9-Deazaguanine and Its Methyl Derivatives: Synthesis, Antitumor Activity in vitro and Effects on Purine Nucleoside Phosphorylase Gene Expression

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9-Deazaguanine 9-DG, 1-methyl-9-deazaguanine AG-19-K1 and 1,7-dimethyl-9-deazaguanine AG-3 were synthesized and their antiproliferative activity against five leukemia and four solid tumor cell lines as well as inhibitory properties vs. calf spleen purine nucleoside phosphorylase (PNP) were tested. Synthesis of 9-DG involves reaction of 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (2) with DMF-dimethylacetal (amount ratio, \( n(2) \)/\( n(\text{DMF-dimethylacetal}) = 1:2.5 \)) and use of the benzyloxymethyl group to protect the N-3 position of 2-(N-dimethylamino)aminomethyleneamino-6-methyl-5-nitropyrimidin-4(3H)-one (4). Reaction of 2 with DMF-dimethylacetal (amount ratio, \( n(2) \)/\( n(\text{DMF-dimethylacetal}) = 1:6 \)) gave the N-3 methyl substituted intermediate 3. Dithionite reduction of this product afforded N-methyl derivatives AG-19-K1 and AG-3. AG-19-K1 and AG-3 were inactive vs. calf spleen PNP at a concentration of 75 \( \mu \)mol dm\(^{-3} \). Cytotoxic effects of 9-deazaguanine derivatives on cell growth were determined by the MTT assay. Investigated derivatives showed moderate antiproliferative activity towards examined tumor cells. At a concentration of 10\(^{-3} \) mol dm\(^{-3} \), AG-19-K1 inhibited the growth of JURKAT, K562 and AGS cells by approximately 80 %. At the same concentration, AG-3 and 9-DG inhibited cell proliferation by 40–50 % of all tested lines, except MOLT-4 and HL-60. The PNP gene expression was changed in treated leukemia cells after exposure to AG-19-K1 and 9-DG in a time-dependent manner.

Keywords

9-deazaguanine derivatives
purine nucleoside phosphorylase
antiproliferative effects
leukemia cells
solid tumors cells

INTRODUCTION

Cancer encompasses many disease states generally characterized by abnormally proliferating cells and is a major and often fatal disease. Chemotherapy is used in attempts to cure or palliate diseases. However, no fundamental trait has been associated with the wide array of cancers known today that would permit an effective, uniform and successful response to this disease. Scientific studies can be broadly separated into those aimed at improving the therapeutic index of currently available compounds and those discovering new agents or approaches. One

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9-deazaguanine itself was not characterized as a PNP inhibitor. This study was therefore also designed so as to explore their effects on cell proliferation and influence on PNP activity. Hence, potent PNP inhibitors that would be able to cause effects similar to PNP deficiency are expected to be useful in treatment of some autoimmune diseases, other T-cell proliferative disorders and also tumors of T-cells.

Mammalian PNP is a trimeric molecule and contains three active sites for ligands binding. The most potent inhibitors of PNP, including transition state analogues inhibitors immucilins, are nucleosides and acyclonucleosides, in which the carbon atom is substituted for the nitrogen atom at position nine of guanine base, resulting in 9-deazaguanine (9-DG) aglycone. Such inhibitors, especially immucilins, are able to significantly block PNP activity. However, there is still need for alternative potent PNP inhibitors and structure-activity relationship studies show that such analogues should contain 9-DG instead of the natural purine ring system, since such analogues, having a hydrogen bond donor at position N(9) of the base, are able to form a strong hydrogen bond with PNP. To have easy access to such 9-deaza-derivatives, we needed an efficient way of synthesizing 9-deazaguanine, so that we could introduce a variety of side chains at position 9 of the base in a region-specific manner. We therefore explored the synthesis of 9-DG according to the literature procedures and, in addition to 9-DG, we also isolated N-methyl derivatives of 9-deazaguanine: 1-methyl-9-deazaguanine AG-19-K1 and 1,7-dimethyl-9-deazaguanine AG-3.

