Design and synthesis of phosphonoacetic acid (PPA) ester and amide bioisosters of ribofuranosylnucleoside diphosphates as potential ribonucleotide reductase inhibitors and evaluation of their enzyme inhibitory, cytostatic and antiviral activity

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Continuing our investigations on inhibitors of ribonucleotide reductase (RNR), the crucial enzyme that catalyses the reduction of ribonucleotides to deoxyribonucleotides, we have now prepared and evaluated 5’-phosphonoacetic acid, amide and ester analogues of adenosine, uridine and cytidine with the aim to verify both substrate specificity and contribution to biological activity of diphosphate mimic moieties. A molecular modelling study has been conducted on the RNR R1 subunit, in order to verify the possible interaction of the proposed bioisosteric moieties. The study compounds were finally tested on the recombinant murine RNR showing a degree of inhibition that ranged from 350 µM for the UDP analogue 5’-deoxy-5’-N-(phosphon-acetyl)uridine sodium salt (amide) to 600 µM for the CDP analogue 5’-O-[(diethyl-phosphon)acetyl]cytidine (ester). None of the tested compounds displayed noteworthy cytostatic activity at 100–500 µM concentrations, whereas ADP analogue 5’-N-[(diethyl-phosphon)acetyl]adenosine (amide) and 5’-deoxy-5’-N-(phosphon-acetyl)adenosine sodium salt (amide) showed a moderate inhibitory activity (EC50: 48 µM) against HSV-2 and a modest inhibitory activity (EC50: 110 µM) against HIV-1, respectively.

Keywords: synthesis, phosphonoacetic ester and amide, diphosphate isosters, RNR inhibitors, cytostatic and antiviral activity, molecular modelling

Introduction

The ribonucleotide reductase (RNR) is a crucial enzyme in the de novo synthesis of DNA; it converts all ribonucleoside diphosphates (NDP) into the corresponding 2′-deoxyribonucleotides (dNDP) in prokaryotic and eukaryotic cells. Viral RNRs are also known: herpes simplex virus (HSV)- and varicella zoster virus (VZV)-infected cells express a viral ribonucleotide reductase, distinct from that present in uninfected cells, which is endowed with a proper enzymatic activity that ensures sufficient 2′-deoxyribonucleotide supply and DNA synthesis (Boehmer et al., 1997; Heineman et al., 1994). Over the last decade, this enzyme has attracted increasing interest as an important target in the control of the replication of neoplastic cells as well as of pathogenic viral agents. The ribonucleotide diphosphate reductase (RDR) of the Escherichia coli, mammalian and HSV is structurally formed by two homodimeric proteins, namely R1 and R2, each of them is composed of two polypeptidic chains characterized by an α2β2 structure. The R1 subunit contains the binding sites for the ribonucleoside diphosphates, which are the specific substrates, and for the allosteric effectors, which control the speed and the specificity of dNDP formation. In previous studies, aimed at discovering new mechanism-based inhibitors of RNR (Manfredini et al., 1999), we pointed out the significant role of the diphosphate moiety of the nucleotide during the interaction with RNR. In fact, molecular modelling studies clearly showed that both natural and nucleoside diphosphate analogues accomplish their strong hydrogen bonds.
with Glu623, Thr624, Ser625 and Thr209 of the RNR R1 subunit, through the diphosphate group and particularly with the beta-phosphate. Eukaryotic class I RNRs recognize natural ribonucleoside diphosphates (NDP) as well as mechanism-based inhibitors with a nucleoside structure (for example, gemcitabine), after their conversion to the corresponding 5'-diphosphate form, by action of specific kinases (Plunkett et al., 1996). However, as widely reported, the nucleoside kinases recognition can be lost when considerable structural modifications, aimed to gain enzyme inhibitors, are accomplished on the natural substrates (Johansson & Eriksson, 1996; Kukhanova et al., 2000). Moreover, it is known that the clinical use of nucleotides is limited by their low stability to non-specific phosphohydrolases (Wagner et al., 2000).

To overcome these drawbacks, different approaches have been reported in the literature (Bazzanini et al., 1999; Manfredini et al., 1999). Among these, interesting results were obtained by SAR studies conducted to discover new bio-analogues of the nucleotide phosphate group with increased stability. An effective application of this approach on the diphosphate nucleotides was reported by Macchia et al. (1994) in the nineties; the authors studied a series of biososters of the diphosphate group in anti-HSV agents such as 5-iododeoxyuridine. A few years before Lambert and co-workers (1989) reported the antiviral activity of phosphonooacetic acid (PAA) and phosphonoformic acid (PFA) esters of 5-bromo-2'-deoxuryridine; these compounds were found to be relatively stable in contact with alkaline and intestinal phosphatase. Charvet et al. (1994) also modified the 2',3'-dideoxy-3'-thiacytidine (3TC, lamivudine) by introduction of PFA and PAA moieties and found these derivatives relatively stable to plasma esterase with a half-life of up to 120 min. Moreover, a well-established approach in medicinal chemistry involves the replacement of ester functions with heterocycles, such as isoxazoles, oxadiazoles and furans. (Manfredini et al., 2000; Manfredini et al., 1996; Manfredini et al., 1996). Design, synthesis, modelling studies and biological evaluation of the synthesized compounds will be herein described.

