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Original Scientific Paper

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(3*H*)-one (**2**) with DMF-dimethylacetal (amount ratio, *n*(**2**) / *n*(DMF-dimethylacetal) = 1:2.5) and use of the benzyloxymethyl group to protect the N-3 position of 2-(*N*-dimethylaminomethylene)amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**4**). Reaction of **2** with DMF-dimethylacetal (amount ratio, *n*(**2**) / *n*(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 μmol 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
synthesis
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.[1] One

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approach is to design novel compounds that could act as antimetabolites and be able to reduce the activity of enzymes important to tumor cells' metabolism. Purine nucleoside phosphorylase (PNP) is an ubiquitous enzyme of purine metabolism with a function in the salvage pathway, enabling cells to utilize purine bases recovered from metabolized purine ribo- and deoxyribo-nucleosides to synthesized purine nucleotides.[2] Biological importance of PNP is well known but the kinetic mechanism seems to be relatively complicated[3] and the molecular mechanism of catalysis has not been fully characterized.[2,4–6] PNP catalytic activity is increased in a number of leukemia cells and colon carcinoma tissues.[7,8] PNP is crucial for the integrity of the immune system, since PNP deficiency in humans leads to defective T-cell response.[2] 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.[2,9]

Mammalian PNP is a trimeric molecule and contains three active sites for ligands binding.[2] The most potent inhibitors of PNP, including transition state analogue inhibitors immucillins, 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.[2,10] Such inhibitors, especially immucillins, are able to significantly block PNP activity.[2,11] 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.[2] 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 Kiselgel 60 F254. Melting points were determined on a Kofler hot-stage apparatus and were uncorrected. UV spectra (λ_{max}/nm, log ε/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 (ν_{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(3*H*)-one (**1**): R_f = 0.12 (EtOAc/MeOH = 3:1); UV (MeOH): λ_{max}/nm: 206, 222, 284, log ε/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), 100.30 (d, C-5), 22.98 (q, CH$_3$).

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

2-Amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**2**) was synthesized following the Pankiewicz procedure.[12] A mixture of nitric acid (5.3 mL), conc. sulfuric acid (5.3 mL) and 2-amino-6-methylpyrimidin-4(3*H*)-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(*H*)-one (**2**): m.p. 280 °C decom; R_f = 0.22 (CH$_2$Cl$_2$/MeOH = 9:1); UV (MeOH): λ_{max}/nm: 203, 296, 326, log ε/dm^3 mol^{-1} cm^{-1}: 3.99, 3.65, 3.52; IR (KBr) ν_{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 Furneaux and Tyler procedure for preparation of 2-(*N*-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-5-nitropyrimidin-4-one.[13] A mixture of 2-amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**2**) (1 g, 5.88 mmol) with dry DMF (12.5 mL) and DMF-dimethylacetal (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; R_f = 0.64 (EtOAc/MeOH = 3:1); UV (MeOH): λ_{max}/nm: 268, 331, 416, log ε/dm^3 mol^{-1} cm^{-1}: 3.97, 4.45, 3.94; IR (KBr) ν_{max}/cm^{-1}: 3448 (w), 2925 (w), 1671 (s), 1629 (s, sh), 1611 (s), 1535 (s), 1511 (s), 1485 (m), 1398 (m), 1384 (m), 1333 (w), 1111 (m), 1055 (s); ^{1}H NMR (DMSO-d$_6$) δ/ppm: 8.83 (s, 1H, CH=N), 8.13 (d, 1H, J = 12 Hz, NHC=CH), 5.37 (d, 1H, J = 12 Hz, NHC=CH), 3.35 (s, 3H, 3-N–CH$_3$); 3.26 (s, 3H, N-CH$_3$), 3.16 (br s, 3H, N–CH$_3$), 3.10 (s, 3H, N–CH$_3$), 2.85 (br s, 3H, N–CH$_3$); ^{13}C NMR (DMSO-d$_6$) δ/ppm: 159.79 (d, HC=N), 157.04 (s, C-4), 156.40 (s, C-2 or C-6), 156.10 (s, C-6 or C-2), 153.36(d, NCH=CH), 133.06 (s, C-5), 88.16 (d, CH=CHN), 41.53 (q, N–CH$_3$), 39.15 (q, N–CH$_3$), 29.31 (q, 3-N–CH$_3$).

