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N^4-[Alkyl-(hydroxyphosphono)phosphonate]-cytidine—New drugs covalently linking antimetabolites (5-FdU, araU or AZT) with bone-targeting bisphosphonates (alendronate or pamidronate)

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ABSTRACT

Amino-bisphosphonates (alendronate, pamidronate) were covalently linked in a three step synthesis, with protected and triazolylated derivatives of therapeutically used nucleoside analogs (5-FdU, araC, AZT) by substitution of their triazolyl residue. From the deprotected and chromatographically purified reaction mixtures N^4-[alkyl-(hydroxyphosphono) phosphonate]-cytidine combining two differently cytotoxic functions were obtained. This new family of bisphosphonates (BPs) contains as novelty an alkyl side chain with a cytotoxic nucleoside. The BPs moiety allows for a high binding to hydroxyapatite which is a prerequisite for bone targeting of the drugs. In vitro binding of 5-FdU-alendronate (5-FdU-ale) to hydroxyapatite showed a sixfold increased binding of these BPs as compared to 5-FdU.

Exploratory cytotoxic properties of 5-FdU-ale were tested on a panel of human tumor cell lines resulting in growth inhibition ranging between 5% and 38%. The determination of IC_{50}-concentrations of the conjugate in Lewis lung carcinoma and murine macrophages showed an incubation time dependent growth inhibition with higher sensitivity towards the tumor cells. We assume that the antimetabolite-BPs can be cleaved into different active metabolites that may exert cytotoxic and other therapeutic effects. However, the underlying mechanisms of these promising new antimetabolite-BPs conjugates remain to be evaluated in future experiments.

1. Introduction

1-Hydroxybisphosphonates (BPs) are analogs of pyrophosphate where the P–O–P linkage is replaced by P–C–P. The carbon atom contains the 1-hydroxy group and variable side chains.[1] The P–C–P bond is relatively stable towards chemicals and enzymatic hydrolysis. The extremely high binding affinity of BPs towards hydroxyapatite in bone matrix is used to treat bone resorption and other bone disorders such as osteoporosis or tumor-induced osteolysis.[2,3] Bones are the most common sites of metastasis in patients with solid tumors arising from breast, prostate, lung, thyroid and kidney.[4] The use of BPs has had a profound beneficial effect on the management of metastatic bone disease and the prevention of treatment-induced bone loss.[5] BPs have also been suggested as bone-targeting vectors for various contrast agents.[6–10] Complexes of radioactive metal ions with BPs have been applied in bone cancer radiotherapy and in palliative settings for pain therapy associated with bone metastasis.[11] BPs were also investigated for their potential use in parasitic diseases.[12,13]

For the activity of BPs the P–C–P structure is a prerequisite and the intensity of the effects are exclusively dependent upon the side chain structure. Small structural changes can lead to extensive alterations in their physico-chemical, biological, therapeutic and toxicologic characteristics. The length of the lateral chain is very important. For example pamidronate with its 3-aminopropyl side chain has an approximately 10-fold lower activity than alendronate carrying a 4-aminobutyl side chain. Highly active BPs contain side chains with terminal nitrogen atoms as amino groups or heterocyclic residues.[1] The replacement of the terminal amino group by an imidazole residue resulting in zoledronate improved drastically the activity of bone resorption inhibition, antimetastatic properties and antitumor activities in respect to pamidronate and alendronate.[2] However, although the side chain structure of BPs has been widely varied only a few BPs with therapeutic and clinical potential were obtained.[12–14]

Several recent studies suggested that BPs have direct effects on tumor cells and that they may enhance the antitumor activities of

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cytostatic drugs.[15–23] Therefore, we hypothesized that the linkage of therapeutically used nucleosides such as Ara-C, 5-FdU or AZT with BPs would create new compounds suitable for bone targeting with potent cytostatic or antiviral properties.[24] Therapeutics like methotrexate,[25,26] doxorubicin,[27] estradiol,[28] prostaglandin E_{230},[29] melphalan[30] or cis-platin[31,32] were directly covalently attached to BPs. As a model for protein targeting to bone bovine serum albumin was modified with a BPs resulting in higher bone retention of the protein as a result of amino-BPs-protein conjugation.[33]

Recently, a synthesis concept for cytostatic nucleoside-5′-triphosphate analogs was described in which the β-and γ-phosphor atoms were linked via a carbon instead of an oxygen atom.[34,35] According to this synthesis developed in milligram scale 5′-fluorouridine-5′-monophosphate and arabinocytosine-5′-monophosphate were coupled with the less active BPs medronate or etidronate. Cytostatic activities of these 5′-triphosphate analogs evaluated in a mouse model were encouraging. However, it was not shown that the more active amino-BPs (e.g., zoledronate, alendronate, and pamidronate) could also be coupled to antimetabolites or other therapeutically active nucleoside analogs.

Here we present a new concept for the coupling of amino-BPs with cytotoxic nucleosides resulting in BPs whose alkyl chain is terminated by antimetabolite residues.

The chemical synthesis, hydoxyapatite binding and first in vitro activities on human and murine tumor cells and macrophages of these nucleoside-BPs are described in the following.

2. Results and discussion

2.1. Synthesis concept of antimetabolite-BPs

For the synthesis of the new antimetabolite-BPs the pyrimidine nucleoside analogs 2′-deoxy-5-fluorouridine (5-FdU), 1-β-D-arabinofuranosyluracil (araU) and AZT (azidothymidine) were chosen as cytotoxic or antiviral and pamidronate and alendronate as the amino-BP components. The conjugation conditions of the amino-BPs with the nucleosides were chosen in a way that compounds with reactive BPs and a tertiary 1-hydroxy group were formed. This synthesis scheme was chosen for the following reasons. It cannot be excluded, that the esterification of only one phosphonate group of BPs could reduce their high affinity to bone.[36] Several mono-, di-, tri- and tetraesters of BPs were shown to be ineffective prodrugs of the parent BPs,[2] whereas free geminal phosphonate groups showed the highest affinity for hydroxyapatite.[13] For this reason the well established esterification of nucleosidic hydroxyl groups with phosphates seemed not to be suited although the method has been used for the conjugation of AZT[37] and other nucleosides to BPs.[38] Therefore, we did not use tetraester protected BPs for the conjugation with nucleosides. It is also known that under alkaline conditions (pH >8) and high temperatures (>60 °C) a rapid rearrangement of the esterified BPs to therapeutically inactive 1-phosphonato-1-phosphonates occurs.[8] The alternative attachment of the BPs moiety to drugs through a linkage between the BPs and a reactive group of the drug such as an amino- or carboxyl function could not be applied without previous derivation of the nucleosides. The carboxylation of the antimetabolites would not only require a considerable synthesis effort, it could also diminish their cytotoxic activity. The linkage of the BPs to the pyrimidine nitrogen through an *N*-alkyl bond as used for the preparation of 5-fluorouracil-BPs is only practicable for the conjugation of nucleobases but not of nucleosides.[39] Under consideration of these aspects the methods available for the conjugation of cytotoxic antimetabolites with BPs are limited. This may also be the reason that nucleoside-BPs conjugates have only been prepared via phosphoester or anhydride bonds taking into account the mentioned disadvantages.