During the course of our attempts to synthesize new nucleoside inhibitors of mammalian PNP, we realized that 9-deazaguanine itself was not characterized as a PNP inhibitor. This study was therefore also designed so as to characterize inhibition properties vs. mammalian PNP of 9-deazaguanine and some of its derivatives, to explore their effects on cell proliferation and influence on PNP gene expression of the tumor cell lines tested.

EXPERIMENTAL

Chemistry in General

Solvents were distilled from appropriate drying agents shortly before use. TLC was carried out on DC-plastikfolien Kieselgel 60 F254. Melting points were determined on a Kofler hotstage apparatus and were uncorrected. UV spectra ($\lambda_{max}$/nm, log $\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$) were taken on a Philips PU8700 UV/Vis spectrophotometer and on an Uvikon 930 (Kontron, Austria) spectrophotometer fitted with a thermostatically controlled cell compartment. The latter apparatus was also used for kinetic measurements with spectrophotometric detection of enzyme activity (see below). IR spectra (v$_{max}$/cm$^{-1}$) were obtained for KBr pellets on a Perkin-Elmer 297 spectrophotometer. The $^1$H and $^{13}$C NMR spectra were recorded on a Varian Gemini 300 spectrometer, operating at 75.46 MHz for the $^{13}$C nucleus. Samples were dissolved in DMSO-$d_6$ and measured at 20 °C in 5 mm NMR tubes. Sample concentrations were 0.1 mol dm$^{-3}$ for $^1$H and 0.2 mol dm$^{-3}$ for $^{13}$C measurements. Chemical shifts (δ/ppm) were referred to DMSO. Digital resolution was 0.3 Hz per point in $^1$H and 0.5 Hz per point in $^{13}$C NMR one-dimensional spectra. The applied techniques were standard $^1$H and $^{13}$C with broadband proton decoupling, $^{13}$C gated decoupling, COSY and NOESY. Waltz-16 modulation was used for proton decoupling. COSY spectra were recorded in the magnitude mode with 1024 points in F2 dimension and 256 increments in F1 dimension, zero-filled to 1024 points. Increments were measured with 16 scans, 4500 Hz spectral width and a relaxation delay of 0.8 s. The corresponding digital resolution was 8.9 Hz per point and 17.6 Hz per point in F2 and F1 dimensions, respectively. NOESY spectra were recorded in a phase-sensitive mode and at mixing times of 0.45–0.80 s. All other measurement parameters were as for the COSY spectra.

2-Amino-6-methylpyrimidin-4(3H)-one (1)

Starting material; Aldrich, 98 %, m.p. > 300 °C. Additional data for 2-amino-6-methylpyrimidin-4(3H)-one (1): R$_f$ = 0.12 (EtOAc/MeOH = 3:1); UV (MeOH): $\lambda_{max}$/nm: 206, 222, 284, log $\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$: 4.26, 4.27, 4.20; $^1$H NMR (DMSO-$d_6$) δ/ppm: 11.35 (br s, 1H, 3-NH), 6.93 (br s, 2H, 2-NH$_2$), 5.41 (s, 1H, H-5), 1.99 (s, 3H, CH$_3$); $^{13}$C NMR (DMSO-$d_6$) δ/ppm: 163.76 (s, C-4), 155.53 (s, C-6 probably with C-2), 141.7 (s, C-2), 135.2 (s, C-1), 123.4 (m, C-3), 121.9 (m, C-15), 116.6 (s, C-7), 111.7 (s, C-8), 110.1 (s, C-9), 55.1 (s, C-11); IR (KBr) v$_{max}$/cm$^{-1}$: 3427 (s), 3304 (s), 3177 (s), 3167 (s), 2760 (m), 1675 (s), 1609 (s), 1579 (s), 1521 (s), 1457 (m), 1417 (s), 1352 (s), 1234 (m), 1167 (m), 1135 (m), 1109 (m), 1094 (m), 1009 (w), 782 (m); $^1$H NMR (DMSO-$d_6$) δ/ppm: 11.63 (s, 1H, 3-NH), 6.5–8.5 (br s, 2H, 2-NH$_2$), 2.26 (s, 3H, CH$_3$); $^{13}$C NMR (DMSO-$d_6$) δ/ppm: 164.84 (s, C-4), 155.78 (s, C-2), 155.41 (s, C-6), 128.98 (s, C-5), 22.67 (q, CH$_3$).