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 (CH_2Cl_2/MeOH = 3:1) to yield 0.624 g (45 %) of 2-amino-3-methyl-3*H*-pyrrolo[3,2-*d*]pyrimidin-4(5*H*)-one (AG-19-K1) and 0.098 g (6 %) of 2-amino-3,5-dimethyl-3*H*-pyrrolo[3,2-*d*]pyrimidin-4(5*H*)-one (AG-3) as white crystals.

*2-Amino-3-methyl-3*H*-pyrrolo[3,2-*d*]pyrimidin-4(5*H*)-one* (*AG-19-K1*): m.p. 252–255 °C; R_f = 0.67 (EtOAc/MeOH = 3:1); UV (EtOH): λ_{max}/nm: 231, 256, 290, log ε/dm^3 mol^{-1} cm^{-1}: 4.39, 3.91, 3.85; IR (KBr) ν_{max}/cm^{-1}: 3452 (s), 3269 (br s), 1686 (s), 1634 (s), 1546 (s), 1419 (m), 1375 (m), 1299 (m), 1333 (w), 1215 (m), 1167 (s) 1055 (s); ^{1}H NMR (DMSO-d$_6$) δ/ppm: 11.44 (br s, 1H, NH-7), 7.15 (t, 1H, J = 2.9 Hz, H-8), 6.31 (br s, 2H, NH$_2$), 5.94 (t, 1H, J = 2.3 Hz, H-9), 3.35 (s, 3H, N-1 CH$_3$); ^{13}C NMR (DMSO-d$_6$) δ/ppm: 153.93 (s, C-6), 151.22 (s, C-2), 144.60 (s, C-4), 127.37 (d, C-8), 112.01 (s, C-5), 100.08 (d, C-9) 27.73 (q, 1-N CH$_3$).

COSY spectra: H-8 ↔ H-9, N-7 ↔ H-8, N-7 ↔ H-9; NOESY spectra: H-8 ↔ H-9, N-7 ↔ H-8, N-7 ↔ H-9 and NH$_2$ ↔ 1-N–CH$_3$.

*2-Amino-3,5-dimethyl-3*H*-pyrrolo[3,2-*d*]pyrimidin-4(5*H*)-one* (*AG-3*): m.p. 235–238 °C; R_f = 0.41 (EtOAc/MeOH = 3:1); UV (EtOH): λ_{max}/nm: 230, 257, 291, log ε/dm^3 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); ^{1}H NMR (DMSO-d$_6$) δ/ppm: 7.43 (d, 1H, J = 3.1 Hz, H-8), 6.41 (br s, 2H, NH$_2$), 5.86 (d, 1H, J = 3.1 Hz, H-9), 3.35 (s, 6H, N-1 and N-7–CH$_3$); ^{13}C NMR (DMSO-d$_6$) 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) 27.92 (q, 1-N and 7-N–CH$_3$).

COSY spectra: H-8 ↔ H-9; NOESY spectra: H-8 ↔ H-9, H-8 ↔ 7-N–CH$_3$.

In vitro *Study*

– *Inhibition of PNP* in vitro *(without cells)*

Inhibition properties of 9-deazaguanine and its derivatives were determined as described[3] earlier on calf spleen PNP, with 7-methylguanosine (7-MG) as variable substrate,[14] 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 (λ_{obs} = 260 nm).[14] 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 trypsine-EDTA were purchased from the Institute of Immunology Inc. (Zagreb, Croatia); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Merck (Darmstadt, Germany, EU) and dimethylsulfoxyde (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-methylgunosine, 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.[3]

– *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), JURKAT (human T-cell leukemia), K562 (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 % CO_2. Trypan blue dye exclusion method was used to assess cell viability.

– Cell Proliferation Assay

Cells were plated into 96-well plates at concentrations of 2×10^4 cells per mL for solid tumor cells and 1×10^5 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.[15] 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 in 10 % SDS with 0.01 mol dm^{-3} HCl. The plate were scanned using an Elisa reader (Stat Fax 2100, Pharmacia Biotech) to measure the extracted dye at 570 nm. All experiments were performed at least three times, with three wells for each concentration of tested compounds. The effect on tumor cells growth of various concentrations of 9-DG derivatives was calculated as:

$$\text{Cells growth / \%} = \frac{(\text{A} - \text{Blank})}{(\text{B} - \text{Blank})} \cdot 100 \qquad (1)$$

A represents the absorbance of cells remaining after treatment with tested substances and *B* is the absorbance of cells without drug stimulation. Blank represents the absorbance of media without cells; for solid tumor cells media was MTT and DMSO, while for leukemia cells media was MTT, 10 % SDS and 0.01 mol dm^{-3} HCl.