On the basis of previously described syntheses,[40–42] which are only successful in organic solvents in respect to high yields, we succeeded to develop a simple, practicable and scalable synthetic route to link the extremely hydrophilic amino-BPs to the nucleobase of protected hydrophobic uridine analogs. The linkage of BPs via their terminal amino groups occurs in water/dioxane by substitution of the triazolyl residue of uracil nucleoside derivatives which had been introduced at the 4-position of uridine resulting in N^4-[alkyl-(hydroxyphosphono) phosphonate]-cytidine. This synthesis concept was applied here for the first time yielding new BPs whose alkyl side chain is terminated by nucleoside analogs.

The new molecules can act on one hand as derivatives of alendronate or pamidronate. On the other hand the nucleoside BPs represent derivatives of therapeutically active nucleoside analogs whose nucleobases contain an *N*-alkylbisphosphonate function. The described synthesis route may also be used for the conjugation of other pyrimidine antimetabolites such as gemcitabine and 3′-ethinylcytidine to amino-BPs.

2.2. Synthetic chemistry

N^4-[Alkyl-(hydroxyphosphono)phosphonate]-cytidine prepared in three steps (Fig. 1) starting with uridine analogs (**1**). In the first synthesis step the free carbohydrate hydroxyl groups of **1** were protected with acetyl residues resulting in **2** at 94–97% yields. Acetylated 5-FdU (**2a**) was obtained as crystalline solid, whereas the acetylation of AZT resulted in solid foam. In the second step the 1,2,4-triazolyl residue was introduced at the 4-position of the protected uracil residues. The O-acetylated and 4-triazolylated uridine analogs **3** were obtained at yields of 77% for **3c** and 88% for **3a**, respectively. Compound **3b** was synthesized according to published methods.[40] The triazolyl residue of **3** was substituted in the third synthesis step by the (aminoalkyl-1-hydroxy-phosphono)phosphonates alendronate (**4**) or pamidronate (**5**) using equimolar amounts of nucleoside and BP resulting in acetylated N^4-[alkyl-(hydroxyphosphono)phosphonate] cytidine nucleosides. The unfavorable equimolar ratio of the reaction partners in view of a desired high yield was chosen as the separation of the hydrophilic nucleoside-BPs from an excess of unreacted and equally hydrophilic BPs was expected to be difficult. The course of the substitution reactions was monitored by thin layer chromatography (TLC). During the reaction the fluorescent spot (360 nm) of **3** disappeared and new, slower migrating dark spots (254 nm) and the desired product remaining as a dark spot at the origin were formed. The isolation and purification of the acetylated reaction product was achieved in two steps. By addition of methanol to the concentrated syrup the major part of the desired product precipitated together with unreacted BP and small amounts of side products. The partially purified residue was obtained by filtration and dissolved in ammonia and kept at room temperature to remove the acetyl protecting groups. The rest of the desired reaction product was obtained by RP-18 chromatography of the filtrate followed by deprotection. The chromatographic separation turned out to be more difficult because the retention time of the nucleoside-BPs and the unreacted BPs were very similar. Using water as eluent the BPs left the column at first, immediately followed by the nucleoside-BPs, whereas the uncoupled nucleoside components and side products remained on the column. Nucleoside-BPs were UV-detectable at 254 nm, whereas the UV-inactive BPs could not be detected. The concentrated and lyophilized eluate containing the nucleoside-BPs afforded white powders with different hydration degrees of **6** and **7**. A possible alternative chromatographic purification using an anion exchange column which is normally used to separate polar nucleotides[43] and which was also be used in the synthesis of 5′-triphosphate analogs[35] would drastically increase the