Materials and methods

Chemistry

Reaction courses were routinely monitored by thin-layer chromatography (TLC) on silica gel precoated Macherey-Nagel durosil-25, with detection under a 254 nm UV lamp and/or by spraying the plates with 10% H2SO4/CH3OH and heating and/or by spraying the plates with ammonium molybdate reagent. Column chromatography was performed with Macherey-Nagel 0.063–0.2 mm/70–230 mesh silica gel. MALDI-MS (matrix-assisted laser desorption ionization time-of-flight) spectra were obtained on a Hewlett-Packard HPG2025A mass spectrometer operative on a positive linear mode. Nuclear magnetic resonance spectra were determined in d6-DMSO, D2O and CDCl3 solution with a Bruker AC-200 spectrometer and chemical shifts are presented in ppm from internal tetramethylsilane as a standard; 31P NMR spectra were determined in d6-DMSO, D2O and CDCl3 with a Bruker AM-200 spectrometer and chemical shifts presented in ppm from internal 85% H3PO4/H2O solution as a standard. Ultraviolet spectra were recorded on a Kontron UVIKON 922 spectrometer. Melting points were determined by Kofler melting point apparatus (Thermovar, C Reichert AG, Vienna) and are uncorrected. All drying operations were performed over anhydrous sodium sulphate or magnesium sulphate; room temperature varied between 22° and 25°C.

Biology

Enzyme

Murine recombinant RNR proteins R1 and R2 were expressed and purified as previously reported (Mann et al., 1991; Thelander et al., 1994). Ribonucleotide reductase activity of recombinant murine protein was measured using the 3H-CDP reduction assay described earlier (Engström et al., 1979) with some modifications. The enzyme (10 and 0.7 micrograms of recombinant R2 and R1 respectively) was incubated at 37°C in 15 µl of a mixture containing 40 mM Hepes-K+, pH 7,6, 10 mM ATP, 6.4 mM MgCl2, 10 µM DDT, 100 mM KCl, 20 µM FeCl3, and 30 µM 3H-CDP (450 cpm/µmol). The reaction was stopped by the addition of 18 µl of 2 M perchloric acid and precipitated proteins were removed by centrifugation. 0.25 µmol of dCMP and CMP were added as carrier to the supernatant. After hydrolysis for 10 min at 100°C, the deoxyctydine monophosphate formed was isolated by thin layer chromatography with the following solvent: 2% boric acid-2 M LiCl (2:1, v/v). The marker spots were located under UV light, cut out and placed in vials with 4 ml of scintillation fluid. The radioactivity was counted in a Beckman liquid scintillation counter and all values were corrected by subtracting the blank values.

When ADP was used as substrate, the reaction mixture (30 µl) was the same as described above except for the substrate and the positive effectors (ADP: 40 µM; ATP: 3 mM and dGTP: 500 µM). After 30 min at 37°C, the reaction was stopped by boiling for 1 min. Precipitated proteins were removed by centrifugation. ADP reduction was determined after separation of the product from the substrate by HPLC chromatography using the BioRad (Hercules, CA) 100 MAPS preparative system. A 4.6×125 mm Partisphere SAX column (Wathman) was used at room temperature under the following conditions: injection volume, 25 µl; detection, UV
260 nm; eluent, buffer A (20 mM ammonium phosphate, pH 3.7) and buffer B (1 M ammonium phosphate, pH 3.7). Gradient conditions were 40 min linear gradient from buffer A to buffer B. The flow rate was 1 ml/min.

**Cytostatic activity of test compounds**

All assays were performed in 96-well microtitre plates. To each well were added 5-7.5x10^4 cells and a given amount of the test compound. The cells were allowed to proliferate for 48 h (murine leukaemia L1210, murine mammary carcinoma FM3A) or 72 h (human lymphocyte CEM and Molt) at 37°C in a humidified CO₂-controlled atmosphere. At the end of the incubation period, the cells were counted in a Coulter counter. The IC₅₀ (50% inhibitory concentration) was defined as the concentration of the compound that reduced the number of viable cells by 50%.

**Antiviral activity of the test compounds**

The compounds were evaluated against a variety of viruses in cell culture. The antiviral assays, other than HIV-1, were based on inhibition of virus-induced cytopathic effect in either E₅₀(SV-1, HSV-2, VV, VSV), HEL (VZV, CMV) or Vero (vesicular stomatitis virus, respiratory syncytial virus, Cosackie virus B4, parainfluenza-3 virus, Sindbis virus, Punta Toro virus, reovirus-1) cell cultures. Briefly, confluent cell cultures in 96-well microtitre plates were inoculated with 100 CCID₅₀ of virus, 1 CCID₅₀ being the virus dose required to infect 50% of the cell cultures. After a 1 h virus adsorption period, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations (400, 200 and 100 µg/ml) of the test compounds. Viral cytopathic effect was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds.

The assay to examine inhibition of HIV-1-induced cytopathic effect in CEM cells was as follows: human CEM (~3x10⁵ cells/ml) cells were infected with 100 CCID₅₀ of HIV-1(11IB)/ml and seeded in 200 µl wells of a microtitre plate, containing appropriate dilutions of the test compounds. After 4 days of incubation at 37°C, virus-induced CEM giant cell formation was examined microscopically.