– 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 10^6 cells was isolated by Tri-reagent. Target RNA was reverse transcribed using oligo$(dT)_{15}$, dNTP mix, and sterile distilled H_2O 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'-ACACTGCAGAATGGCTTCTG-3' from nucleotides 144 to 163 (sense primer in exon 2) and 5'-GGTACCCTTCATACATGTGG- 3' from nucleotides 383 and 364 (antisense primer in exon 3).[16,17] 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:[18] 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).[19] 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 ImageQuant^{TL} 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.[20]

– 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^{TM} 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 deazaguanine 9-DG (Scheme 1). In the first route,[13] 2-amino-6-methyl-5-nitropyrimidin-4(3*H*)-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*-dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-3-methyl-5-nitropy rimidin-4-one (**3**) as the sole product (73 % yield). Dithionite reduction of this product **3** afforded *N*-methyl derivatives; 1-methyl-9-dezaguanine AG-19-K1 as the main product in 45 % yield and 1,7-dimethyl-9-deazaguanine AG-3 (6 % yield).

The second route[21] for the 9-DG synthesis involves reaction of 2-amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**2**) with DMF-dimethylacetal (amount ratio, *n*(**2**) / *n*(DMF-dimethylacetal) = 1:2.5) and use of the benzyloxymethyl group to protect the N-3 position of 2-(*N*-dimethylaminomethylene)amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**4**). Treatment of **4** with benzyl chloromethyl ether in DMF in the presence of 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) at room temperature, followed by condensation with DMF-dimethylacetal in DMF gave compound **5**. Subsequent reductive cyclization of **5** produced the protected 9-deazaguanine derivative **6**. The removal of the protecting group by catalytic hydrogenation gave the desired 9-DG.

Results of the antiproliferative activity of AG-19-K1, AG-3 and 9-DG on several leukemia and solid tumor

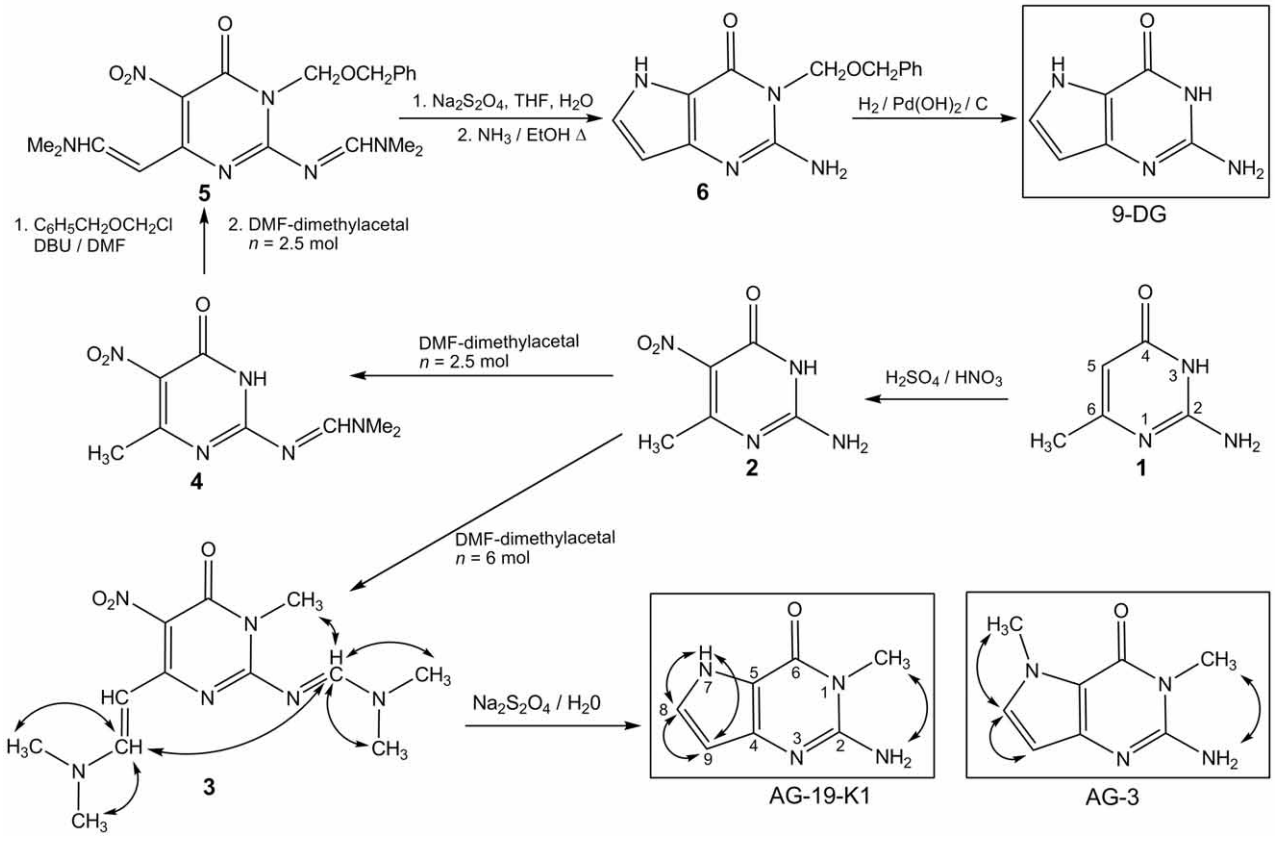
cell lines are shown in Figure 1. Investigated compounds show moderate antiproliferative activity towards the examined cell lines. Compound AG-19-K1 at a concentration of 10^{-3} mol dm^{-3} has the strongest inhibitory effect on the tumor cells growth when compared to the two other compounds tested. 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-methylgunosine 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 PNPs.

