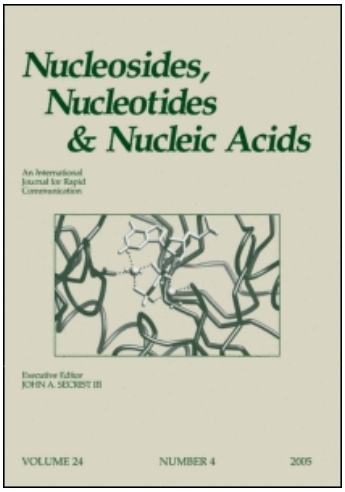

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Synthesis and Antitumor Activity of a Heterodinucleotide of BVDU and Gemcitabine

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SYNTHESIS AND ANTITUMOR ACTIVITY OF A HETERODINUCLEOTIDE OF BVDU AND GEMCITABINE

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□ *A heterodinucleotide comprising BVDU and Gemcitabine bound together by a 5′,5′-pyrophospate bridge (BVDUp$_2$dFdC) has been synthesized and evaluated as antitumor agent against AH13 rat sarcoma cells. BVDUp$_2$dFdC showed a cytotoxicity similar to that of Gemcitabine.*

Keywords BVDU; gemcitabine; dinucleotides; antitumor activity

INTRODUCTION

Nucleoside analogues (NA) constitute an important class of antimetabolites used in the treatment of several types of tumor diseases. These antitumor agents, as mimics of physiological nucleosides, are incorporated after phosphorylation into newly synthesized DNA resulting in DNA synthesis inhibition and chain termination. The mechanism of action of some of these drugs also includes the inhibition of key enzymes involved in the purine and pyrimidine nucleotide biosynthetic pathways and RNA synthesis, and apoptotic activity through caspase cascade activation.[1] However, the antitumor efficacy of these nucleosides could be weakened by the development of resistant tumor cells. The resistance to NA could arise from an insufficient intracellular concentration of NA di- or triphosphates, which may result from reduced levels of phosphorylating enzymes.[2] Chemoresistance could be overcome by the combination of two or more drugs that interact with different targets in order to interfere with multiple alterated pathways.[3]

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Various hetero- and homodinucleotides containing nucleosides endowed with antiviral or antitumor activity have been synthesized in the last years as an alternative strategy of the standard chemotherapy.[4–15] These dinucleotides have some advantages compared to the administration of nucleoside analogues as single drugs: 1) can act as prodrugs for a slow delivery of monomers in circulation; 2) can overcome the limiting phosphorylating activities of target tumor cells; 3) can have the advantage of a combination therapy with the administration of a single drug.

On these bases, we designed a new heterodinucleotide comprising the chemotherapeutic agents BVDU and Gemcitabine as a single drug.