**Molecular modelling**

Computational studies were conducted on a Silicon Graphics Origin 200, using the molecular modelling packages Cerius2 (v. 4.2) Accelrys Inc., San Diego (Calif., USA), and AMBER (v. 6.0) (Case et al., 1999; Pearlman et al., 1995). The starting 3D model of the R1 subunit of ribonucleotide reductase (RNR) was based on its X-ray crystallographic structure downloaded from the Protein Data Bank (PBD entry code 4R1R; Eriksson et al., 1997), data 4, C292A mutant with bound GDP and dTTP, and reduced active site. Water molecules in the coordinate file were removed and hydrogen was added to the protein backbone and side chains with the PARSE module of AMBER. All ionizable residues were considered in the standard ionization state at neutral pH. The all-atom force field (FF) parameters by Cornell et al. (1995) (in parm94.dat file of the AMBER 6.0 code) was applied for protein relaxation. The primary cut-off distance for non-bonded interactions was set to 12 Å and the cut-off tapers for the Coulomb and van der Waals interactions were 1.2 and 2, respectively. The GB/SA continuum solvation model (Jayaram et al., 1998; Weiser et al., 1999) was used to mimic a water environment. Geometry refinement was carried out using the SANDER module via a combined steepest descent-conjugate gradient algorithm, using as a convergence criterion for the energy gradient the root-mean-square of the Cartesian elements of the gradient equal to 0.01 kcal/(mol Å). As expected, no relevant structural changes were observed between the R1 subunit relaxed active site and the original 3D structure. To prevent global conformational changes of the enzyme, the backbone of the protein binding site were constrained by a harmonic force constant of 100 kcal/mol, whereas the amino acid side chains and the ligands were allowed moving without any constraint. Each nucleotide analogue/enzyme complex resulting from the procedure described above was further refined in the AMBER suite using the quenched molecular dynamics (QMD) method. In this case, 100 ps MD simulation at 298 K were employed to sample the conformational space of the ligand-enzyme complex. The integration step was equal to 1 fs. After each ps, the system was cooled to 0 K, the structure was extensively minimized and stored. The lowest energy structure for each ligand/receptor complex was selected for further analysis.

**2',3'-O-isopropyliden-5'-O-{(diethyl-phosphon) acetyl}-adenosine (3a)** Compound 1a (Hampton, 1961) (200 mg, 0.651 mmol), DCC (322.4 mg, 1.562 mmol) and 4-DMAP (7.95 mg, 0.0651 mmol) were dissolved in DMF (8 ml) and, to the stirred solution, diethyl phosphonacetic acid (210 ml, 1.302 mmol) was slowly added under argon atmosphere. After 20 h at room temperature (TLC: CH₃Cl/MeOH, 9:1), the reaction was filtered. The solvent was evaporated and co-evaporated with EtOH (3x10 ml). The residue was dissolved in CH₃Cl (15 ml) and the organic phase was washed with H₂O (20 ml), dried, filtered and evaporated to dryness. The resulting oil was purified by silica gel column chromatography (CH₃Cl/MeOH 8:2 with 5 drops/l of TEA) to give 220 mg of compound 3a: yield 70%, yellow oil. H NMR (DMSO-d₆): δ 1.14–1.26 (m, 6H, 2xCH₃); 1.32 (s, 3H, CH₃-C); 1.54 (s, 3H, CH₃-C); 3.11 (2H, Jₐₜ=24 Hz, CH₂-P); 3.91-4.07 (m, 5H, 2xCH₂ and H₅'); 4.22–4.36 (m, 2H, H₄' and H₅'); 5.02–5.06 (m, 1H, H₃'); 5.02–5.06 (m, 2H, 2xCH₃); 5.02–5.06 (m, 2xCH₃); 5.02–5.06 (m, 2xCH₃); 5.02–5.06 (m, 2xCH₃).
1H, H3′); 5.45–5.50 (m, 1H, H2′); 6.19 (d, 1H, J=2.63 Hz, H1′); 7.37 (sbr, 2H, NH2); 8.16 (s, 1H, H2′); 8.35 (s, 1H, H8); 31P NMR (DMSO-d6): δ -20.75; MALDI-TOF MS: m/z 485 Da (M+Na)+, 461 Da (M+K)+ and 485 Da (M+Na+K)+. C19H28N5O8P requires 485.484. Anal. (C19H28N5O8P) C, H, N calcd. 4.12; found 4.10.

5′-O-(phosphon-acetyl)adenosine sodium salt (7a)

To a solution of compound 3a (188.8 mg, 0.389 mmol) in CHCl3 (8 ml), TMSBr (1 ml, 7.78 mmol) was added and the solution was stirred under argon atmosphere and at room temperature for 9 h (TLC: iPrOH/H2O/NH4OH 6:3:1). The solution was evaporated and the residue was purified by column chromatography on DEAE Sephadex A-25 (2×30 cm column), using as eluent TEAB (linear gradient from 0.01 to 1 M), at pH 7.5 and with a flow rate of 50 ml/h. The collected fractions, containing the expected product, were evaporated and the residue dissolved in water. Tetra-n-butyl ammonium cation was exchanged for sodium by passing the solution through a Dowex AG 50W-X2 column (50–100 mesh, Na+ form). The solution was freeze-dried to give 45 mg of 7a, yield 30%; white solid, mp198°–201°C. 1H NMR (D2O): δ 2.81 (d, 2H, J=1.27; MALDI-TOF MS: m/z 469 Da (M+Na)+ and 461 Da (M+K)+). C16H24N5O8P requires 433.038. Anal. (C16H24N5O8P) C, H, N calcd. 5.89; found 5.86.

5′-O-(diethyl-phosphon)acetyl)-adenosine sodium salt (8a)

The compound 8a was prepared as described for 7a starting from 4a (104.5 mg, 0.226 mmol). The residue, after work-up, was purified by DEAE Sephadex A-25 (2×30 cm) column using TEAB as eluent to give 28 mg of compound 8a, yield 30%; yellow solid, mp >300°C. 1H NMR (D2O): δ 2.77 (d, 2H, J=2.63 Hz, CH22′); 3.92–4.41 (m, 5H, H5′, H5″, H4′ and H4″); 5.31 (d, 1H, J=4.85 Hz, H1″); 8.19 (s, 1H, H2); 8.36 (s, 1H, H8); 31P NMR (DMSO-d6): δ 12.76; MALDI-TOF MS: m/z 390 Da (M+H)+; 412.3 Da (M+Na)+; C19H28N5O8P requires 433.038. Anal. (C19H28N5O8P) C, H, N calcd. 4.12; found 4.12.