2-Amino-6-methyl-5-nitropyrimidin-4(3H)-one (2)

2-Amino-6-methyl-5-nitropyrimidin-4(3H)-one (2) was synthesized following the Pankiewicz procedure. A mixture of nitric acid (5.3 mL), conc. sulfuric acid (5.3 mL) and 2-amino-6-methylpyrimidin-4(3H)-one (1) (1.78 g, 98 %, 14.2 mmol) gave 2 (1.75 g, 72 %) as light brown crystals.

Additional data for 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (2): m.p. 280 °C dec; R$_f$ = 0.22 (CH$_3$Cl/MeOH = 9:1); UV (MeOH): $\lambda_{max}$/nm: 203, 296, 326, log $\varepsilon$/dm$^3$ mol$^{-1}$ cm$^{-1}$: 3.99, 3.65, 3.52; IR (KBr) v$_{max}$/cm$^{-1}$: 3427 (s), 3304 (s), 3177 (s), 3167 (s), 2760 (m), 1675 (s), 1609 (s), 1567 (s), 1499 (s), 1465 (s), 1417 (s), 1352 (s), 1234 (m), 1094 (m), 1009 (w), 782 (m); $^1$H NMR (DMSO-$d_6$) δ/ppm: 11.63 (s, 1H, 3-NH), 6.5–8.5 (br s, 2H, 2-NH$_2$), 2.26 (s, 3H, CH$_3$); $^{13}$C NMR (DMSO-$d_6$) δ/ppm: 164.84 (s, C-4), 155.78 (s, C-2), 155.41 (s, C-6), 128.98 (s, C-5), 22.67 (q, CH$_3$).
2-[(N-Dimethylaminomethylene)amino]-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (3)

This reaction was performed following the Fumraine and Tyler procedure for preparation of 2-(N-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-5-nitropyrimidin-4-one. A mixture of 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (2) (1 g, 5.88 mmol) with dry DMF (12.5 mL) and DMF-dimethylacetil (3.75 mL, 35 mmol) was stirred at 100 °C for 24 h and then cooled. Acetone (25 mL) was added, and the mixture was filtered and washed with acetone, affording 2-(N-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (3) as an orange solid (1.1 g, 64 %); m.p. 196–198 °C; Rf = 0.64 (EtOAc/MeOH = 3:1); UV (MeOH): λ_{max}/nm: 268, 331, 416, log ε/dm³ mol⁻¹ cm⁻¹: 3.97, 4.45, 3.94; IR (KBr) ν_{max}/cm⁻¹: 3448 (w), 2925 (w), 1671 (s), 1629 (s), 1535 (s), 1511 (s), 1485 (m), 1398 (m), 1384 (m), 1333 (w), 1111 (m), 1055 (s); ¹H NMR (DMSO-d₆) δ/ppm: 8.83 (s, 1H, CH=N), 8.13 (d, 1H, J = 12 Hz, NH=CH), 5.37 (d, 1H, J = 12 Hz, NH=CH), 3.35 (s, 3H, 3-N–CH₃); 3.26 (s, 3H, N-CH₃), 3.16 (br s, 3H, N-CH₃), 3.10 (s, 3H, N–CH₃); 2.85 (br s, 3H, N–CH₃); ¹³C NMR (DMSO-d₆) δ/ppm: 159.79 (d, HC=CH), 157.04 (s, C-4), 156.40 (s, C-2 or C-6), 156.10 (s, C-5), 147.62 (s, C-8), 135.36 (d, N=CH=CH), 133.06 (s, C-5), 88.16 (d, CH=CH), 41.53 (q, N–CH₃), 39.15 (q, N–CH₃), 29.31 (q, 3-N–CH₃).