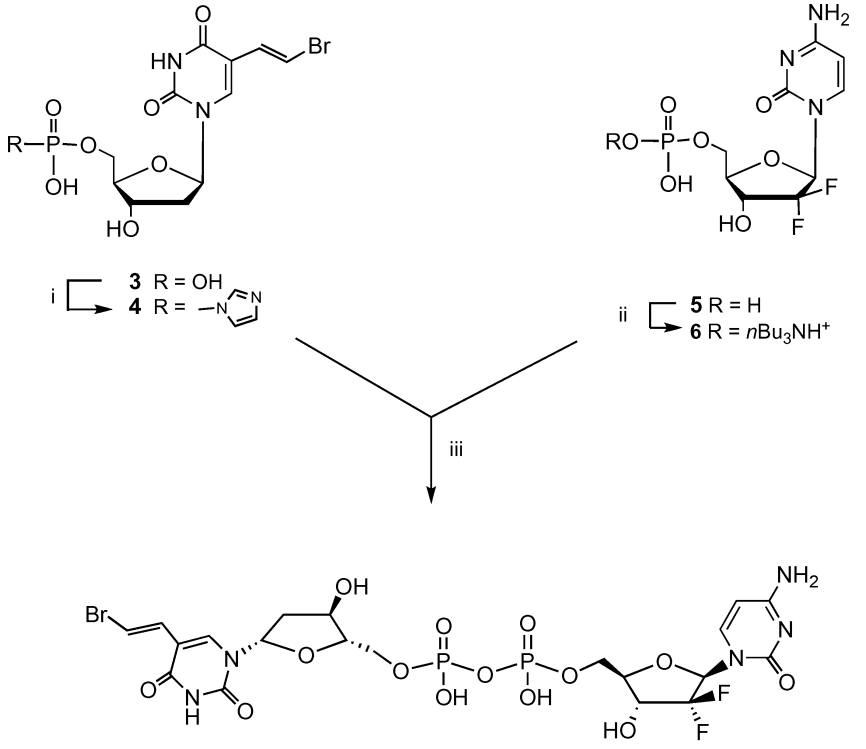
BVDU [(*E*)-5-(2-bromovinyl)-2′-deoxyuridine, RP101] is a highly potent antiviral drug endowed with selective activity against herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) infections. Moreover, several other herpes viruses and varicella viruses are sensitive to BVDU. The high selectivity of BVDU toward HSV-1 and VZV depends primarily on a specific phosphorylation of BVDU to its 5′-diphosphate by the virus-encoded thymidine kinase (TK). After further phosphorylation to the 5′-triphosphate, the drug interferes with the viral DNA polymerase.[16] The specific phosphorylation by the HSV-1 and VZV-induced TK also explains the marked cytostatic activity of BVDU against tumor cells that have been transduced by the viral TK gene. It also has been found that BVDU cotreatment with standard chemotherapy prevents the acquisition of chemoresistance.[3] Moreover, combination therapy of BVDU with the antitumor agent Gemcitabine improves the efficacy of chemotherapy in pancreas carcinoma cell lines and pancreatic cancer patients.[17] An improvement of chemotherapy in vitro also was showed by the combination of BVDU with Mitomycine C on AH13 sarcoma cells.[3] So, BVDU could be used as a co-treatment with cytostatic drugs to give a broader range of chemotherapy treatment options. This drug has demonstrated promising results in a clinical Phase I/II pilot study in pancreatic cancer patients.[17]

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine, dFdC) is a very promising new anticancer drug that is most commonly used to treat non-small cell lung cancer, pancreatic, bladder and breast cancer.[18] Gemcitabine is metabolized intracellularly to its active metabolites 5′-diphosphate (dFdCDP) and triphosphate (dFdCTP).[18] dFdCDP is a potent inhibitor of ribonucleotide reductase (RR), that is increased in several model of acquired resistance to Gemcitabine.

On the basis of these findings we prepared the heterodinucleotide BVDUp$_2$dFdC (**1**) comprising BVDU and Gemcitabine bound together by a 5′,5′-pyrophospate bridge. In this article, we report on the synthesis and antitumor evaluation of this dinucleotide. The homodinucleotide of BVDU (BVDUp$_2$BVDU, (**2**)) also was investigated.