5′-O-(phosphon-acetyl)-uridine (6a)

Compound 6a was prepared as described for 5a starting from compound 4a (50 mg, 0.108 mmol). After usual work-up, the residue was purified by silica gel column chromatography (linear gradient from CHCl3/MeOH 9:1 to 7:3) to give 26 mg of compound 6a, 57% yield; colourless foam. 1H NMR (DMSO-d6): δ 1.19–1.26 (m, 6H, 2×CH2); 3.2 (d, 2H, J=1.27; MALDI-TOF MS: m/z 445 Da (M+Na)+, 461 Da (M+K)+ and 485 Da (M+Na+K)+. C19H28N5O8P requires 422.324. Anal. (C19H28N5O8P) C, H, N calcd. 5.89; found 5.86.

2′-3′-O-isopropyliden-5′-O-(diethyl-phosphon)acetyl)-uridine (4a)

Compound 2a (Hampton, 1961) (200 mg, 0.704 mmol) was reacted with diethyl phosphonacetic acid (114 ml, 0.704 mmol) as described for compound 3a. After the usual work-up, the residue was purified by silica gel column chromatography (CH2Cl2/MeOH 9:1) to give 26 mg of compound 2a, 72% yield; colourless foam. 1H NMR (CDCl3): δ 1.29–1.36 (m, 3H, CH22C, CH2-C); 3.0 (2H, JPH=21.17 Hz, CH2-P); 4.08–4.23 (m, 4H, 2×CH2); 4.30–4.40 (m, 3H, H4′, H5′ and H5″); 4.83–4.88 (m, 1H, H3); 4.99–5.02 (m, 1H, H2′); 5.73–5.77 (m, 2H, H1′ and H5); 7.43 (d, 1H, J=8.05 Hz, H6); 8.0 (sbr, 1H, NH); 31P NMR (CDCl3): δ -19.91; MALDI-TOF MS: m/z 462 Da (M+) and 501 Da (M+K)+. C14H27N2O10P requires 462.388. Anal. (C14H27N2O10P) C, H, N calcd. 5.89; found 5.86.

2′-3′-O-isopropyliden-5′-O-(diethyl-phosphon)acetyl)-uridine (4a)

Compound 2a (Hampton, 1961) (200 mg, 0.704 mmol) was reacted with diethyl phosphonacetic acid (114 ml, 0.704 mmol) as described for compound 3a. After the usual work-up, the residue was purified by silica gel column chromatography (CH2Cl2/MeOH 9:1) to give 56 mg of compound 2a, 72% yield; colourless foam. 1H NMR (CDCl3): δ 1.29–1.36 (m, 3H, CH22C, CH2-C); 3.0 (2H, JPH=21.17 Hz, CH2-P); 4.08–4.23 (m, 4H, 2×CH2); 4.30–4.40 (m, 3H, H4′, H5′ and H5″); 4.83–4.88 (m, 1H, H3); 4.99–5.02 (m, 1H, H2′); 5.73–5.77 (m, 2H, H1′ and H5); 7.43 (d, 1H, J=8.05 Hz, H6); 8.0 (sbr, 1H, NH); 31P NMR (CDCl3): δ -19.91; MALDI-TOF MS: m/z 462 Da (M+) and 501 Da (M+K)+. C14H27N2O10P requires 462.388. Anal. (C14H27N2O10P) C, H, N calcd. 5.89; found 5.86.
Compounds 4b and 5b were prepared as described for 3a, starting from 2b (236 mg, 0.833 mmol) (MacCoss et al., 1980). After work-up, the residue was purified by silica gel column chromatography (CH$_2$Cl$_2$/MeOH 9:1) to give 320 mg of compound 4b, yield 83%; yellow oil.

$^1$H NMR (CDCl$_3$): δ 1.22-1.33 (m, 9H, CH$_3$-C, 2x CH$_2$); 1.49 (s, 3H, CH$_3$-C); 2.9 (d, 2H, J$_{2p}$=21.43 Hz, CH$_2$-P); 4.48–4.49 (m, 1H, H$_4$); 5.39–5.41 (m, 1H, H$_5$); 5.89 (d, 1H, J=4.42 Hz, H$_1$); 6.31 (sbr, 2H, NH$_2$); 7.95 (s, 1H, H$_8$); 8.36 (s, 1H, H$_6$); 8.52–8.54 (m, 1H, NH); 11.4 (sbr, 1H, NH); $^{31}$P NMR (CDCl$_3$) δ 22.2. MALDI-TOF MS: m/z 485.5 Da (M+H$^+$); 507 Da (M+Na$^+$).

MALDI-TOF MS: m/z 444.6 Da (M+Na$^+$); 465.0 Da (M+K$^+$). $^{31}$H$_2$N$_3$O$_7$P requires 432.14. Anal. (C$_{12}$H$_{25}$N$_6$O$_7$P) C,H,N: H calcd. 9.93; found 9.92.