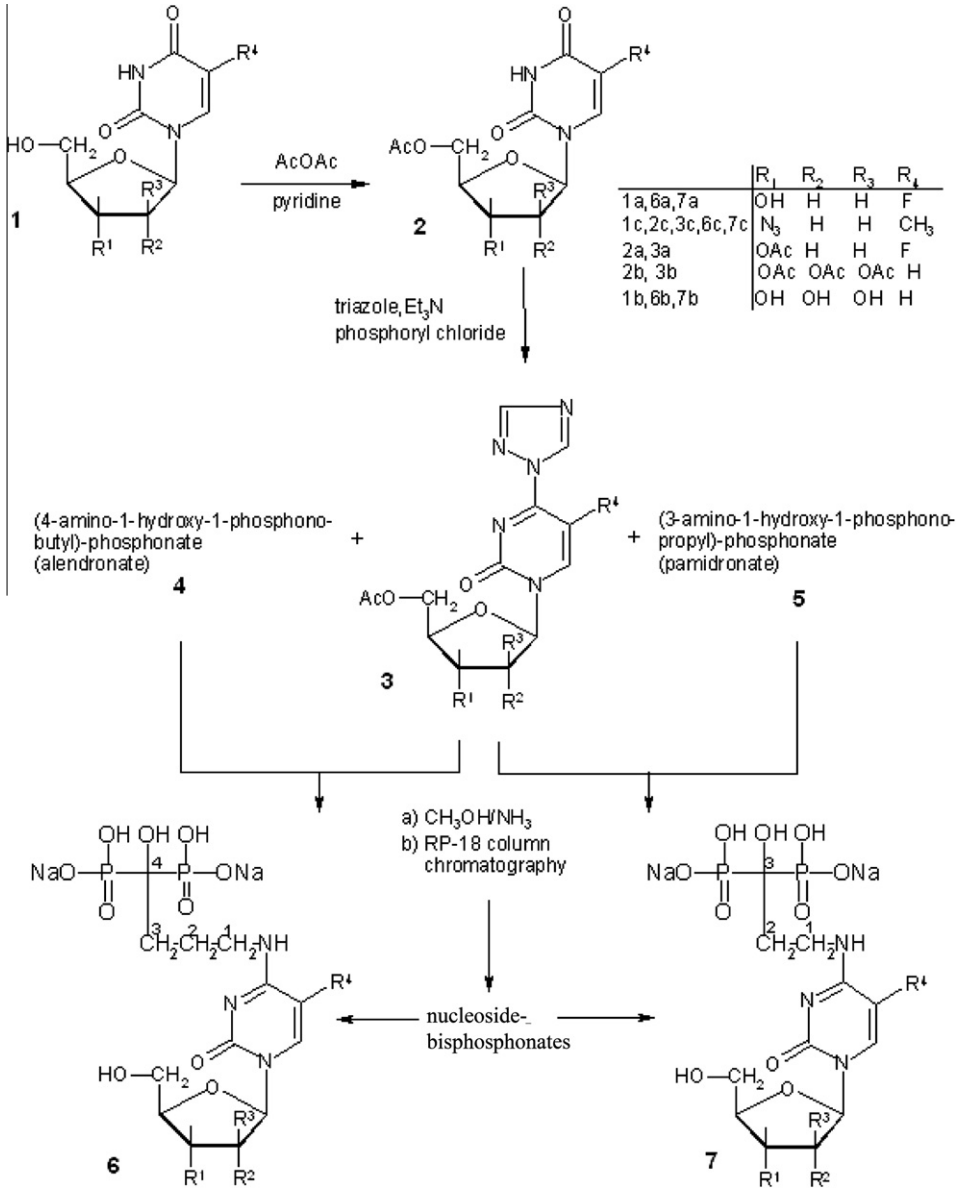

Figure 1. Synthesis route of N^4-[alkyl-(hydroxyphosphono)phosphonate]-cytosines. After protection and derivation of the starting nucleosides 2′-deoxy-5-fluorouridine (5-FdU, **1a**), 1-β-D-arabinofuranosyluracil (araU,**1b**) or 3′-azido-2′,3′-dideoxythymidine (AZT, **1c**), the triazolylated compounds **3** were obtained. Subsequent substitution of the triazolyl residue with the (aminoalkyl-1-hydroxy)bisphosphonates alendronate (**4**) and pamidronate (**5**) followed by deacetylation and chromatographic purification resulted in 5-FdU-ale (**6a**), araU-alendronate (**6b**), AZT-alendronate (**6c**) and the corresponding drugs (**7a–c**) linking pamidronate instead of alendronate.

retention time of the negatively charged compounds without improving the separation of the compounds. Beside this, the eluents used for anion exchange chromatography contain salts which have to be removed from the chromatographed compounds in time consuming procedures.

BPs were obtained at rather low yields ranging between 35% and 55%. Another reason could be the unfavorable conditions for the substitution reaction. The solubility of the highly polar BPs being only soluble in water in respect to the protected non-polar nucleoside derivatives which were nearly insoluble in water is not an optimal condition for a homogenous reaction. The used reaction medium water/dioxane did not provide a suitable environment favoring the substitution at high yields. Reversible protection by esterification of the phosphate residues of the BPs improve their solubility in organic solvents which could increase the yield, however, the attempt to achieve this protection would also drastically increase the synthesis effort and the use of esterified BPs could cause the above mentioned undesired rearrangements of the BPs to inactive derivatives. The chemical structure and the analytical purity of the synthesis products were confirmed by NMR-mass spectroscopy and elementary analysis. Elementary analysis of **2c** and **3a** was not performed because these substances were only obtained as syrup or foam.

2.3. Hydroxyapatite binding of nucleoside-BPs

It is known that the BPs moiety of conjugates preserves its high affinity for the bone matrix even when coupled to a bulk residue.[33,44] The high affinity to the bone matrix is based on the strong binding properties of BPs to hydroxyapatite.[36,45,46] For this reason, we analyzed the adsorption properties of 5-FdU-ale and 5-FdU to hydroxyapatite.

As summarized in Table 1, only 15% of 5-FdU were adsorbed to hydroxyapatite, whereas 5-FdU-ale adsorption was approximately sixfold higher (87%). The binding study was also used for an indirect assessment of the stability of the nucleoside-BPs in acidic

Table 1
Binding of 5-FdU and 5-FdU-alendronate (5-FdU-ale) to hydroxyapatite

Drug	Drug (mg)	Hydroxy-apatite (g)	UV-absorption			Bound drug	
			A_{260}-units	Ratio		mg	%
				250/260	280/260		
5-FdU	246	0	8050	0.61	1.01	0	0
5-FdU	246	7	6860	0.63	1.10	37	15
5-FdU-ale	475	0	7590	1.00	1.27	0	0
5-FdU-ale	475	5	4500	1.02	1.23	195	41
5-FdU-ale	475	7	990	1.04	1.17	414	87
5-FdU-ale*	475	7	1950	1.00	1.23	352	74

The A_{260}-units of aqueous solutions/suspensions (100 ml) containing 5-FdU or 5-FdU-ale were measured before and after the addition of solid hydroxyapatite. Amounts of drug bound were calculated on the basis of the decreased A_{260}-units of the supernatant.
* Acid pretreatment (see Section 3.3.2).

conditions. Binding was reduced by 13% compared to the untreated compound when a solution of 5-FdU-ale was acidified to pH 2 and kept at 50 °C during 24 h prior to the adsorption to hydroxyapatite. The unchanged A250/260 and 280/260 UV-ratio indicate that no cleavage of 5-FdU-ale into the monomer 5-FdU+ale occurs during treatment with hydroxyapatite under the used conditions.

A TLC analysis of the supernatant did not reveal any UV-active compounds like 5-fluorouracil or 5-FdU. Thus, the absence of UV-active compounds allows to conclude that 5-FdU-ale is very stable in acidic conditions.