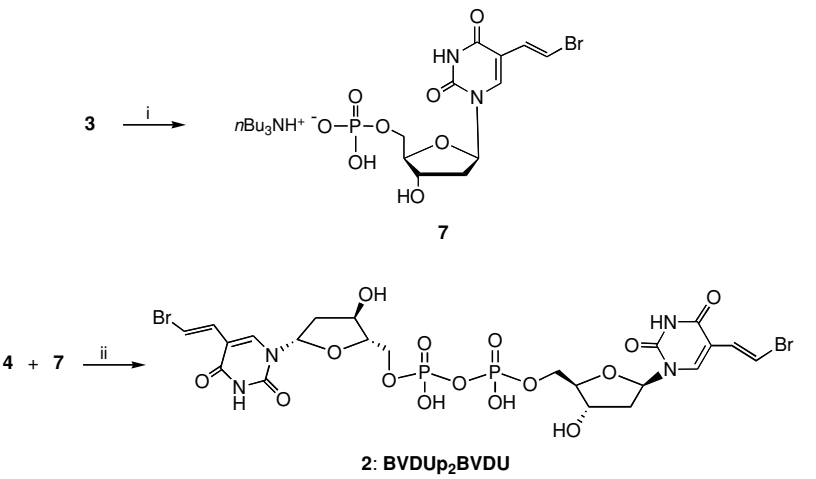
RESULTS AND DISCUSSION

The synthesis of compound **1** (BVDUp$_2$dFdC) was performed as reported in Scheme 1 by coupling of the 5-bromovinyl-2′-deoxyuridine-5′-monophosphate (BVDU-MP) (**3**) as imidazolide derivative (**4**) with the mono *n*-tributylammonium salt of 2′-deoxy-2′,2′-difluorocytidine-5′-monophosphate (**6**). BVDU-MP, was prepared as ammonium salt following the literature procedure of Yoshikawa et al.[19] This compound was converted to free acid by treatment with Dowex H^+ form and then activated as imidazolide (**4**) with an excess of 1,1′-carbonyldiimidazole (CDI) in dimethylformamide in quantitative yield. 2′-Deoxy-2′,2′-difluorocytidine-5′-monophosphate (**5**) was prepared as reported by Bonjouklian et al.[20] with some modifications, and treated with *n*-tributylamine to give the mono *n*-tributylammonium salt **6**. The coupling of **4** with **6** was performed in dry dimethylformamide at room temperature affording the heterodinucleotide



Reagents and conditions: i) 1,1'-carbonyldiimidazole, DMF a., rt, 4 h; ii) tributylamine, MeOH a., 30 min; iii) DMF a., rt, 18 h.

SCHEME 1 Synthesis of BVDUp$_2$dFdC



Reagents and conditions: i) tributylamine, MeOH a., 30 min; ii) DMF a., 40 °C, 3 h.

SCHEME 2 Synthesis of BVDUp$_2$BVDU.

1, which was purified on a silica gel column. In the same reaction, homodinucleotide **2** [P^1,P^2-bis(5-bromovinyl-2′-deoxyuridine-5′-yl)pyrophosphate] also was obtained (5% as determined by HPLC).

Homodinucleotide **2** (BVDUp$_2$BVDU) also was synthesized by reaction of BVDU-MP imidazolide (**4**) with BVDU-MP *n*-tributylammonium salt (**7**) (Scheme 2).

Antitumor activity of dinucleotides **1** and **2** was evaluated in in vitro experiments on AH13 rat sarcoma cells. AH13 cells were subjected to stepwise increasing doses of Gemcitabine and passaged serially. In parallel, cells were treated with increasing doses of BVDUp$_2$dFdC (equimolar). Enhanced dosage of either cytostatic compound resulted in decreasing cell numbers as expected (Figure 1). An advantage of BVDUp$_2$dFdC over Gemcitabine was not observed in this set of experiments.

AH13 cells were subjected to stepwise increasing doses of Mitomycine C (MMC) (30, 42, 49, 56 ng/ml) and passaged serially. In parallel, cells were treated with a combination of Mitomycine C + BVDUp$_2$BVDU or Mitomycine C + BVDU (30 μM). As BVDUp$_2$BVDU was toxic at 30 μM, and still toxic to 15 μM, the dosage was reduced to 7.5 μM. BVDU showed no toxicity at 30 μM. After 4 passages (17 days of treatment), the following cell numbers were recorded:

	A13H Cells/mL
MMC alone	846667
MMC + BVDU	609333
MMC + BVDUp$_2$BVDU	846333

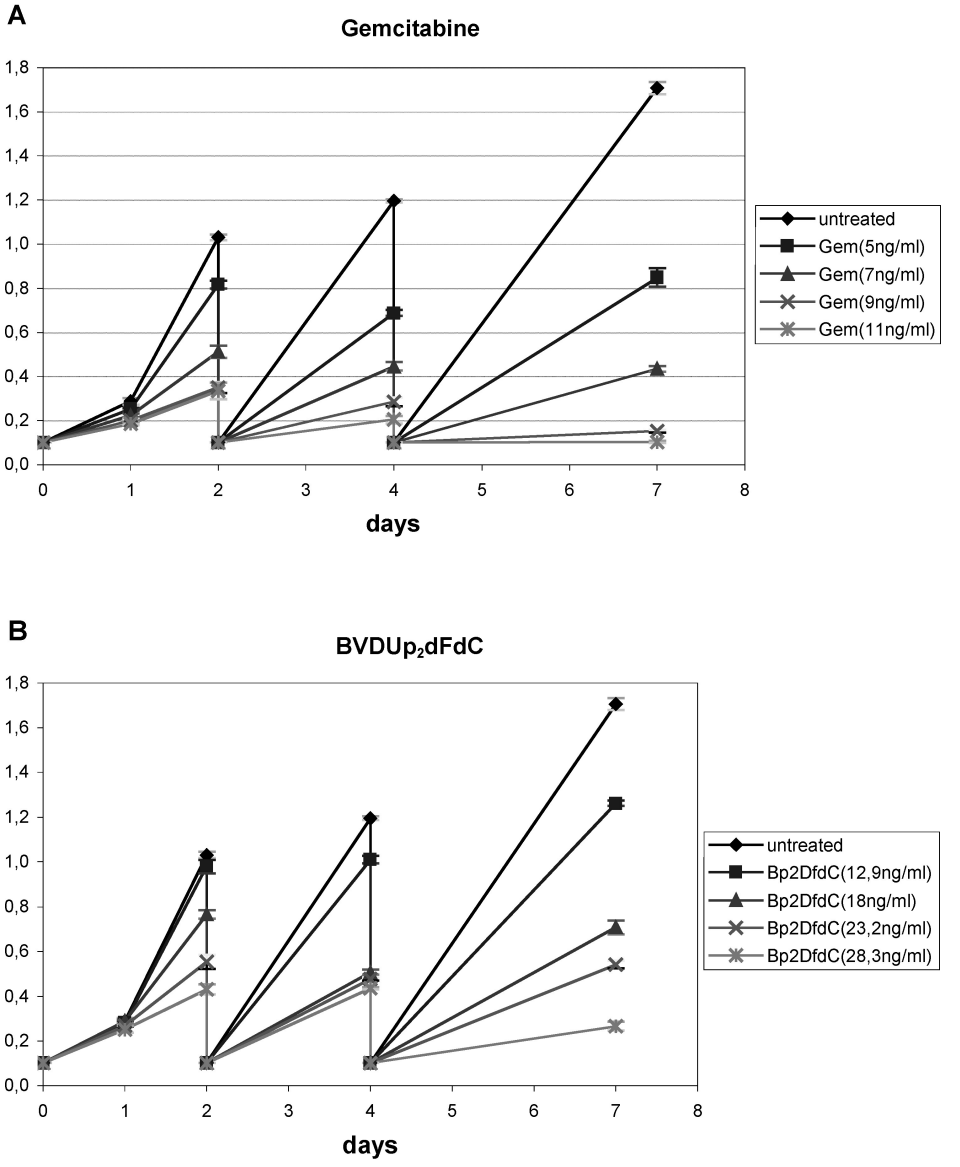


FIGURE 1 In vitro experiments. Effect of Gemcitabine (A) and BVDUp$_2$dFdC (B) on cell numbers in AH13 rat sarcoma cells.

Once the dose of BVDUp$_2$BVDU was reduced to non-toxic levels no more enhancement of the MMC effect was observed.