5'-deoxy-5'-N-(phosphon-acetyl)uridine sodium salt (7b)
The compound 7b was prepared as for 7a, starting from 3b (90.3 mg, 0.187 mmol). After work-up the residue was purified by column chromatography on DEAE Sephadex A-25 (2×30 cm) column with TEAB as eluent, to give 16 mg of compound 7b, yield 22%; white solid; m.p. >300°C. $^1$H NMR (D$_2$O): δ 2.71 (d, 2H, J$_{2p}$=20 Hz, CH$_2$-P); 3.16–3.69 (m, 3H, H$_5$, H$_8$ and H$_4$); 4.29–4.36 (m, 2H, H$_3$, H$_2$); 6.02 (d, 1H, J=5.6 Hz, H$_1$); 8.32 (s, 1H, H$_8$); $^{31}$P NMR (DMSO-$d_6$) δ 18.18; MALDI-TOF MS: m/z 397.9 Da (M+H$^+$); 411.7 Da (M+Na$^+$). $^{31}$H$_2$N$_3$O$_7$P requires 432.054. $^{31}$H$_2$N$_3$O$_7$P requires 388.09. Anal. (C$_{12}$H$_{25}$N$_6$O$_7$P) C,H,N: H calcd. 9.11; found 9.06.

5'-N-[(diethyl-phosphon)acetyl]adenosine sodium salt (8b)
The compound 8b was prepared as for 7a, starting from 4b (121 mg, 0.262 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2×30 cm) column with TEAB to give 45 mg of compound 8b, yield 47%; yellow solid; m.p. >300°C. $^1$H NMR (D$_2$O-$d_6$): δ 2.75 (d, 2H, J$_{2p}$=20.3 Hz, CH$_2$-P); 3.31–3.36 (m, 2H, H$_5$, H$_8$); 3.77–4.05 (m, 2H, H$_4$, H$_3$ and H$_2$); 4.8–5.3 (m, 2H, OH$_3$, OH$_2$); 5.60 (d, 1H, J=8.1 Hz, H$_5$); 5.79 (d, 1H, J=5.8 Hz, H$_8$); 5.95 (sbr, 1H, NH$_2$); 11.4 (sbr, 1H, NH); $^{31}$P NMR (DMSO-$d_6$) δ 18.46; MALDI-TOF MS: m/z 365 Da (M+Na$^+$); 388 Da (M+K$^+$). $^{31}$H$_2$N$_3$O$_7$P requires 432.24. $^{31}$H$_2$N$_3$O$_7$P requires 365.223. Anal. (C$_{12}$H$_{25}$N$_6$O$_7$P) C,H,N: H calcd. 9.97; found 9.92.

5'-O-terbutyl-dimethyl-silyl-2',3'-O-isopropyliden-5'-N-(benzyl-oxy-carbonyl)cytidine (10a)
Compound 9 (602 mg, 1.25 mmol) was dissolved in pyridine (20 ml) and benzyl chloroformate (429 µl, 3.043 mmol) was added drop-wise at 0°C. The mixture was stirred at room temperature under positive argon pressure, and the reaction was monitored by TLC (CH$_2$Cl$_2$/MeOH 9:1). After 15 h the mixture was evaporated and co-evaporated with EtOH (3×10 ml) and the residue was dissolved in CH$_2$Cl$_2$ (15 ml). The organic phase was then washed with H$_2$O (2×20 ml), dried, filtered and evaporated to dryness. The resulting oil was purified by silica gel column chromatography (CH$_2$Cl$_2$/MeOH 9:1) to give 679 mg of compound 10a, yield 78%; yellow oil. $^1$H NMR (CDCl$_3$);
δ 0.07 (s, 6H, (CH3)2-Si); 0.87 (s, 9H, tBut-Si); 1.35 (s, 3H, CH3-C); 1.58 (s, 3H, CH3-C); 3.81–3.93 (m, 2H, H2′, H2′′); 4.40–4.41 (m, 1H, H4′); 4.72–4.80 (m, 2H, H3′, H′′); 5.15–5.25 (m, 2H, CH2-Ph); 5.95 (s, 1H, H1′); 7.21 (d, 1H, J=7.5 Hz, H3); 7.30–7.45 (m, 5H, Ph); 8.09 (d, 1H, J=7.5 Hz, H6); 8.6 (sbr, 1H, NH); MALDI-TOF MS: m/z 532 Da (M+H)+; 554 Da (M+Na)+; 570 Da (M+K)+. C26H37N3O7Si requires 531.673. Anal. (C26H37N3O7Si) C,H,N: H calcd. 5.55; found 5.52.

Compound 11b was purified by silica gel column chromatography (linear gradient from CH2Cl2/MeOH 9.9:0.1 to 9.8:0.2) to give 247 mg of compound 11b, yield 85%; white foam. 1H NMR (DMSO-d6): δ 1.28 (s, 3H, CH3-C); 1.45 (s, 9H, tBut-O); 1.48 (s, 3H, CH3-C); 3.57–3.61 (m, 2H, H3′ and H′′); 4.16–4.18 (m, 1H, H4′); 4.75–4.77 (m, 1H, H3); 4.84–4.85 (m, 1H, H2); 5.05–5.10 (m, 1H, OH5); 5.80 (d, 1H, J=1.9 Hz, H1′); 7.0 (d, 1H, J=7.5 Hz, H5); 8.15 (d, 1H, J=7.5 Hz, H6); 10.4 (sbr, 1H, NH); MALDI-TOF MS: m/z 385 Da (M+H)+; 406 Da (M+Na)+; 444 Da (M+K)+. C21H33N3O11P requires 595.535. Anal. (C21H33N3O11P) C,H,N: N calcd. 10.00; found 10.05.

