive to most antimalarial drugs and W2 (Indochina) line, resistant to chloroquine and pyrimethamine, were maintained in RPMI-1640-LPLF complete medium, containing 10% human plasma, at 4% haematocrit and 1% to 8% parasitaemia as previously described.[26] Cultures were routinely synchronised using D-sorbitol.[27] To evaluate the antimalarial activity of the ANPs, the [^3H]-hypoxanthine growth

inhibition assay[28] was utilized, where the uptake of [^{3}H]-hypoxanthine by malaria parasites is used as a surrogate marker for parasite growth. For these assays, stock solutions of ANPs were made to concentrations of 20-40 mM in DMSO or water and subsequently diluted in hypoxanthine-free complete media prior to assay. The assays (in 96-well plate format) were initiated when the majority of parasites (>90%) at early trophozoite (ring) stage. Parasite cultures (100 μL per well) at 0.5% initial parasitemia and 2% hematocrit in hypoxanthine-free RPMI1640-LPLF medium were exposed to ten 2-fold serial dilutions of the ANPs and chloroquine (CQ) (reference drug) for 96 hours, with [^{3}H]-hypoxanthine (0.2 μCi/well) added ~48 hours after beginning of the experiment. The [^{3}H]-hypoxanthine incorporation data were analyzed and sigmoidal growth inhibition curves were produced by non-linear regression analysis of the [^{3}H]-hypoxanthine incorporation data versus log-transformed concentrations of the compounds using Graphpad Prism V5.0 software (GraphPad Software Inc. USA), from which the inhibitory concentration (IC_{50}) that cause 50% of parasite growth were determined. The IC_{50} values were based on at least two independent experiments with mean ± SD calculated.

Cytotoxicity assays in human cell lines

The inhibitory effect of the test compounds on cell proliferation was determined in three human cell lines (purchased from the American Type Culture Collection): A549 lung carcinoma cells; C32 melanoma cells, and C32-TG mutant cells, which were selected under 6-thioguanine and are deficient in HGPRT activity due to an exon 2 deletion.[25] To determine the cytostatic effect of the test compounds, the cells were seeded in 96-well plates at 7,500 (A549) or 15,000 (C32 and C32-TG) cells per well and, 24 hours later, the compounds were added at serial dilutions. After four days incubation at 37°C, the cells were trypsinized and counted with a Coulter Counter apparatus. The CC_{50} values, or compound concentrations at which cell proliferation was 50% compared to that in untreated cells, were calculated by extrapolation. Data presented are the mean ± SEM of two or three independent tests.

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References:

1. World Health Organization. World malaria report **2014**. World Health Organization, Geneva, Switzerland.
2. Saunders, D.; Vanachayangkul, P.; Lon, C.; U.S. Army Military Malaria Research Program, National Center for Parasitology, Entomology, and Malaria Control (CNM), Royal Cambodian Armed Forces. *N. Engl. J. Med.* **2014**, *371*, 484.
3. Muller, I. B.; Hyde, J. E. *Future Microbiol*. **2010**, *5* (12), 1857.
4. de Jersey, J.; Holý, A.; Hocková, D.; Naesens, L.; Keough, D. T.; Guddat, L. W. *Curr. Top. Med. Chem*. **2011**, *11* (16), 2085.
5. Ducati, R. G.; Namanja-Magliano, H. A.; Schramm, V. L. *Future Med. Chem*. **2013**, *5* (11), 1341.
6. Downie, M. J.; Kirk, K. Mamoun, B. C. *Eukaryotic Cell* **2008**, *7* (8), 1231.
7. Xu, Y.; Eads, J.; Sacchettini, J. C.; Grubmeyer, C. *Biochemistry* **1997**, *36*, 3700.
8. De Clercq, E. *Med. Res. Rev*. **2013**, 33 (6), 1278.
9. Keough, D. T.; Hocková, D.; Holý, A.; Naesens, L.; Skinner- Adamsm, T.; de Jersey, J.; Guddat, L. W. *J. Med. Chem.* **2009**, *52*, 4391.
10. Česnek, M.; Hocková, D.; Holý, A.; Dračínský, M.; Baszczyňski, O.; de Jersey, J.; Keough, D. T.; Guddat, L. *Bioorg. Med. Chem*. **2012**, *20,* 1076.
11. Hocková, D.; Holý, A.; Masojídková, M.; Keough, D. T.; de Jersey, J.; Guddat, L. W. *Bioorg. Med. Chem.* **2009**, *17*, 6218.
12. Keough, D. T.; Hocková, D.; Krečmerová, M.; Česnek, M.; Holý, A.; Naesens, L.; Brereton, I.; Winzor, D. J.; de Jersey, J.; Guddat, L. W. *Mol. Biochem. Parasitol.* **2010**, *173*, 165.
13. Keough, D. T.; Špaček, P.; Hocková, D.; Tichý, T.; Vrbková, S.; Slavětínská, L.; Janeba, Z.; Naesens, L.; Edstein, M. D.; Chavchich, M.; Wang, T.-H.; de Jersey, J.; Guddat, L. W. *J. Med. Chem.* **2013**, *56*, 2513.
14. Hocková, D.; Keough, D. T.; Janeba, Z.; Wang, T.-H.; de Jersey, J.; Guddat, L. W. *J. Med. Chem*. **2012**, *55*, 6209.

15. Keough, D. T.; Hocková, D.; Janeba, Z.; Wang, T.-H.; Naesens, L.; Edstein, M. D.; Chavchich, M.; Guddat, L. W. *J. Med. Chem*. **2015**, *58*, 827.
16. Pertusati, F.; Serpi, M.; McGuigan, C. *Antiviral Chem. Chemo.* **2012,** *22* (5), 181.
17. Pradere, U.; Garnier-Amblard, E. C.; Coats, S. J.; Amblard, F.; Schinazi, R. F. *Chem. Rev*. **2014**, *114*, 9154.
18. Oliveira, F. M.; Barbosa, L. C. A.; Ismail, F. M. D. *Rsc. Adv.* **2014,** *4* (36), 18998.
19. Tsai, C-Y.; Ray, A. S.; Tumas, D. B.; Keating, M. J.; Reiser, H.; Plunkett, W. *Clinical. Cancer Res*. **2009**, *15* (11), 3760.
20. Jansa, P.; Baszczynski, O.; Dracinsky, M.; Votruba, I.; Zidek, Z.; Bahador, G.; Stepan, G.; Cihlar, T.; Mackman, R.; Holý, A.; Janeba, Z. *Eur. J. Med. Chem*. **2011**, *46*, 3748.
21. Hazleton, K. Z., Ho, M. C., Cassera, M. B., Clinch, K., Crump, D. R., Rosario, I., Jr., Merino, E. F., Almo, S. C., Tyler, P. C. & Schramm, V. L. *Chem Biol* **2012***, 19*, 721.
22. Zhou, D.; Lagoja, I. M.; Van Aerschot, A.; Herdewijn, P. *Collect. Czech. Chem. Commun*. **2006**, *71*, 15.
23. Mackman, R. L.; Cihlar, T. *Annu. Rep. Med. Chem*. **2004**, *39*, 305.
24. Hecker, S. J.; Erion, M. D. *J. Med. Chem.* **2008**, *51*, 2328.
25. Chen, T. R. *Cytogenet. Cell Genet.* **1983,** *35* (3), 181.
26. Trager, W.; Jensen, J. B. *Science* **1976**, *193*, 673.
27. Lambros, C.; Vanderberg, J. P. *J. Parasitol.* **1979**, *65* (3), 418.
28. Desjardins, R. E.; Canfield, C. J.; Haynes, J. D.; Chulay, J. D. *Antimicrob. Agents Chemother*. **1979**, *16* (6), 710.

Graphical abstract