In conclusion, no advantages of heterodinucleotide BVDUp$_2$dFdC over Gemcitabine alone and of homodinucleotide BVDUp$_2$BVDU over BVDU against AH13 tumor cells were observed.

EXPERIMENTAL

Chemistry

Thin layer chromatography (TLC) was run on silica gel 60 F_{254} plates (Merck); silica gel 60 (70–230 Merck) for column chromatography was used. Ion exchange chromatography was carried out on (diethylamino)ethyl (DEAE)-A25 Sephadex column. Nuclear magnetic resonance spectra were recorded on a Varian Mercury AS400 spectrometer (Palo Alto, CA, USA) with TMS as the internal standard for ^{1}H NMR and external H_3PO_4 for ^{31}P NMR. Chemical shift values are expressed in δ values (parts per million) as s (singlet), d (doublet), t (triplet), dd (double doublet), q (quartet), m (multiplet), or brs (broad singlet). High-resolution mass spectra were recorded on an Agilent TOF II TOF/MS instrument equipped with either an ESI or APCI interface (Agilent Technologies, Santa Clara, CA, USA). HPLC analysis was carried out on a HP-1100 (Agilent Technologies GmbH, Waldbroon, Germany), using a LiChroCART 125–3 (Merck) RP18 endcapped (5 mm) Purospher column with a flow rate of 1mL/min of buffer A (250 mM CH_3COONH_4, 10 mM tetrabutylammonium phosphate monobasic and NH_4OH till to pH 7.2) 60%, and B (MeOH) 40%.

5-Bromovinyl-2′-deoxyuridine-5′-monophosphate **(3).** To a cooled mixture of BVDU (275 mg, 0.82 mmol) and $(CH_3O)_3PO$ (4.7 mL), $P(O)Cl_3$ (750 μL, 8.2 mmol) was added dropwise and the mixture was stirred at -5°C for 20 hours. Ice deionized water (5.0 mL) was added, the solution was neutralized with concentrated NH_4OH and then washed with $CHCl_3$ (3 × 15 mL). Concentration of the aqueous layer in vacuo gave the crude compound, which was purified by DEAE-Sephadex chromatography eluting with a linear gradient of NH_4HCO_3 (0–0.5 M). The pure compound (308 mg, 0.69 mmol) was dissolved in H_2O (11 mL) and treated with Dowex 50W-X8 (H^+ form) ion exchange resin (580 mg) stirring for 30 minutes. The resin was filtered off, the filtrate was concentrated in vacuo and coevaporated with anhydrous EtOH (3 × 10 mL) to give a solid (270 mg, 79% yield). ^{1}H NMR (400 MHz, D_2O): δ 2.20 (m, 2H, H-2′); 3.87 (m, 2H, H-5′); 4.0 (m, 1H, H-4′); 4.40 (m, 1H, H-3′); 6.13 (t, J = 7.0 Hz, 1H, H-1′); 6.77 (d, J = 14.1 Hz, 1H, vinyl); 7.08 (d, J = 13.7 Hz, 1H, vinyl); 7.78 (s, 1H, H-6). ^{31}P NMR (162 MHz, D_2O): δ 1.87 (s).

2′-Deoxy-2′,2′-difluorocytidine-5′-monophosphate **(5).** To a solution of $(CH_3O)_3PO$ (3.4 mL), and $P(O)Cl_3$ (2.3 mL, 25 mmol) at 5°C, was added 2′-deoxy-2′,2′-difluorocytidine (150 mg, 0.57 mmol) in small portions with rapid stirring (reaction time 2 hours). Ice deionized water (15 mL) was added, and the reaction mixture was extracted with $CHCl_3$ (2 × 15 mL). The aqueous layer was treated with concentrated NH_4OH to pH 6.5, taking care to keep the solution below 30°C and then extracted a third time with $CHCl_3$. The aqueous layer was concentrated under vacuo at 30°C or less. The residue was stirred with 30 mL of MeOH for 1 hour and filtered.