2.4. Growth inhibition of tumor cell lines incubated with 5-FdU-alendronate

Preliminary evaluations of the in vitro cytostatic activity of the nucleoside-BPs were performed with 5-FdU-ale using the 60-cell line in vitro screening panel of the National Cancer Institute (NCI, USA). In these standard screens the growth percent inhibition caused by short 48 h incubation and using only one concentration (10 μM) of 5-FdU-ale was determined (additional details of the NCI screening can be found at http://www.dtp.nci.nih.gov). Under these not optimized screening conditions in respect to testing of prodrugs it was not surprising that the growth of only 11/59 cell lines was reduced in the range between 5% and 38%, whereas the other cell lines (48/59) remained unaffected. The growth inhibition values of the 11 sensitive cell lines are listed in Table 2. Nevertheless, these preliminary results showed that the antimetabolite-BPs represent a new family of BPs with cytotoxic activities which are comparable to those of amino-BPs. In a selection of human solid tumor cell lines zoledronate showed the best cytotoxic activity with an IC_{50} of 17 μM, whereas for ale the IC_{50} was 67 μM and for clodronate 105 μM, respectively.[22] In another study the combination of zoledronic acid with gemcitabine showed cytotoxic synergy in a panel of 8 human tumor cell lines. The IC_{50} of zoledronate ranged between 2.3 and 86.6 μM in these tumor cells.[15]

Table 2
In vitro growth inhibition of tumor cell lines with 5-FdU-ale

Panel/cell line	Growth% 5-FdU-ale
Leukemia	
HL-60 (TB)	62
Non-small cell lung cancer	
EKVX	82
HOP-62	93
NCI-H322 M	89
CNS cancer	
SF-268	87
SF-539	95
SNB-75	87
Melanomas	
MALME-3 M	89
MDA-MB-435	87
Ovarian cancer	
OVCAR-3	88
Renal cancer	
CAKI-1	79

The cytostatic activity of 5-FdU-ale is expressed by percent growth compared to untreated control cells (100%) after incubation treatment for 48 h with 10 μM 5-FdU-ale.

Table 3
Drug concentrations (μM) of 5-FdU, ale or 5-FdU-ale resulting in 50% growth inhibition (IC_{50}) of the Lewis lung cancer (LLC) or the macrophage RAW.264.7 (RAW) cell lines

Drug	Incubation (h)	IC_{50} (μM)	
		LLC	RAW
5-FdU	24	14.2	—*
	72	2	—*
Alendronate	24	0.43	—*
	72	2.62	30 (11)
5-FdU-ale	24	—*	—*
	72	0.15	25 (167)

* IC_{50} not reached, in parentheses: ratio of IC_{50} RAW/IC_{50} LLC.

A second cell viability test confirmed the cytotoxic activity of 5-FdU-ale and showed a distinct dependence between the IC_{50} values and the duration of incubation on Lewis lung cancer (LLC) cells and RAW 264.7 macrophages (Table 3). To reach the IC_{50} for LLC cells an incubation of 24 h was sufficient for 5-FdU and ale. However, for 5-FdU-ale this incubation time was too short and a prolonged incubation of 72 h was required. IC_{50} values of the less sensitive RAW cells were only achieved with ale and 5-FdU-ale during 72 h of incubation, whereas 5-FdU was inactive.

2.4.1. Possible causes and mechanisms resulting in cytostatic activities of antimetabolite-BPs

The coupling of pamidronate or ale to an antimetabolite results in new molecules combining two different cytostatic functions. The first example of an active BP with a dual function linked sulfur mustard to a BP.[47] The relatively stable anchoring of the BPs to the N^4-position of cytidine may contribute to the accumulation of nucleoside-BPs in vivo in the bone before its complete metabolic transformation and therefore these BPs could act in vivo as a depot of different antimetabolites enriched in metastatic bones. In contrast, due to the low affinity of cytotoxic nucleosides to bone, their specific targeting and accumulation in bone or bone metastases cannot be achieved with mixtures of antimetabolites and BPs.

It is conceivable that the intact molecules act as modified BPs before they are metabolized as they exert weak cytotoxic effects which are comparable to those of therapeutically used amino-BPs. It is not described that nucleoside derivatives which contain highly polar N^4-residues show any cytotoxic activity[42,48] the nucleoside BPs could only be active after enzymatic hydrolysis within the bone matrix could inhibit osteoclast activity and effectively kill tumor cells.

In case that the N-glycosidic bond between carbohydrate and nucleobase is enzymatically cleaved, new N-containing BPs are formed, whose alkyl chain carry either a pyrimidinone (from araU), 5-fluoropyrimidinone (from 5-FdU) or a 5-methylpyrimidinone (from AZT) residue at the terminal end of the alkyl chain. The hydrolytic cleavage at the N^4-alkyl-linkage can lead to amino-BPs, respectively to uridine analogs (5-FdU, AraU, AZT). This cleavage pathway would result in a mixture of drugs with particularly

strong antitumor or antiviral effects (5-FdU, AZT). The cytostatic effects of the formed metabolites could be caused by the fact that the antimetabolites either disrupt vital functions of DNA and RNA or that the effects of the amino-BPs occur by BP-induced ATP-analog formation or inhibition of farnesyl diphosphate synthase in the mevalonate pathway.[49]

The rather modest growth inhibitory effects of 5-FdU-ale which resulted from the NCI screening suggest that the conjugate was not metabolized to 5-FdU or 5-FU and that the cytotoxic activity originated from the BPs moiety of the intact compound.

We assume that the antimetabolite-BPs can be cleaved into different active metabolites that may exert cytotoxic and other therapeutic effects. However, the underlying mechanisms of the new conjugates remain to be assessed in future experiments.