1-Methyl-9-deazaguanine AG-19-K1 and 1,7-dimethyl-9-deazaguanine AG-3

A mixture of 2-(N-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (3) (2.54 g, 8.63 mmol) and sodium dithionite (5.69 g, 85 %) in water (25 mL) was heated under reflux for 2 h. The suspension was hot filtered and the residue was purified by silica gel column chromatography (CH₂Cl₂/MeOH = 3:1) to yield 0.624 g (45 %) of 2-amino-3-methyl-3,5-dimethyl-3-pyrrolo[3,2-d]pyrimidin-4(3H)-one (AG-19-K1) and 0.098 g (6 %) of 2-amino-3-methyl-3,5-dimethyl-3-pyrrolo[3,2-d]pyrimidin-4(3H)-one (AG-3) as white crystals.

2-Amino-3-methyl-3H-pyrrrolo[3,2-d]pyrimidin-4(3H)-one (AG-3): m.p. 235–238 °C; Rf = 0.41 (EtOAc/MeOH = 3:1); UV (EtOH): λ_{max}/nm: 230, 257, 291, log ε/dm³ mol⁻¹ cm⁻¹: 4.25, 3.85, 3.72; IR (KBr) ν_{max}/cm⁻¹: 3485 (m), 3383 (s), 3251 (w), 1678 (s), 1636 (s), 1560 (s), 1469 (w), 1426 (w), 1393 (w), 1363 (w), 1287 (s), 1239 (m), 1196 (m), 1143 (m), 1056 (s); ¹H NMR (DMSO-d₆) δ/ppm: 7.43 (d, 1H, J = 3.1 Hz, H-8), 6.41 (br s, 2H, NH₂), 5.86 (d, 1H, J = 3.1 Hz, H-9), 3.35 (s, 6H, N-1 and N-7–CH₃); ¹³C NMR (DMSO-d₆) δ/ppm: 152.70 (s, C-6 or C-2), 152.04 (s, C-2 or C-6), 147.62 (s, C-4), 131.11 (d, C-8), 110.72 (s, C-5), 98.90 (d, C-9) 29.72 (q, 1-N and 7-N–CH₃).


In vitro Study

– Inhibition of PNP in vitro (without cells)

Inhibition properties of 9-deazaguanine and its derivatives were determined as described earlier on calf spleen PNP, with 7-methylguanosine (7-MG) as variable substrate, and at two concentrations of the co-substrate phosphate: 50 mmol dm⁻³ (saturating concentration) and 1 mmol dm⁻³, which is supposed to be close to the concentration in cells. Experiments were conducted at 25 °C in 50 mmol dm⁻³ Hepes buffer, pH = 7.0, by the initial velocity method using spectrophotometric assay (λₑₒₛ = 260 nm). In all kinetic experiments, the concentration of 9-DG was determined spectrophotometrically on the basis of the extinction coefficient for the neutral ionic form of the compound (present in the pH range 7–9): ε (266 nm) = 6 600 dm⁻³ mol⁻¹ cm⁻¹.

– Chemicals

Media, RPMI-1640 and DMEM with 10 % heat inactivated fetal bovine serum (FBS), penicillin and streptomycin, and trypsin-EDTA were purchased from the Institute of Immunology Inc. (Zagreb, Croatia); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Merck (Darmstadt, Germany, EU) and dimethylsulfoxide (DMSO) was purchased from Eurobio (France, EU). Superscript II reverse transcriptase was purchased from Invitrogen (UK, EU), and oligo(dT)₁₅ and dNTP mix from Roche Molecular Diagnostics (Germany, EU). Glutamine, Tri-reagent, 7-methylguosine, calf spleen purine nucleoside phosphorylase and all other chemicals were obtained from Sigma Chem. Co. (St. Louis, USA). The enzyme obtained as suspension in ammonium sulphate was desalted as previously described.

– Cell Culture

Four solid tumor cell lines: SW620 (cells from lymph node metastasis of colon adenocarcinoma), HT-29 (colon carcinoma), AGS (gastric adenocarcinoma) and HeLa (cervical carcinoma) and five leukemia cell lines: HL-60 (human acute myeloid leukemia), U937 (human chronic myeloid leukemia in blast crisis), RAJI (Burkitt lymphoma) and MOLT-4 (human T-cell leukemia) were used in the study.