The filtrate was evaporated under vacuo to a solid which was purified by chromatography on a silica gel column eluting with *i*PrOH/NH_4OH/H_2O (7:2:1). The obtained solid (153 mg, 0.4 mmol) was dissolved in H_2O (1.5 mL) and treated with Dowex 50W-X8 (H^+ form) ion exchange resin (325 mg) stirring for 30 minutes. The resin was filtered off, the filtrate was concentrated in vacuo and coevaporated with anhydrous EtOH (3 × 10 mL) to a solid (100 mg, 53% yield). ^{1}H NMR (400 MHz, D_2O): δ 3.94 (2dd, J = 3.0, 5.6 Hz, 2H, H-5′); 4.10 (m, 1H, H-4′); 4.32 (m, 1H, H-3′); 6.10 (d and t, 2H, H-5, H-1′); 7.92 (d, J = 8.1 Hz, 1H, H-6). ^{31}P NMR (162 MHz, D_2O): δ 1.11 (s).

2′-Deoxy-2′,2′-difluorocytidine-5′-monophosphate tri-butylammonium salt (6). A mixture of **5** (92 mg, 0.27 mmol) and tributylamine (1 eq.) in dry MeOH (1.9 mL) was stirred for 30 minutes. Evaporation in vacuo gave the title compound as a white amorphous solid (125 mg, 88% yield). ^{1}H NMR (400 MHz, D_2O): δ 0.74 (t, J = 7.5 Hz, 9H, CH_3CH_2CH_2CH_2); 1.18 (m, 6H, CH_3CH_2CH_2CH_2); 1.48 (m, 6H, CH_3CH_2CH_2CH_2); 2.95 (m, 6H, CH_3CH_2CH_2CH_2); 3.94 (2dd, J = 3.0, 5.6 Hz, 2H, H-5′); 4.10 (m, 1H, H-4′); 4.28 (m, 1H, H-3′); 5.96 (d, J = 7.7 Hz, 1H, H-5); 6.06 (t, J = 7.3 Hz, 1H, H-1′); 7.74 (d, J = 7.7 Hz, 1H, H-6). ^{31}P NMR (162 MHz, D_2O): δ 1.22 (s).

P^1-5′-[5-Bromovinyl-2′-deoxyuridine]-P^2-5′-[2′-Deoxy-2′,2′-difluorocytidine] pyrophosphate (1). To a solution of **3** (94 mg, 0.23 mmol) in anhydrous DMF (4.6 mL), CDI (184 mg, 1.13 mmol) was added and the mixture was stirred at room temperature for 4 hours. Dry MeOH (95.2 μL) was added to hydrolyze excess CDI stirring for 30 minutes. To obtained imidazolide **4**, compound **6** (120 mg, 0.23 mmol) was added and the mixture was stirred at room temperature for 18 hours. The solvent was removed in vacuo and the residue was dissolved in H_2O (10 mL) and extracted with $CHCl_3$ (5 × 5 mL). The aqueous layer was concentrated in vacuo and the residue was purified by chromatography on a silica gel column eluting with *i*PrOH/NH_4OH/H_2O (8:1:1). Evaporation of the appropriate fractions gave the desired compound as a white solid (diammonium salt, 56 mg, 32% yield). ^{1}H NMR (400 MHz, D_2O): δ 2.14 (m, 2H, H-2′ (BVDU)); 4.0 (m, 5H, H-4′ (Gem), H-5′ (Gem), H-5′ (BVDU)); 4.28 (m, 2H, H-3′ (Gem), H-4′ (BVDU)); 4.40 (m, 1H, H-3′ (BVDU)); 5.90 (d and t, 2H, H-5 (Gem), H-1′ (Gem)); 6.12 (t, J = 6.8 Hz, 1H, H-1′ (BVDU)); 6.70 (d, J = 13.7 Hz, 1H, vinyl); 7.10 (d, J = 13.7 Hz, 1H, vinyl); 7.60 (d, J = 7.7 Hz, 1H, H-6 (Gem)); 7.66 (s, 1H, H-6). ^{31}P NMR (162 MHz, D_2O): δ -10.35 (dd, J = 20.74, 40.18 Hz). HRMS (ESI-): calcd for $C_{20}H_{23}BrF_2N_5O_{14}P_2$ (M-H) 735.9868, found 7359872.