Therefore, instead of the inhibition constant, parameter IC_{50} was determined on the basis of these data, since such analysis is not based on the Michaelis-Menten ki-

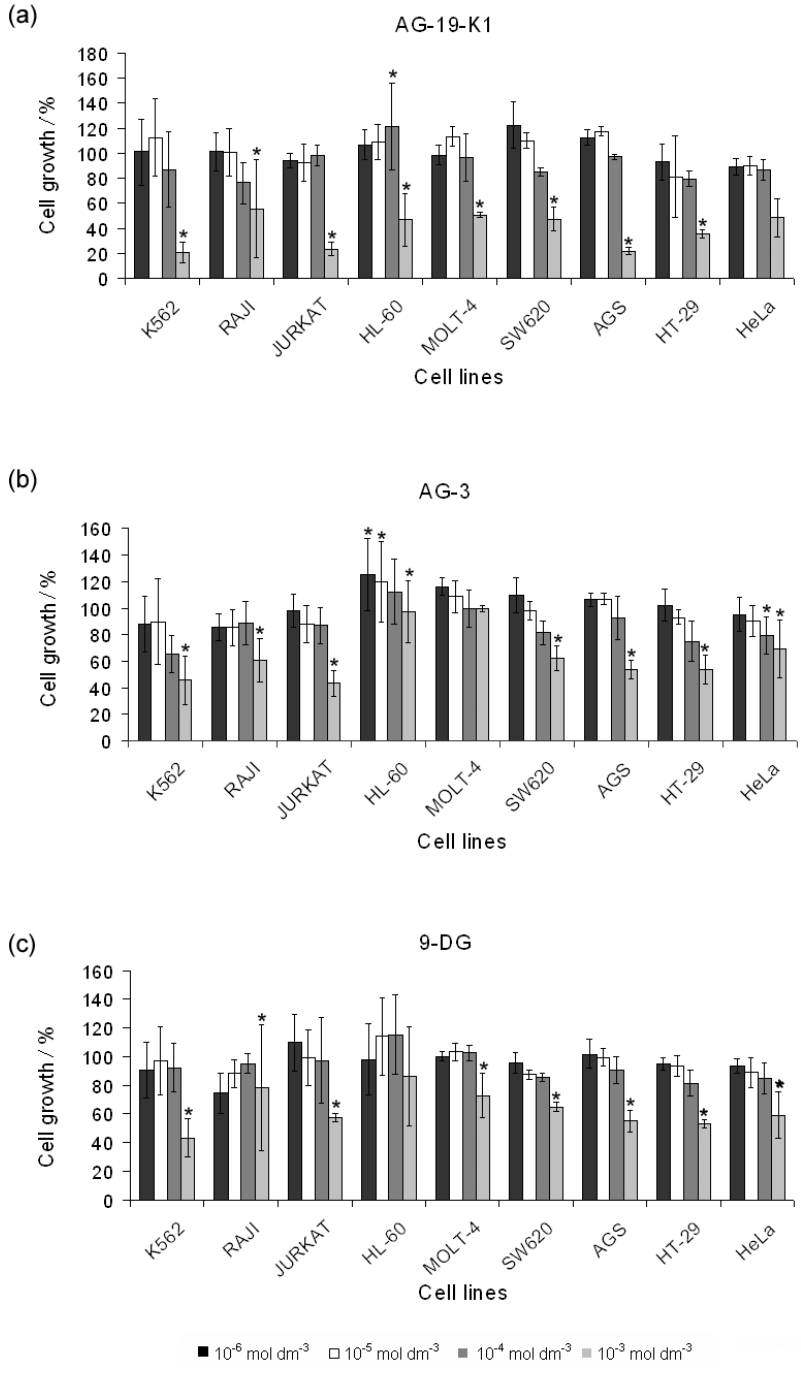


Figure 1. Effects of AG-19-K1 (a), AG-3 (b), and 9-DG (c) on the growth of different solid tumor and leukemia cells. Exponentially growing cells were treated with different concentrations of investigated compounds during a 72 h period. Cytotoxicity was analyzed using the MTT survival assay. All experiments 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.

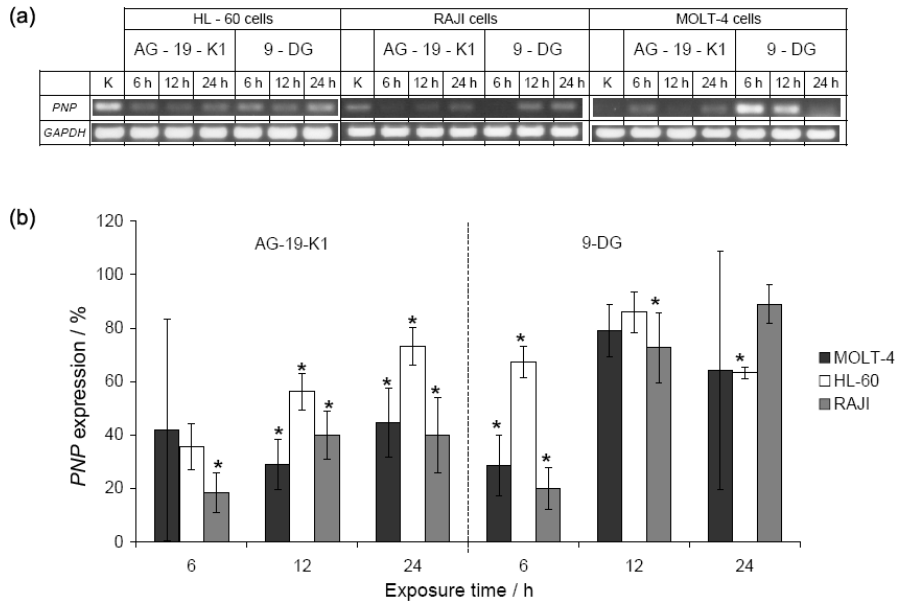


Figure 2. Effects of 9-DG and AG-19-K1 on *PNP* gene expression. (a) Agarose gel electrophoresis of PCR products. Total mRNA was obtained from three different leukemia cell lines (MOLT-4, HL-60 and RAJI) after treatment by tested compounds (AG-19-K1 and 9-DG) at concentration of 10^{-3} mol dm^{-3} and from control (nontreated) cells. After amplification with specific primers, PCR products of cDNA were analyzed using ImageQuant^{TL} software. (b) Results of *PNP* gene expression in treated cells after quantification with respect to the *PNP* gene expression in control cells. *PNP* gene expression was normalized with expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Statistically significant differences from control ($P \leq 0.05$) are marked with asterisks.