Solid tumor cells were grown as a monolayer in DMEM. Leukemia cells were grown in suspension, in RPMI 1640. DMEM and RPMI 1640 were supplemented with 10 %
fetal bovine serum, 2 mmol dm⁻³ glutamine, 100 U/mL penicillin (≈ 60 µg/mL) and 100 µg/mL streptomycin. All tested cell lines were grown at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. Trypan blue dye exclusion method was used to assess cell viability.

– Cell Proliferation Assay

Cells were plated into 96-well plates at concentrations of 2 × 10⁴ cells per mL for solid tumor cells and 1 × 10⁵ cells per mL for leukemia cells. All cell lines were treated with investigated compounds in a concentration range 10⁻³–10⁻⁶ mol dm⁻³ and incubated at 37 °C for 72 h. Control cells were grown in the same conditions but without addition of test compounds. Cytotoxic effects of 9-DG derivatives on cell growth were measured using the MTT assay. After 3 days of incubation, MTT was added to each well and incubated for the subsequent 4 h. Precipitate of solid tumor cells was dissolved in DMSO and that from leukemia cell lines were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Trypan blue dye exclusion method was used to assess cell viability.

– Extraction of mRNA and Preparation of cDNA and Purine Nucleoside Phosphorylase (PNP) Gene Expression

Three leukemia cell lines (RAJI, MOLT-4 and HL-60) were treated with 10⁻³ mol dm⁻³ 9-DG and AG-19-K1 for 6, 12 and 24 h. The mRNA from 10⁶ cells was isolated by Tri-reagent. Target RNA was reverse transcribed using oligo(dT)₁₅, dNTP mix, and sterile distilled H₂O added to the total RNA and the resulting solution was annealed at 65 °C for 5 min. Then, the 5X First-Strand Buffer and DTT were added and the reaction mixture was incubated at 42 °C for 2 minutes. In the end, the reverse transcriptase was added and the mixture was incubated at 42 °C for 50 min followed by incubation at 70 °C for 15 minutes. cDNA for PNP gene was amplified using Progene Thermal Cycler (Techne Cambridge Ltd., UK) with primers 5'-ACACTGCAAGATGGCTCTCG-3' from nucleotides 144 to 163 (sense primer in exon 2) and 5'-GGTACCTTTACATGATGG-3' from nucleotides 383 and 364 (antisense primer in exon 3). The amplification primers used were: GAPDH 5'-CCA TCA ATG ACC CCT TCA TTG ACC-3' sense, 5'-GAA GGC CAT GCC AGT GAG CTT CC-3' antisense; PNP Standard PCR conditions were applied; denaturation at 94 °C, 60 s; annealing at 51 °C, 120 s; extension at 72 °C, 3 min; 28 cycles followed by one cycle with 7-minutes extension.

As an internal reference gene was used gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). cDNA for GAPDH gene was amplified with primers 5'-CCA TCA ATG ACC CCT TCA TTG ACC-3' (sense primer) and 5'-GAA GGC CAT GCC AGT GAG CTT CC-3' (antisense primer). The PCR conditions were 95 °C, 68 °C, and 72 °C for 30, 90 and 60 seconds, respectively. Products of the PCR reaction were separated by 1 % agarose gel electrophoresis. The intensity of the bands was analyzed using ImageQuantTL software (Pharmacia Biotech, Sweden) and normalized to internal reference gene GAPDH. Three independent experiments were performed. The effects of 9-DG and 9-DG derivatives on GAPDH gene expression was not significant during all tested period of exposure, indicating that GAPDH could be used as a stable housekeeping gene in this experiment.

– Statistical Analysis

The Kolmogorov-Smirnov test, a normality distribution test, was applied. The differences between groups were assessed by a non-parametric Kruskal-Wallis test (p<0.05). Statistical analyses were performed with STATISTICA™ software (version 7.0). Data are presented as mean values ± SD of three separate experiments.