5-Bromovinyl-2′-deoxyuridine-5′-monophosphate tri-butylammonium salt (7). The title compound was obtained from **3** (110 mg, 0.27 mmol) as described for **6** (white amorphous solid, 134 mg, 83% yield). ^{1}H NMR (400 MHz, D_2O): δ 0.74 (t, J = 7.5 Hz, 9H, CH_3CH_2CH_2CH_2); 1.18 (m, 6H,

$CH_3CH_2CH_2CH_2$); 1.48 (m, 6H, $CH_3CH_2CH_2CH_2$); 2.20 (m, 2H, H-2′); 2.94 (m, 6H, $CH_3CH_2CH_2CH_2$); 3.90 (m, 2H, H-5′); 4.03 (m, 1H, H-4′); 4.40 (m, 1H, H-3′); 6.15 (t, $J = 6.8$ Hz, 1H, H-1′); 6.78 (d, $J = 13.7$ Hz, 1H, vinyl); 7.10 (d, $J = 13.7$ Hz, 1H, vinyl); 7.78 (s, 1H, H-6). ^{31}P NMR (162 MHz, D_2O): δ 1.60 (s).

P*^{1},*P*^{2}*-Bis[5-bromovinyl-2′-deoxyuridine]-5′-pyrophosphate (2). To a solution of **3** (60 mg, 0.14 mmol) in anhydrous DMF (3.0 mL), CDI (117 mg, 0.72 mmol) was added and the mixture was stirred at room temperature for 3 hours. Dry MeOH (61 μL) was added stirring for 30 minutes. To the mixture, compound **7** (130 mg, 0.217 mmol) was added stirring at 40°C for 3 hours. The solvent was removed in vacuo and the residue was dissolved in H_2O (10 mL) and extracted with $CHCl_3$ (5 × 5 mL). The aqueous layer was concentrated in vacuo and the residue was purified by chromatography on a silica gel column eluting with *i*PrOH/NH_4OH/H_2O (8:1:1). Evaporation of the appropriate fractions gave compound **2** as a white solid (diammonium salt, 56 mg, 46% yield). ^{1}H NMR (400 MHz, D_2O): δ 2.14 (m, 4H, H-2′); 4.0 (m, 6H, H-4′, H-5′); 4.37 (m, 2H, H-3′); 6.0 (t, $J = 6.8$ Hz, 2H, H-1′); 6.72 (d, $J = 13.7$ Hz, 2H, vinyl); 7.05 (d, $J = 13.7$ Hz, 2H, vinyl); 7.62 (s, 2H, H-6). ^{31}P NMR (162 MHz, D_2O): δ -10.39 (s). HRMS (ESI-): calcd for $C_{22}H_{25}Br_2N_4O_{15}P_2$ (M-H) 804.9159, found 804.9163.

Biological Evaluation

Treatment of AH13R Sarcoma Cells in Culture. AH13r cells, a subline of the rat Yoshida sarcoma, were obtained from the Cell and Tumor Bank of the West German Cancer Center, University Essen, Medical School, Essen, Germany. Cells were grown in DMEM (FG 0415, Biochrom AG, Berlin, Germany) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Logarithmically growing cells were seeded at a density of 100,000 cells/ml and incubated with different cytostatic drugs in combination with or without BVDU. After 2–4 days (unless otherwise indicated), cells were counted using the Cell Counter and Analyser System CASY TT (Schärfe System GmbH, Reutlingen, Germany) and serially passaged.

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