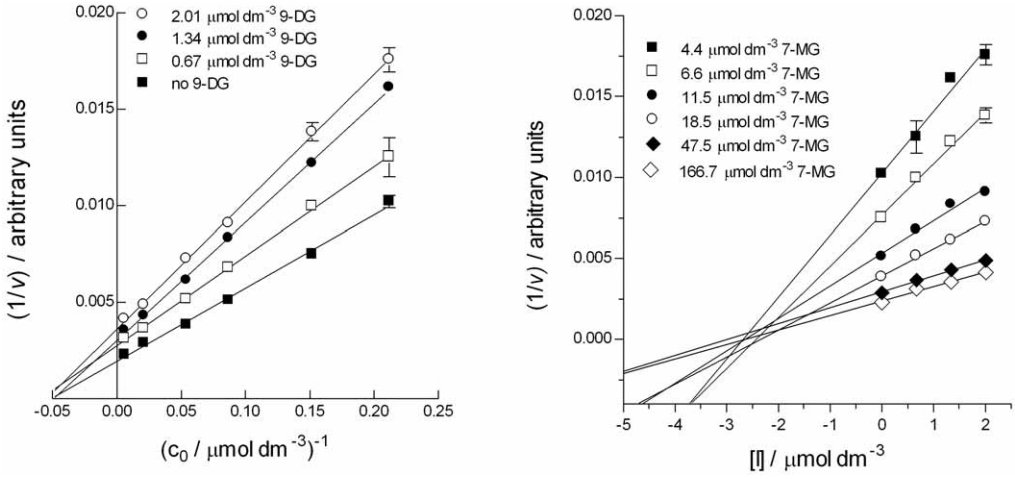


Figure 3. Inhibition of calf spleen PNP by 9-DG shown in the form of the Lineweaver-Burk plot (left panel) and the Dixon plot (right panel). Experiments were conducted at 25 °C in 50 mmol dm^{-3} Hepes buffer, pH = 7.0, by the continuous spectrophotometric method[14] (λ_{obs} = 260 nm) with 7-methylguanosine (7-MG) as substrate and at constant, 1 mmol dm^{-3} phosphate concentration.

netic equation. Results of such analysis can be summarized as follows: 9-deazaguanine inhibits calf spleen PNP with IC_{50} of about (2.3 ± 0.5) μmol dm^{-3} at 1 mmol dm^{-3} phosphate and (12 ± 3) μmol dm^{-3} at 50 mmol dm^{-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^{-3} at 1 mmol dm^{-3} phosphate (data not shown).

DISCUSSION

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

There are relatively few routes to the pyrrolo[3,2-*d*]-pyrimidine ring system, and the most commonly used ap-

proach starts from pyrimidines with appropriate functional groups at the C-5 and C-6 positions to allow elaboration of the pyrrole ring (Scheme 1). Otherwise, the pyrimidine ring can be constructed onto a substituted pyrrole.[28] Our synthetic plan was to synthesize 9-DG on a multigram scale and we explored an improved protocol[13] which, however, in our hands did not lead to the expected compound. Instead, methylation on the heterocycle was observed, as also reported by other researchers.[29] The *N*-methylation as well as formation of imine was observed when 2-amino-6-methyl-5-nitropyrimidin-4(3*H*)-one (**2**) was treated with DMF-dimethylacetal (amount ratio, *n*(**2**) / *n*(DMF-dimethylacetal) = 1:6), Scheme 1. 2-(*N*-Dimethylaminomethylene)amino-6-(2-dimethylaminovinyl)-3-methyl-5-nitropyrimidin-4-one (**3**) was isolated as the sole product. In ^{1}H as well as ^{13}C NMR spectra of **3**, methyl group at position N-3 was identified at δ = 3.35 ppm (s, 3H) and δ = 29.31 ppm (q), respectively. Dithionite reduction of product **3** afforded *N*-methyl derivatives; 1-methyl-9-dezaguanine AG-19-K1 as the main product in 45 % yield and 1,7-dimethyl-9-deazaguanine AG-3 (6 % yield). The structure of 9-deazaguanine derivatives substituted with methyl group was assigned on the basis of ^{1}H NMR spectra. The N-1–H proton signal at 10.44 ppm (characteristic of 9-DG in DMSO) was not observed in the case of AG-19-K1, and in the case of AG-3, the N-1–H proton signal at 10.44 ppm and also the N-7–H proton signal at 11.42 ppm (DMSO) were missing. This assignment was supported by the ^{13}C NMR spectra (carbon 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 (δ = 7.15 ppm) and NH-7 (δ = 11.44 ppm), and NH_2-2 (δ = 6.31 ppm) and CH_3-1 (δ = 3.35 ppm) support the N-1 metylation of the 9-deazaguanine base. The NOESY spectrum of AG-3 showed strong NOE interactions between H-8 (δ = 7.43 ppm) and CH_3-7 (δ = 3.35 ppm), which together with the CH_3-1 and CH_3-7 protons signal at δ = 3.35 ppm (s, 6H in DMSO) in ^{1}H NMR spectra support the N-1 and N-7 metylation of the 9-deazaguanine base.