3. Experimental

3.1. General chemistry

Unless otherwise noted, starting materials were purchased from commercial sources and were used as is. Hydroxyapatite (1.2 mg/g dry material binding capacity, DNA) was obtained from Sigma–Aldrich. AZT, **1c** was isolated from expired drugs[37,50] 4-(1,2,4-triazol-1-yl)-1-(β-D-2′,3′,5′-tri-*O*-acetyl-arabinofuranosyl)pyrimidine-2(1*H*)one (**3b**) was synthesised according to published methods.[40] (4-Amino-1-hydroxy-1-phosphono-butyl)phosphonate monosodium salt trihydrate (alendronate) (**4**) and (3-amino-1-hydroxy-1-phosphonopropyl)phosphonate monosodium salt monohydrate (pamidronate) (**5**) were synthesized, as described, however, markedly lower yields were obtained.[51] The nucleoside derivatives and impurities were detected using UV-light and spray reagents as developing agents as previously described.[42] TLC was performed on pre-coated silica gel plates 60_{F254} (0.25 mm, Merck). Preparative column chromatography of the obtained mixtures (>2 g) was carried out at room temperature by flash chromatography on self-packed RP-18 reversed phase (LiChroprep, 40–60 μm, Merck) columns. The desired compounds were identified using UV-absorption. The eluates were collected in 20 ml fractions. The fractions of the desired product were concentrated to syrup and the product precipitated by adding methanol. Smaller amounts (<2 g) were chromatographed using commercially available RP-18 reversed phase columns (Lobar B, LiChroprep RP-18, 40–63 μm, Merck). The eluted product was isolated by lyophilization of the combined and concentrated fractions. Aliquots of the BPs-derivatives were purified to p.a. quality by twofold reversed phase chromatography and lyophilization of the collected fractions. The concentration of the reaction mixtures, organic layers and eluted fractions were done in vacuo at a bath temperature of about 45 °C. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker A250 spectrometer at 250 and 62.9 MHz or on a Bruker Avance 400 spectrometer at 400 and 100 MHz, respectively. $CDCl_3$ and D_2O were used as solvents and Me_4Si as internal standard. ^{31}P NMR data were obtained on a Bruker Avance 400 spectrometer at 161 MHz, using H_3PO_4 as the external standard. Mass spectra were measured on a Finnigan TSQ 70 or on a Finnigan MAT 95 instrument. For FAB Mass spectra, all compounds were measured in a NBA or glycerine matrix.

3.2. Synthesis of N^4-[alkyl-(hydroxyphosphono)phosphonate]-cytidine nucleosides

3.2.1. Synthesis of 3′,5′-di-*O*-acetyl-2′-deoxy-5-fluorouridine (2a)

2′-Deoxy-5-fluorouridine (**1a**) (24.6 g, 0.1 mol) was dissolved in dry pyridine (200 ml) and acetic anhydride (47 ml, 0.5 mol) was added. The resulting solution was stirred under exclusion of moisture for 8 h at room temperature and subsequently concentrated until crystallisation occurred. The concentrate was co-evaporated with toluene (100 ml) and the resulting residue dissolved by heating (60 °C) in ethanol (250 ml) followed by crystallization at 4–7 °C. The precipitate was filtered, washed with cold ethanol and dried to yield 32.4 g (97%) of **2a**; mp: 151 °C; R_F = 0.35 ($CHCl_3$/MeOH, 95:5, v/v); MS (FAB$^+$) 331. [M−H$^+$]. Anal. Calcd $C_{13}FH_{15}N_2O_7$ (330.3): C, 47.28; H, 4.58; N, 8.48. Found: C, 47.43; H, 4.78; N, 8.20. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 2.13 (s, 3H, –CH_3), 2.15 (s, 3H, –CH_3), 2.16–2.25 (m, 1H, H2′α), 2.55 (ddd, 1H, J_1 = 2.3 Hz, J_2 = 5.7 Hz, J_3 = 14.3 Hz, H2′β), 4.28 (m, 1H, H4′), 4.32 (dd, 1H, J_1 = 3.1 Hz, J_2 = 12.2 Hz, H5′β), 4.41 (dd, 1H, J_1 = 3.6 Hz, J_2 = 12.1 Hz, H5′α), 5.24 (m, 1H, H3′), 6.31 (m, 1H, H1′), 7.7 (d, 1H, J = 6.2 Hz, H6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 20.8 ($COCH_3$), 20.9 ($COCH_3$), 37.8 (C2′), 63.8 (C5′), 73.9 (C3′), 82.5 (C4′), 85.4 (C1′), 123.2 (d, J_{CF} = 34.5 Hz, C6), 139.7 and 142.1 (d, J_{CF} = 238 Hz, C5), 149.2 (C2), 156.9 (d, J_{CF} = 26.7 Hz, C4), 170.3 (C=O), 170.5 (C=O).

3.2.2. Synthesis of 5-fluoro-4-(1,2,4-triazol-1-yl)-1-(β-D-3′,5′-di-*O*-acetyl-2′-deoxyribofuranosyl)-pyrimidine-2(1*H*)-on (3a)

1,2,4-Triazole (34.5 g, 0.5 mol) was dissolved under stirring and heating in dry acetonitrile (150 ml). The mixture was cooled to 10 °C under vigorous stirring and to the resulting suspension phosphoryl chloride (10.3 ml, 0.11 mol) was added slowly dropwise to prevent temperature increase over 25 °C. Then, the reaction mixture was cooled to approx. 10 °C and triethylamine (70 ml, 0.5 mol) was added dropwise under stirring and cooling. To this mixture 3′,5′-di-*O*-acetyl-5-fluorouridine (**2a**) (16.5 g, 50 mmol) dissolved in dry acetonitrile (90 ml) was slowly added and the reaction mixture was stirred at ambient temperature for 4 h. After addition of triethylamine (70 ml) and water (17 ml) the solution was concentrated to a syrup which was diluted with $CHCl_3$ (150 ml) and extracted with 5% sodium hydrogen carbonate (2× 50 ml). The organic layer was concentrated and dried resulting in the crude product (16.7 g, 88%) of **3a** as a solid foam which was used in the following synthesis step without further purification. R_F = 0.35 ($CHCl_3$/MeOH, 95:5, v/v). The R_F values **2a** and **3a** are very similar. Compound **3a** shows under UV-light (366 nm) violet fluorescence, whereas **2a** is not fluorescent and appears as dark spot (254 nm) on the TLC plate. MS (FAB$^+$) 382.1 [M−H$^+$]; ^{1}H NMR (250 MHz, $CDCl_3$): δ = 2.13 (s, 3H, $COCH_3$), 2.14 (s, 3H, $COCH_3$), 2.10–2.15 (m, 2H, H2′), 2.30 (dt, 1H, J_1 = 6.7 Hz, J_2 = 14.5 Hz, H2′α), 2.90, (ddd, 1H, J_1 = 3.3 Hz, J_2 = 5.9 Hz, J_3 = 14.5 Hz, H2′β), 4.25–4.50 (m, 3H, H5′ + H4′), 5.26 (m, 1H, H3′), 6.24 (dd, 1H, J_1 = 6.1 Hz, J_2 = 6.5 Hz, H1′), 8.25 (s, 1H, N=CH–N), 8.48 (d, 1H, J = 6.5 Hz, H6), 9.25 (s, 1H, NCH–N). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 22.7 ($COCH_3$), 22.8 ($COCH_3$), 38.9 (C2′), 63.3 (C5′), 73.3 (C3′), 83.5 (C1′), 88.1 (C4′), 133.8 (d, J_{CF} = 34.8 Hz, C6), 135.0, 137.5, 140.8 (d, J_{CF} = 237 Hz, C5), 145.0 (NCN), 151.5, 154.8 (C2), 156.9 (d, J_{CF} = 26.5 Hz, C4), 170.2 (C=O), 170.3 (C=O).