2′, 3′-O-isopropyliden-5′-O-[(diethyl-phosphon)acetyl]4-N-(benzyl-oxycarbonyl)cytidine (12b)
Compound 12b was prepared as described for compound 3a starting from 11b (450 mg, 0.576 mmol). After work-up, the residue was purified by silica gel column chromatography (linear gradient from hexane/acetone 1:1 to acetone 100% with 5 drops/l of TEA) to give 300 mg of compound 12b, yield 35%; yellow oil. 1H NMR (CDCl3): δ 1.16–1.27 (m, 9H, 2×CH2, CH3-C); 1.38–1.47 (m, 12H, CH3-C, tBut-O); 2.05 (d, 2H, JPH=33.3 Hz, CH2-P); 3.36–3.82 (m, 2H, H3′ and H′′); 4.04–4.28 (m, 5H, 2×CH2, H4′); 4.85–4.90 (m, 1H, H3′); 5.20–5.23 (m, 2H, CH2-Ph); 5.41 (s, 1H, H1′); 5.72 (s, 1H, H1); 7.26 (d, 1H, J=7.5 Hz, H5); 7.34–7.45 (m, 5H, Ph); 7.78 (d, 1H, J=7.5 Hz, H6); 7.9 (sbr, 1H, NH); 31P NMR (CDCl3): δ 19.87; MALDI-TOF MS: m/z 596 Da (M+H)+; 618 Da (M+Na)+; 634 Da (M+K)+. C21H33N3O11P requires 595.535. Anal. (C21H33N3O11P) C,H,N: N calcd. 10.00; found 10.05.
heated at reflux conditions for 3 h (TLC: CH₂Cl₂/MeOH 9:1). The suspension was filtered on a celite pad and the solution evaporated to dryness. The residue was purified by silica gel column chromatography (CH₂Cl₂/MeOH 9:1) to give 120 mg of compound 13a: yield 34%, colourless oil. ¹H NMR (CDCl₃): δ 1.27–1.39 (m, 9H, 2xCH₃, CH₂-C₃) 1.52 (s, 3H, CH₂-C); 2.99 (d, 2H, Jₚp=29.1 Hz, CH₂-P); 4.06–4.20 (m, 4H, 2xH₂, H₂); 3.91–3.93 (m, 2H, 2xH₃, H₃); 7.31 (d, 1H, J=7.3 Hz, H₅); 7.86 (d, 1H, J=7.65 Hz, H₆); 8.75 (s, 1H, H7); 31P NMR (CDCl₃): δ 14.25; MALDI-TOF MS: m/z 422.5 Da (M+H)⁺; 444.5 Da (M+Na)⁺; 460 Da (M+K)⁺.

Compound 13a was prepared as described for compound 15a starting from 13 (64 mg, 0.139 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2x30 cm) column with TEAB, to give 160 mg of compound 15a: yield 82%, yellow foam. ¹H NMR (D₂O): δ 0.97 (t, 3H, J=7.05 Hz, CH₃); 2.61 (d, 2H, Jₚp=15.0 Hz, CH₂-P); 3.72–3.78 (m, 2H, 2xH₃, H₃); 4.05–4.15 (m, 2H, 2xH₃, H₃); 6.11 (d, 1H, J=3.06 Hz, H1); 6.27 (d, 1H, J=7.72 Hz, H5); 7.70 (d, 1H, J=7.3 Hz, H6); 31P NMR (D₂O): δ 16.25; MALDI-TOF MS: m/z 365.4 Da (M+H)⁺; 387 Da (M+Na)⁺; 409 Da (M+K)⁺.

Compounds 15a and 15b were finally deprotected by using two different deprotection methods. Compounds 13b, 14b and 15b were obtained by reaction with trimethylsilyl bromide (TMSBr) followed by treatment with Dowex Na⁺ Form, followed by reaction with triethylammonium chloride (TEAB) to give 45 mg of compound 15b: yield 30%, white solid, mp >300°C.

The compound 15b was prepared as described for compound 7a starting from 14b (240 mg, 0.57 mmol). After work-up, the residue was purified by a DEAE Sephadex A-25 (2x30 cm) column with TEAB, to give 160 mg of compound 15b: yield 70%; white solid; mp >300°C. ¹H NMR (D₂O): δ 7.11 (d, 2H, Jₚp=23.64 Hz, CH₂-P); 3.15–3.35 (m, 2H, 2xH₃, H₃); 3.68–3.71 (m, 1H, H₄); 4.07–4.32 (m, 2H, 2xH₃, H₃); 3.87 Da (M+Na)⁺; 403.5 Da (M+K)⁺. C₁₁H₁₅N₃Na₂O₉P requires 409.20 Da; C₁₁H₁₇N₄O₈P requires 408.20 Da; C₁₁H₁₅N₃NaO₇P requires 365.2 Da (M+Na)⁺; 387 Da (M+K)⁺.

Compounds 14 and 15 were obtained by reaction with trimethylsilyl bromide (TMSBr) followed by treatment with Dowex Na⁺ Form, followed by reaction with triethylammonium chloride (TEAB) to give 45 mg of compound 15b: yield 30%, white solid, mp >300°C.

Results

Chemistry

In order to obtain nucleotide the 5′phosphonoacetic acid derivatives, uridine and adenosine were protected at the 2′ and 3′ positions (1a, 2a), as described by Hampton (1961). To prepare the corresponding 5′-amino-5′-deoxy nucleotide derivatives (1b, 2b), we adapted the synthetic strategy described by MacCoss et al. (1980) starting from 1a and 2a. The protected compounds 1a, 2a and 1b, 2b were then reacted with phosphonoacetic acid (PAA) in the presence of dicyclohexyl carbodiimide (DCCI) and DMF (Figure 1).

The resulting ester (3a, 4a) and amide (3b, 4b) derivatives were finally deprotected by using two different deprotection methods. Compounds 5a, b and 6a, b were obtained by treatment with TFA, whereas compounds 7a, b and 8a, b were obtained by reaction with trimethylsilyl bromide (TMSBr) followed by treatment with Dowex Na⁺ Form, (50x2–100).