The most versatile approach for the synthesis of 9-deazaguanine (9-DG) was described by Sartorelli and co-workers,[21] 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 PNPs. Absence of inhibition observed with 1-methyl-9-deazaguanine AG-19-K1 and 1,7-dimethyl-9-deazaguanine AG-3 results from the fact that the hydrogen at position N-1–H defines the specificity of the enzyme.[2,30] In inhibition studies with calf spleen PNP, errors are very big since the inhibitor data cannot be properly analyzed by a pure competitive, uncompetitive, noncompetitive or even mixed inhibitor model since, as we have shown earlier, calf spleen PNP kinetics does not obey the Michaelis-Menten model.[2,3,31] Therefore, a more reliable parameter characterizing interactions of inhibitors with calf spleen and other trimeric PNPs is a dissociation constant. For the complex 9-deazaguanine/calf spleen PNP, this was obtained earlier form the steady-state fluorescence titration experiments and is (0.17 ± 0.02) μmol dm^{-3} at pH = 7.0 in the absence of phosphate.[32]

The antiproliferative activity of 9-DG and its methyl derivatives AG-19-K1 and AG-3 was tested against a panel of tumor cell lines. Results of our study show that these compounds display moderate antiproliferative effects on investigated tumor cells. There is no significant difference in their cell growth inhibition activity between the treated leukemia and solid tumor cell lines. In comparison to the 9-DG, methylated derivatives AG-19-K1 and AG-3 more potently decrease treated tumor cells growth, at a relatively high dose (10^{-3} mol dm^{-3}). The maximum of growth inhibitory efficacy (80 %) on the growth of K562, JURKAT and AGS is achieved using 10^{-3} mol dm^{-3} AG-19-K1. AG-3, the compound with two methyl groups, maximally inhibits cell proliferation of K562 and JURKAT cells by 55 %, and AGS and HT-29 by 45 %. Recent studies on nucleosides with 9-DG aglycone showed their positive antiproliferative effects on tumor cells and significant inhibitory potential on catalytic activity of PNP.[33,34] In comparison with these results, substances investigated in this study do not display pronounced antitumor effects. Despite the potent PNP inhibitory potential of 9-DG**,** no differences were observed between the effects on the growth of tumor cells with increased PNP activity (such as human adult T-cell leukemia and colon carcinoma cells) and tumor cells, where PNP enzyme activity is unchanged.[7,35,36] On the other hand, the two methyl derivatives AG-19-K1 and AG-3 (both with substituents at position N-1 of the purine base, which makes these analogues unable to form hydrogen bond *via* N-1–H position with PNP), were inactive *vs.* calf spleen PNP but they displayed better cytotoxic activity against all treated tumor cells compared to 9-DG.

While some purine nucleoside analogues down-regulate expression of *PNP* gene[37,38] and, 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 *PNP* gene 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(3*H*)-ona (**2**) s DMF-dimetilacetalom (množinski omjer, *n*(**2**) / *n*(DMF-dimetilacetal) = 1 : 6) i zaštitu N-3 položaja 2-(*N*-dimetilaminometilen)amino-6-metil-5-nitropirimidin-4(3*H*)-ona (**4**) s benziloksimetilnom skupinom. Reakcijom spoja **2** s DMF-dimetilacetalom (množinski omjer, *n*(**2**) / *n*(DMF-dimetilacetal) = 1 : 2,5) nastaje (*N*-3)-metilni spoj **3**. Ditionitna 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*.