3.2.3. Synthesis of 5-methyl-4-(1,2,4-triazol-1-yl)-1-(β-D-5′-*O*-acetyl-3′-azido-2′,3′-dideoxyribofuranosyl)-pyrimidine-2(1*H*)-on (3c)

3′-Azido-2′,3′-dideoxythymidine (**1c**) (9 g, 34 mmol) was dissolved in dry pyridine (70 ml). Acetic anhydride (16 ml, 0.17 mol) was added and the solution was stirred under exclusion of moisture for 8 h at ambient temperature, subsequently concentrated to a syrup which was co-evaporated with toluene (40 ml) and dried to a solid foam (9.8 g, 94%) of 5′-acetyl-3′-azido-2′,3′-dideoxythymidine (**2c**) which was used without further purification in the following triazolylation, performed in analogy to Section 3.2.2 using **2c** (9.8 g, 32 mmol) instead of **2a.** The organic layer obtained after neutralization of the reaction mixture was concen-

trated to syrup. After addition of ethanol (120 ml) the solution was concentrated to 50 ml and kept overnight in a refrigerator (6 °C). The crystalline precipitate was collected by filtration, washed with a small amount of cold ethanol and dried resulting in 8.8 g (77%) of **3c**. Mp: 142–143 °C. R_F = 0.49 ($CHCl_3$/MeOH, 95:5, v/v); MS (FAB^+) 361 [M−H^+]. Anal. Calcd $C_{14}H_{16}N_8O_4$ (360.33): C, 46.66; H, 4.48; N, 31.10. Found: C, 46.72; H, 4.58; N, 31.65. ^{1}H NMR (250 MHz, $CDCl_3$): δ = 2.16 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 2.43–2.55 (m, 1H, H2′), 2.81 (dt, 1H, J_1 = 6.4 Hz, J_2 = 14.1 Hz, H2′b), 4.14–4.28 (m, 2H, H3′ und H4′), 4.40 (dd, 1H, J_1 = 3.1 Hz, J_2 = 12.4 Hz, H5′α), 4.50 (dd, 1H, J_1 = 4.1 Hz, J_2 = 12.4 Hz, H5′β), 6.15 (dd, 1H, J_1 = 4.8 Hz, J_2 = 6.5 Hz, H1′), 8.1 (s, 1H, NCHN), 8.13 (s, 1H, NCHN), 9.28 (s, 1H, H6). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ = 16.9 (CH_3), 20.5 ($COCH_3$), 38.3 (C2′), 59.5 (C5′), 62.7 (C3′), 82.6 (C1′), 87.5 (C4′), 105.3 (C5), 144.7 (C6), 145.8 (2 × NCN), 153.2 (C2), 158.0 (C4) 169.9 (C=O).

3.2.4. Synthesis of N^4-[butyl-(4-hydroxy-4-phosphono)phosphonate]-5-fluoro-2′-deoxycytidine (5-FdU-alendronate) (6a)

To a stirred and heated (85 °C) suspension of **4** (16.2 g, 50 mmol) in water (90 ml) **3a** (19 g, 50 mmol) dissolved in dioxane was added. The pH value of the suspension was adjusted to 8–9 by successively adding triethylamine. The progress of the substitution reaction was monitored by TLC. The fluorescent spot of the triazolyl compound (**3a**) disappeared during the reaction and new, slower migrating fluorescent spots were formed. When the substitution was completed after 3–4 h the reaction mixture was allowed to cool down to room temperature. Precipitated compounds were removed by filtration and the filtrate was evaporated. To the obtained syrup methanol (150 ml) was added and the formed fine precipitate was filtered off and dried resulting in the raw material part I. The filtrate was evaporated to syrup resulting in a raw material part II. I and II were separately purified. I was suspended in acetonitrile (100 ml), stirred at 70 °C for 3 h, filtrated, dried and deprotected according to the following procedure. To the obtained fine powder 25% ammonium hydroxide (300 ml) was added. The stirred reaction mixture was kept closed at ambient temperature for 3 days, followed by concentration. The resulting syrup formed by addition of methanol and vigorous stirring a fine precipitate, which was filtered and dried. The obtained residue was dissolved in a small volume of water and purified by flash chromatography on RP-18 column (LiChroprep, 40–60 μm) using distilled water as eluent. The desired compounds of the fractionated eluate were identified by UV absorption (254 nm). The combined fractions of the UV-absorbing main peak eluted first were evaporated to syrup which formed a fine precipitate by adding methanol and vigorous stirring. The filtered precipitate was dried resulting in the first part of **6a**. The purification of II was performed according to the following procedure. By adding water to II a precipitate was formed and removed by filtration. The filtrate was evaporated and the residue was dissolved in a small volume of water and purified on a RP-18 column as described for I. Deprotection was done using 25% ammonium hydroxide according to the described procedure resulting in the second part of **6a**. The isolated products I and II were combined resulting 15.6 g **6a** in a yield of 55%. MS (FAB^-) 476.0, [M−H^-], 498.0 [M+Na^-], 520.1 [M+2Na^-]. Anal. Calcd $C_{13}H_{20}FN_3Na_2O_{11}P_2$*2.5$H_2O$ (566.29); C, 27.57, H, 4.45, N, 7.42. Found: C, 27.31, H, 4.33, N, 7.57. ^{1}H NMR (400 MHz, D_2O): δ = 1.87–2.05 (m, 4H, 2× $-CH_2-$, H2″, H3″), 2.19–2.44 (m, 2H, H2′) 3.43 (m, 2H, H1″) 3.74 (dd, 1H, J_1 = 5.0 Hz, J_2 = 12.5 Hz, H5′α), 3.82 (dd, 1H, J_1 3.6 Hz, J_2 = 12.5 Hz, H5′β), 3.99–4.04 (m, 1H, H3′), 4.39–4.44 (m, 1H, H4′), 6.21 (m, 1H, H1′), 7.83 (d, 1H, J_{HF} = 6.7 Hz, H6); ^{13}C NMR (100 MHz, D_2O: δ = 23.2 (C3″), 31.2 (C2″), 39.2 (C2′), 41.1 (C1″), 61.2 (C5′), 70.4 (C3′), 73.9 (t, J_{CP} = 131 Hz, P–C–P), 85.9 (C1′), 86.6 (C4′), 123.4 (d, J_{CF} = 33.4 Hz, C6), 138.1 (d, J_{CF} = 243.4 Hz, C5), 155.8 (C2), 160.0 (C4); ^{31}P NMR (161 MHz, D_2O) δ = 18.4 ppm.