RESULTS

Two synthetic routes were chosen for the synthesis of deaza-guanine 9-DG (Scheme 1). In the first route, 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (2) was treated with DMF-dimethylacetal (amount ratio, n(2) / n(DMF-dimethylacetal) = 1:6) and in our hands gave N-3 methyl substituted intermediate 2-(N-dimethylaminomethylamino)-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (3) as the sole product (73 % yield). Dithionite reduction of this product afforded N-methyl derivatives: 1-methyl-9-deazaguanine AG-19-K1 as the main product in 45 % yield and 1,7-dimethyl-9-deazaguanine AG-3 (6 % yield).

The second route for the 9-DG synthesis involves reaction of 2-amino-6-methyl-5-nitropyrimidin-4(3H)-one (2) with DMF-dimethylacetal (amount ratio, n(2) / n(DMF-dimethylacetal) = 1:2.5) and use of the benzyl-oxymethyl group to protect the N-3 position of 2-(N-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (3) as the sole product (73 % yield). Dithionite reduction of this product afforded 6-N-methyl derivatives: 1-methyl-9-deazaguanine AG-19-K1 as the main product in 45 % yield and 1,7-dimethyl-9-deazaguanine AG-3 (6 % yield).

Results of the antiproliferative activity of AG-19-K1, AG-3 and 9-DG on several leukemia and solid tumor...
cell lines are shown in Figure 1. Investigated compounds show moderate antiproliferative activity towards the examined cell lines. Compound AG-19-K1 at a concentration of \(10^{-3}\) mol dm\(^{-3}\) has the strongest inhibitory effect on the tumor cells growth when compared to the two other compounds tested. AG-19-K1 (\(10^{-3}\) mol dm\(^{-3}\)) inhibits growth of leukemia (JURKAT, K562) and gastric adenocarcinoma (AGS) cell lines by approximately 80% and all other tested cell lines by about 50% (Figure 1a). At a concentration of \(10^{-4}\) mol dm\(^{-3}\), AG-19-K1 inhibits proliferation of SW620 and HT-29 cells in a range of 15–25% and has no influence on the proliferation of other treated cell lines.

Compound AG-3 displays similar moderate antiproliferative capacity (Figure 1b). At a concentration of \(10^{-3}\) mol dm\(^{-3}\), it inhibits cell proliferation of JURKAT and K562 cells by approximately 55%. Inhibition effect was 30–45% for other cell lines, i.e., SW620, RAJI, HT-29, HeLa, and AGS cells. At a concentration of \(10^{-4}\) mol dm\(^{-3}\), AG-3 slightly inhibits growth of investigated cell lines.

Compound 9-DG, in a concentration of \(10^{-3}\) mol dm\(^{-3}\) also inhibits cell growth in a range of 35–47% in the case of solid tumor cell lines and by 15–55% in the case of leukemia cells (Figure 1c). 9-DG applied in lower concentrations has no influence on tumor cells growth.

Investigation of the \(PNP\) gene expression after 6, 12 and 24 h exposure of leukemia cells to \(10^{-3}\) mol dm\(^{-3}\) AG-19-K1 and 9-DG showed a significant decrease in a time-dependent manner (Figure 2). Compared to the controls, AG-19-K1 evokes drop in \(PNP\) expression of MOLT-4 cells by 60%, 70%, and 55% after 6, 12, and 24 hours of treatment respectively. Under the same conditions, 9-DG significantly affects \(PNP\) expression after 6 h of treatment (70%), but after 12 and 24 h of exposure, \(PNP\) mRNA level begin to rise. The investigated gene expression in treated HL-60 was quite changed compared to the controls. In comparison to the previously described treated cell lines, AG-19-K1 induces the best inhibition effect on the \(PNP\) gene expression in RAJI cells (81%) after 6 h of treatment. 9-DG also leads to a reduction in the \(PNP\) gene expression (80%) after 6 h of treatment in the same cells, but after 12 and 24 h of exposure, negligible inhibitory effects were observed.