In the case of cytidine, previous protection of the N₄ position of the pyrimidine ring was required to avoid competition during the coupling step. Indeed, the related 2’,3’,5’-protected compound 9 (Griffey & Poulter 1983),
was treated with benzyloxychloroformiate in pyridine or with di-tBut-dicarbonate (BOC) in THF/dioxane to give the compounds 10a and 10b, respectively (Figure 2). The corresponding phosphonic acid derivatives (12a, b) were obtained, as described above for uridine and adenosine (Figure 1), after the removal of the t-butyl-dimethylsilyl (TBDMS) protecting group at the 5′-position by treatment with TEA × 3HF (11a, b). The final synthetic step involved the deprotection of the N 4 position of the pyrimidine ring and the 2′,3′ positions of the sugar moiety. Hydrogenation of 12a over Pd/C gave the partially deprotected compound 13a, which was subsequently treated with 50% TFA to give 14a. Finally, direct treatment of the BOC-protected derivative 12b with TFA 50% led to compound 14b (Figure 2).

The disodium salt derivatives 15a, b were obtained by reaction with TMSBr followed by treatment with Dowex Na+ (50 × 2–100) starting from compounds 13a and 14b.

### Biology

#### RNR inhibition

The synthesized compounds were evaluated for their in vitro inhibitory activity on murine recombinant RNR proteins R1 and R2, showing lower inhibitory activity than expected: IC₅₀ values ranged from 350 µM for the UDP analogue 8b (amide) to 600 µM for the CDP analogue 14a (ester). The IC₅₀ value is defined as the concentration of the compound that inhibits the reduction of the substrate by 50% in the assay conditions (Table 1).

#### Cytostatic and antiviral activity

The cytostatic activity of the prepared nucleosides was evaluated against murine leukemia L1210, mammalian carcinoma FM3A and human T4-lymphocyte Molt and CEM cells. None of the studied compounds were cytostatic at 100–500 µM (Table 2), whereas ADP analogues showed moderate activity (EC₅₀: 48 µM, 5b, amide) against herpes simplex virus type 2 (HSV-2) in E6SM cell cultures and modest activity (EC₅₀: 110 µM, 7b, amide) against HIV-1 in CEM cell cultures (Tables 2 and 3).

The virtual lack of biological activity, in absence of any other different mechanism, at the concentration range used for the tested compounds, is in agreement with the observed poor inhibition of the isolated enzyme.

#### Molecular modelling

The enzyme was studied with different isosteric nucleotide analogues (heterocycles, phosphonic acid, esters and amides) of the four natural substrates (ADP, GDP, CDP and UDP). These were docked into the binding site of the R1 subunit by modifying the 5′-position of the GDP contained in the crystallographic structure, by substitution of the diphosphate group with the appropriate bioisosteric

### Table 1. Inhibitory activity (IC₅₀) and binding energies (ΔG) of the study compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC₅₀ (µM)</th>
<th>ΔG (Kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8a</td>
<td>655</td>
<td>−5.35</td>
</tr>
<tr>
<td>8b</td>
<td>350</td>
<td>−7.74</td>
</tr>
<tr>
<td>5a</td>
<td>500</td>
<td>−6.29</td>
</tr>
<tr>
<td>5b</td>
<td>N.I.</td>
<td>−5.15</td>
</tr>
<tr>
<td>6a</td>
<td>760</td>
<td>−5.08</td>
</tr>
<tr>
<td>6b</td>
<td>N.I.</td>
<td>−4.94</td>
</tr>
<tr>
<td>7a</td>
<td>880</td>
<td>−5.20</td>
</tr>
<tr>
<td>7b</td>
<td>N.I.</td>
<td>−4.70</td>
</tr>
<tr>
<td>14</td>
<td>600</td>
<td>−6.14</td>
</tr>
<tr>
<td>14b</td>
<td>N.I.</td>
<td>−5.04</td>
</tr>
<tr>
<td>15</td>
<td>773</td>
<td>−5.06</td>
</tr>
<tr>
<td>15b</td>
<td>N.I.</td>
<td>−4.92</td>
</tr>
</tbody>
</table>

N.I., not inhibitory.

---

**Figure 1.** Synthesis of 5′−N or 5′−O−diethyl phophonoacetyl derivatives of adenosine and uridine and of corresponding sodium salts

1,3,5,7: Base=adenine; 2,4,6,8: base=uracil
a: X=O; b: X=NH
i: DCCI, PPA-diethyl ester, DMF; ii: TFA 50%; iii: TMSBr, CH₂Cl₂, Dowex 50W × 2 –100, Na⁺ form.
residue. Figures 3a and b report, as a graphic example, the comparison between the structures of the 5a/R1 and the crystallographic GDP/R1 complexes, respectively. The resulting structures of the enzyme complexed with the considered nucleotide analogues, even though not superimposed to the corresponding reference X-ray structure of the GDP/R1 assembly, presented interesting data on the energy's point of view. In fact, the relevant calculations indicated high stability of the substrate–enzyme complexes in the case of the amide and ester derivatives; in particular, a detailed analysis of these structures revealed an increased number of hydrogen bonds as compared to the natural substrate suggesting a potential inhibition activity. In the case of the heterocyclic isosters, the steric hindrance limited the docking of the analogues into the active site. Therefore, the phosphonic acid ester and amide bioisosters were selected for the synthetic studies.