3.2.5. Synthesis of N^4-[butyl-(4-hydroxy-4-phosphono)phosphonate]-1-β-D-arabino-furanosylcytosine (araU-alendronate) (6b)

This BPs derivate was obtained by linking **3b** (21 g, 50 mmol) and **4** (16.2 g, 50 mmol) in the same manner as described above. Yield 11.7 g (42%) of **6b**; MS (FAB^-) 474.1 [M−H^-], 496.1 [M+Na^-], 518.2 [M+2Na^-]. Anal. Calcd $C_{13}H_{21}N_3Na_2O_{12}P_2$*2$H_2O$ (555.29) C, 28.12, H, 5.54; N, 7.57; Found C, 28.18; H, 4.75; N, 7.90. ^{1}H NMR (400 MHz, D_2O): δ = 1.84–2.09 (m, 4H, H2″, H3″), 3.30–3.41 (m, 2H, H1″), 3.78–3.94 (m, 2H, H5′), 3.95–4.04 (m, 1H, H4′), 4.06–4.16 (m, 1H, H3′), 4.37–4.43 (m, 1H, H2′), 4.8 (s, br, OH + NH + D_2O), 5.93–6.0 (m, 1H, H1′), 6.15–6.22 (m, 1H, H5), 7.62–7.69 (m, 1H, H6). ^{13}C NMR (100 MHz, D_2O: δ = 23.1 (C1″), 31.0 (C2″), 41.3 (C3″), 60.7 (C5′), 75.4 (C2′), 75.5 (C3′), 82.9 (C4′), 85.8 (C1′), 96.4 (C5), 140.6 (C6), 157.3 (C2), 163.6 (C4). ^{31}P NMR (161 MHz, D_2O) δ = 18.1 ppm.

3.2.6. Synthesis of N^4-[butyl-(4-hydroxy-4-phosphono)-phosphonate]-5-methyl-3′-azido-2′,3′-dideoxycytidine (AZT-alendronate) (6c)

This BPs derivate was obtained by linking **3c** (3.6 g, 10 mmol) and **4** (3.24 g 10 mmol) in the same manner as described above. Yield 2.0 g (35%) of **6c**; MS (FAB^-) 497.0 [M−H^-]; 519.1 [M+Na$^-$]; 541.1 [M+2Na^-]. Anal. Calcd $C_{14}H_{22}N_6Na_2O_{10}P_2$*3$H_2O$ (596.35), C, 28.20; H, 4.73, N, 14.09. Found: C, 27.92; H, 4.55; N, 14.45. ^{1}H NMR (400 MHz, D_2O): δ = 1.86–2.05 (m, 7H, H7, H2″, H3″), 2.35–2.52 (m, 2H, H1″), 3.38–3.52 (m, 2H, H2′), 3.78 (dd, 1H, J_1 = 4.7 Hz, J_2 = 12.5 Hz, H5′a), 3.86 (dd, 1H, J_1 = 3.6 Hz, J_2 = 12.5 Hz, H5′b), 4.0 (m, 1H, H3′), 4.31 (dt, 1H, J_1 = 5.4 Hz, J_2 = 7.2 Hz, H4′), 4.8 (s, br, D_2O + NH + OH), 6.18 (dd, 1H, J_1 = J_2 = 6.4 Hz, H1′), 7.49 (s, 1H, H6). ^{13}C NMR (100 MHz, D_2O): δ = 12.4 (CH_3), 22.4 (C3″), 31.2 (C2″), 36.5 (C1″), 41.6 (C2′), 60.0 (C5′), 61.0 (C3′), 73.9 (t, J_{CP} = 131 Hz, P–C–P), 83.9 (C1′), 85.4 (C4′), 105.5 (C5), 136.4 (C6), 157.5 (C2), 163.5 (C4). ^{31}P NMR (161 MHz, D_2O) δ = 18.5 ppm.