Inhibition of mammalian PNP by 9-deazaguanine and its two derivatives AG-19-K1 and AG-3 was determined using calf spleen enzyme and 7-methylguanosine as variable substrate at two concentrations, 50 mmol dm\(^{-3}\) and 1 mmol dm\(^{-3}\) of the co-substrate, phosphate. Trimeric PNPs are known for their non-Michaelis kinetics, especially when the concentration of natural nucleoside substrates (inosine, guanosine) or phosphate is varied.\(^2\)\(^,\)\(^3\) We

Scheme 1. Synthesis of 9-deazaguanine derivatives (double arrows indicate the intramolecular NOE effects).
have previously shown that, by contrast, kinetics of 7-
methylguanosine may be in some cases analyzed using
the Michaelis-Menten model.3 However, data shown in
Figure 3 clearly indicate that when 9-DG is used as an
inhibitor, the inhibition constant cannot be determined by
standard kinetic approaches and procedures (i.e., slope of
the Lineveawer-Burk plot, line crossing of the Dixon plot,
etc.) derived for the Michaelis kinetics. As shown in
Figure 3, lines on $1/v$ vs. $1/c$ plot and those on $1/v$ vs. $[I]$ plot do not cross at one point. It should be underlined
that this does not result from experimental errors but from
the kinetic properties of the enzymatic reaction catalyzed
by trimeric PNP.

Therefore, instead of the inhibition constant, param-
eter IC$_{50}$ was determined on the basis of these data, since
such analysis is not based on the Michaelis-Menten ki-

![Figure 1](image-url)

**Figure 1.** Effects of AG-19-K1 (a), AG-3 (b), and 9-DG (c) on the growth of different solid tumor and leukemia cells. Exponentially
growing cells were treated with different concentrations of investigated compounds during a 72 h period. Cytotoxicity was analyzed using
the MTT survival assay. All experiments were performed at least three times. Tumor cells growth was calculated by Eq. (1). Statistically
significant differences from control ($P \leq 0.05$) are marked with asterisks.
netic equation. Results of such analysis can be summarized as follows: 9-deazaguanine inhibits calf spleen PNP with IC\textsubscript{50} of about (2.3 ± 0.5) μmol dm\textsuperscript{-3} at 1 mmol dm\textsuperscript{-3} phosphate and (12 ± 3) μmol dm\textsuperscript{-3} at 50 mmol dm\textsuperscript{-3} phosphate concentration. Two 9-deazaguanine derivatives, AG-19-K1 and AG-3, both with substituents at position N-1 of the purine base, are inactive vs. calf spleen PNP in the concentration of 75 μmol dm\textsuperscript{-3} at 1 mmol dm\textsuperscript{-3} phosphate (data not shown).

**DISCUSSION**

As part of our ongoing program directed towards the synthesis of some novel nucleosides\textsuperscript{22–27} with potentially useful biological activity, we were exploring the synthesis of 3H,5H-pyrrrolo[3,2-d]pyrimidines (9-deazaguanine derivatives).

There are relatively few routes to the pyrrrolo[3,2-d]pyrimidine ring system, and the most commonly used ap-
3.35 ppm), which together with the CH$_3$-1 and CH$_3$-7 proton signal at 11.42 ppm (DMSO) were missing. This was supported by the N-1–H proton signal at 10.44 ppm and also the N-7–H proton signal for CH$_3$- group at 27 ppm, quartet) and 2D NMR NOESY spectra (in Scheme 1 double arrows indicate the intramolecular NOE effects). In the NOESY experiment with AG-19-K1, strong NOE interactions between H-8 ($\delta = 7.15$ ppm) and NH$_2$-2 ($\delta = 6.31$ ppm) and CH$_2$-1 ($\delta = 3.35$ ppm) support the N-1 methylation of the 9-deazaguanine base. The NOESY spectrum of AG-3 showed strong NOE interactions between H-8 ($\delta = 7.43$ ppm) and CH$_2$-7 ($\delta = 3.35$ ppm), which together with the CH$_2$-1 and CH$_2$-7 protons signal at $\delta = 3.35$ ppm (s, 6H in DMSO) in $^1$H NMR spectra support the N-1 and N-7 methylation of the 9-deazaguanine base.