Discussion

Nucleoside diphosphates represent the only substrate forms recognized by RNR. Notwithstanding that the potential therapeutic use of nucleotide analogues, as widely demonstrated (Plunkett et al., 1996), is prevented by their instability in biological fluids and, when administered in a non-phosphorylated form, strongly conditioned by metabolic activation by specific kinases (Balzarini, 1993; Meier, 1998). Our previous studies (Manfredini et al., 1999) pointed out that the principal interactions between the active site and the natural and nucleoside analogue diphosphates concern the diphosphate group (Eriksson et al., 1997).

Taking all this into account, our investigation started with a molecular modelling study on the RNR R1 subunit, which aimed to explore the possibility of substituting the GDP/R1 assembly, presented interesting data on the energy's point of view. In fact, the relevant calculations indicated high stability of the substrate–enzyme complexes in the case of the amide and ester derivatives; in particular, a detailed analysis of these structures revealed an increased number of hydrogen bonds as compared to the natural substrate suggesting a potential inhibition activity. In the case of the heterocyclic isosters, the steric hindrance limited the docking of the analogues into the active site. Therefore, the phosphonic acid ester and amide bioisosters were selected for the synthetic studies.
diphosphate group of natural substrates by bioisosteric modifications. This approach is well-known from the literature: the pyrophosphate (PP) analogue foscarnet (PFA) that inhibits viral DNA polymerase activity, interferes with cytomegalovirus, herpes virus and HIV replication (Kudlacek et al., 2001). In this work we considered heterocycles, PAA esters and amides. As described in the modelling section, calculations indicated the PAA amide and ester as the best candidates for bioisosteric replacement of the diphosphate. Thus, PAA-amides and esters of cytidine, uridine and adenosine were prepared and investigated for their inhibitory activity on RNR and for their cytostatic and antiviral activities. Among these compounds, only the UDP analogue 8b (amide), ADP analogue 5a (ester) and CDP analogue 14a (ester) were endowed with a moderate inhibitory activity against the isolated enzyme (IC₅₀ 350, 500 and 600 µM, respectively). None of the compounds showed any cytostatic activity at 100–500 µM, whereas compound 5b (ADP analogue, amide) showed a moderate activity (EC₅₀ 48 µM) against HSV-2 and compound 7b (ADP analogue, amide) a modest but interesting activity (EC₅₀ 110 µM) against HIV-1. As discussed above a significant degree of interaction with the enzyme’s active site, comprising two orders of DG values magnitude, was predicted by the modelling study for the designed molecules (Table 1). Thus, although modelling studies predicted improvements in the binding capabilities by replacement of

Table 3. Antiviral activity of test compounds in cell culture

<table>
<thead>
<tr>
<th>Comp.</th>
<th>EC₅₀ (µM)</th>
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<tbody>
<tr>
<td></td>
<td>E₆SM cell cultures</td>
</tr>
<tr>
<td></td>
<td>HSV-2</td>
</tr>
<tr>
<td>8a</td>
<td>&gt;40</td>
</tr>
<tr>
<td>8b</td>
<td>&gt;80</td>
</tr>
<tr>
<td>5a</td>
<td>240</td>
</tr>
<tr>
<td>5b</td>
<td>240</td>
</tr>
<tr>
<td>6a</td>
<td>&gt;400</td>
</tr>
<tr>
<td>6b</td>
<td>240</td>
</tr>
<tr>
<td>7a</td>
<td>&gt;200</td>
</tr>
<tr>
<td>7b</td>
<td>&gt;200</td>
</tr>
<tr>
<td>14a</td>
<td>&gt;80</td>
</tr>
<tr>
<td>14b</td>
<td>&gt;200</td>
</tr>
<tr>
<td>15a</td>
<td>&gt;80</td>
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<tr>
<td>15b</td>
<td>&gt;200</td>
</tr>
<tr>
<td>BVDV</td>
<td>0.07</td>
</tr>
<tr>
<td>GCV</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*Data are for CMV strain AD-169 and Davis, and for VZV strains YS, OKA, 07/1 (TK-deficient) and YS/R (TK-deficient). CMV, cytomegalovirus; CS B4, coxsackie B4; EC₅₀, 50% effective concentration, or compound concentration required to inhibit virus-induced cytopathicity by 50%; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; PI 3, para-influenza 3; PTV, punta toro virus; RV-1, reovirus-1; RSV, respiratory syncytial virus; VSV; vesicular stomatitis virus; VV, vaccinia virus; VZV, varicella-zoster virus

Figure 3. The active-binding site cleft of the RNR R1 subunit with the docked nucleotide analogue 5a (A) and with the natural substrate molecule GDP (B)*

*The docked molecules are shown in stick representation.
the diphosphate moiety for a corresponding PAA amide and ester bioisoster, only a fair agreement exists for the most potent inhibitors 8b, 5a and 14a, and all the other compounds were inactive. However, some final conclusions can be drawn. It is worth noting that the compounds 5a and 14a, notwithstanding the lack of acid phosphate moiety characteristic of natural substrates, were also endowed with a inhibitory activity on RNR, although modest. Moreover, the only compounds provided with a limited activity (antiviral) in tumour cell lines, 5b and 7b, both PAA-amides and ADP analogues, were found not inhibitory against the isolated enzyme. Thus, it may be assumed that the observed activity should be ascribed to a mechanism different than RNR inhibition or to interaction with RNKs different than the human one (Boehmer et al., 1997; Heineman et al., 1994). Finally, as both 5b and 7b are PAA-amides, the reversible nature of the ester linkage may also play a role in determining the inactivity of the corresponding PAA-ester ADP analogues in cell cultures, possibly due to degradation by cellular enzymes.

Acknowledgements

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