3.2.7. Synthesis of N^4-[propyl-(3-hydroxy-3-phosphono)phosphonate]-5-fluoro-2′-deoxycytidine (5-FdU-pamidronate) (7a)

This BPs derivate was synthesised by coupling **3a** (9.5 g, 25 mmol) and **5** (6.9 g, 25 mmol) in analogy to the synthesis route of **6a** resulting in **7a**. Yield 6.5 g (48%). MS (FAB^-) 462.0 [M−H^-]; 484.0 [M+Na^-]; 505.9 [M+2Na^-]. Anal. Calcd $C_{12}H_{18}FN_3$-$Na_2O_{11}P_2$*2H_2O (543.26) C, 26.53, H, 4.08, N, 7.73. Found: C, 26.28, H, 4.26, N, 8.22. ^{1}H NMR (400 MHz, D_2O): δ = 2.19–2.45 (m, 4H, H2′ + H2″), 3.68–3.87 (m, 4H, H5′ + H1″), 3.99–4.05 (m, 1H, H3′), 4.39–4.50 (m, 1H, H4′), 6.18–6.25 (m, 1H, H1′), 7.83 (d, 1H, J_{HF} = 6.0 Hz, H6). ^{13}C NMR (100 MHz, D_2O): δ = 32.3 (C2″), 36.6 (C1″), 39.2 (C2′), 61.2 (C5′), 70.4 (C3′), 72.9 (t, J_{CP} = 132.7 Hz, P–C–P), 85.9 (C1′), 86.6 (C4′), 123.4 (d, J_{CF} = 33.4 Hz, C6), 138.1 (d, J_{CF} = 243 Hz, C5), 155.6 (d, J_{CF} = 13.9 Hz, C4), 155.9 (C2). ^{31}P NMR (161 MHz, D_2O) δ = 17.8 ppm.

3.2.8. Synthesis of N^4-[propyl-(3-hydroxy-3-phosphono)-phosphonate]-1-β-D-arabino-furanosylcytosine (araU-pamidronate) (7b)

The substitution of **3a** (42 g, 0.1 mol) with **5** (27.4 g, 0.1 mol) in analogy to the preparation of **6a** resulted in **7b**. Yield 25.3 g, (45%). MS (FAB^-) 460.1 [M−H^-]; 482.2 [M+Na^-]. Anal. Calcd $C_{12}H_{20}N_3NaO_{12}P_2$*2$H_2O$*1.5$NH_3$ (562.86) C, 26.46, H, 5.27, N, 11.57. Found: C, 26.95, H, 5.56, N, 11.80. ^{1}H NMR (400 MHz, D_2O): δ = 1.93–1.98 (m, 2H, H2″), 2.76–2.81 (m, 2H, H1″), 3.75–3.92 (m, 2H, H5′), 3.92–4.01 (m, 1H, H4′), 4.04–4.39 (m, 1H, H3′), 4.34–4.39 (m, 1H, H2′), 5.86–5.92 (m, 1H, H1′), 6.12–6.18 (m, 1H,

H5), 7.64–7.75 (m, 1H, H6); ^{13}C NMR (100 MHz, D_2O): δ = 32.4 (C1″), 38.4 (C2″), 60.8 (C5′), 75.4 (C2′), 75.5 (C3′), 83.6 (C4′), 85.6 (C1′), 96.5 (C5), 140.7 (C6), 157.2 (C2), 163.2 (C4). ^{31}P NMR (161 MHz, D_2O) δ = 17.8 ppm.

3.2.9. Synthesis of N^4-[propyl-(3-hydroxy-3-phosphono)-phosphonate]-5-methyl-3′-azido-2′,3′-dideoxycytidine (AZT-pamidronate) (7c)

By substitution of **3c** (1.8 g, 5 mmol) with **5** (1.4 g, 5 mmol) in analogy to the preparation of **6a** the BPs derivat **7c** was obtained. Yield 1.2 g (41%). MS (FAB$^-$) 483.0 [M−H$^-$]; 505.1 [M+Na$^-$], 527.1 [M+2Na$^-$]. Anal. Calcd $C_{13}H_{20}N_6Na_2O_{10}P_2$*3.5$H_2O$ (591.34). C, 26.41, H, 4.60, N, 14.21. Found: C, 25.98, H, 4.46, N, 14.75. ^{1}H NMR (400 MHz, D_2O): δ = 1.93 (s, 3H, CH_3), 2.17–2.31 (m, 2H, H2″), 2.35–2.52 (m, 2H, H1″), 3.69–3.89 (m, 4H, H2′ + H5′), 3.97–4.03 (m, 1H, H3′), 4.28–4.35 (m, 1H, H4′), 4.8 (s, br, OH + NH + D_2O), 6.16–6.22 (m, 1H, H1′), 7.49 (s, 1H, H6). ^{13}C NMR (100 MHz, D_2O): δ = 12.3 (C7), 32.6 (C2″), 36.4 (C1″), 37.2 (C2′), 59.9 (C5′), 61.0 (C3′), 73.2 (t, J_{CP} = 130 Hz), 83.9 (C1′), 85.3 (C4′), 105.6 (C5), 136.3 (C6), 157.5 (C2), 163.1 (C4). ^{31}P NMR (161 MHz, D_2O) δ = 18.0 ppm.

3.3. Hydroxyapatite binding

3.3.1. 5-FdU and untreated 5-FdU-alendronate

5-FdU (246 mg, 1 mmol) or 5-FdU-ale (475 mg) were dissolved in distilled water (100 ml). The A_{260}-units of the obtained solution and the absorbance ratios of 250/260 and 280/260 were determined using an UV-spectrophotometer. After addition of solid hydroxyapatite as listed in Table 1 the obtained suspension was stirred at room temperature for 24 h. Then, an aliquot (1 ml) of the suspension was filtered through a sterile filter and the A_{260}-units of the filtrate were measured. The amount of drug bound to hydroxyapatite was calculated on the basis of the decreased A_{260}-units of the suspension.

3.3.2. Acidified pretreatment 5-FdU-alendronate

A solution of 5-FdU-ale (475 mg) in 100 ml H_2O was acidified to pH 2 by adding HCl and stirred at 50 °C for 24 h, followed by neutralization with solid sodium carbonate. UV-active degradation products were not detectable at 254 nm by TLC-analysis with $CHCL_3$/MeOH (80:20) using silica gel plates. The hydroxyapatite binding of the pretreated 5-FdU-ale was analyzed as described above.

3.4. In vitro cytotoxicity testing

3.4.1. Cell viability assay

Lewis Lung carcinoma cells (LLC) were cultivated in RPMI-140 with L-glutamine and RAW 264.7 macrophages were cultivated in DMEM (Dulbecco's Modified Eagle's Medium) containing 4.5 mg/L glucose. Both media were supplemented with 10% foetal bovine serum (FBS) and with 1% antibiotics (10,000 U/ml penicillin, 10,000 μg/ml streptomycin).

For the cell viability tests the cells were seeded at 5–10,000 cells per well in 96- or 48-well plates, respectively. Cell viability was determined with a resazurin assay.[52]

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