The most versatile approach for the synthesis of 9-deazaguanine (9-DG) was described by Sartorelli and co-workers, wherein the 9-DG was generated in five steps after reductive cyclization of the key-intermediate 5 (Scheme 1). Following this procedure, we have prepared 9-DG in the overall yield of 50%.

9-DG and its two methyl derivatives were tested as potential inhibitors of mammalian PNP. Results of the in vitro study performed on calf spleen PNP are fully in line with all that we know about the active site of calf spleen enzyme and other trimeric PNP.

**Effect of 9-DG and its derivatives on the growth of tumor cells.**

While some purine nucleoside analogues down-regulate expression of PNP gene, to our knowledge, the effect of 9-DG on PNP gene expression has not yet been investigated. The influence of 9-DG and AG-19-K1 on modulation of PNP gene expression was also tested. In comparison with the other two treated cell lines, AG-19-K1 (after 6 and 12 h of treatment) showed the strongest and prolonged inhibition effects on PNP gene expression in human T cell leukemia (MOLT-4) cells and affected
their growth by approximately 50%. After 6 h of treatment with 9-DG, expression of the target gene in MOLT-4 and RAJI cell lines decreased by approximately 50%. In comparison with 9-DG, regardless of the exposure time, AG-19-K1 caused stronger inhibition effects on the PNP gene expression in all tested cell lines, except in RAJI cells after 24 h of exposure. The results of our study show that the tested substances modulate PNP gene expression but further research is needed for a better insight.

In conclusion, the presented 9-deazaguanine and its methyl derivatives, synthesized as pools for the future synthesis of novel purine nucleosides (9-deazaguanine derivatives), slightly affect the growth of solid tumor and leukemia cells and significantly modulate expression in leukemia cells. 9-deazaguanine is a potent inhibitor of mammalian PNP.

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SAŽETAK

9-deazagvanin i njegovi metilni derivati: Sinteza, protutumorsko djelovanje i učinci na ekspresiju gena za purin-nukleozid-fosforilazu

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Sintetizirani su 9-deazagvanin (9-DG), 1-metil-9-deazagvanin (AG-19-K1) i 1,7-dimetil-9-deazagvanin (AG-3). Ispitivane su njihove antiproliferativne sposobnosti na pet leukemijskih i 4 stanične linije dobivene iz čvrstih tumora, te njihov inhibicijski potencijal za purin-nukleozid-fosforilazu (PNP) iz slezene teleta. Sinteza 9-DG uključuje reakciju 2-amino-6-metil-5-nitropirimidin-4(3H)-ona (2) s DMF-dimetilacetalom (množinski omjer, \( n(2) / n(\text{DMF-dimetilacetal}) = 1 : 6 \)) i zaštitu N-3 položaja 2-(N-dimetilaminometilen)amino-6-metil-5-nitropirimidin-4(3H)-ona (4) s benziloksimetilnom skupinom. Reakcijom spoja 2 s DMF-dimetilacetalom (množinski omjer, \( n(2) / n(\text{DMF-dimetilacetal}) = 1 : 2,5 \)) nastaje (N-3)-metilni spoj 3. Ditonitna redukcija tog produkta daje N-metilne derivate AG-19-K1 i AG-3. AG-19-K1 i AG-3 u koncentraciji 75 μmol dm⁻³ nisu inhibirali PNP. Citotoksični učinci derivata 9-DG na rast tumorskih stanica ispitani su MTT-testom. Istraživani spojevi pokazali su slabe učinke na rast tumorskih stanica. Rezultati pokazuju da AG-19-K1 u koncentraciji od 10⁻³ mol dm⁻³ inhibira rast stanica JURKAT, K562 i AGS u prosjeku za 80 %. U istoj koncentraciji, AG-3 i 9-DG inhibiraju za 40–50 % staničnu proliferaciju svih ispitanih staničnih linija MOLT-4 i HL-60. U ovisnosti o vremenu izlaganja, u leukemijskim stanicama izloženim djelovanju AG-19-K1 i 9-DG dolazi do značajnih promjena u ekspresiji gena PNP.