7-(2-Thienyl)-7-deazaadenosine (AB61), a new potent nucleoside cytostatic with a complex mode of action

Pavla Perliková,1 Gabriela Rylová,2 Petr Nauš,1 Tomáš Elbert,1 Eva Trloušťová,1 Aurelie Bourderiouxf,1 Lenka Poštovoť Slavětinská,1 Kamil Motyka,3 Dalibor Doležal,2 Pawel Znojek,2 Alice Nová,2 Monika Harvanová,2 Petr Džubák,2 Michal Šiller,2 Jan Hlaváč,2,3 Marián Hajdúch,2* Michal Hocek1,4*

1 Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Gilead Sciences & IOCB Research Center, Flemingovo nam. 2, CZ-16610 Prague 6, Czech Republic; 2 Institute of Molecular and Translational Medicine, Palacky University and University Hospital in Olomouc, Faculty of Medicine and Dentistry, Hněvotínská 5, CZ-77515 Olomouc, Czech Republic; 3 Department of Organic Chemistry, Faculty of Natural Sciences, Palacky University, 17. listopadu 1192/12, CZ-77146 Olomouc, Czech Republic; 4 Department of Organic Chemistry, Faculty of Science, Charles University in Prague, Hlavova 8, CZ-12843 Prague 2, Czech Republic.

Corresponding authors: biology: M. Hajdúch, Institute of Molecular and Translational Medicine, Palacky University and University Hospital in Olomouc, Faculty of Medicine and Dentistry, Hněvotínská 5, CZ-77515 Olomouc, Czech Republic, marian.hajduch@upol.cz; medicinal chemistry and biochemistry: M. Hocek, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Gilead Sciences & IOCB Research Center, Flemingovo nam. 2, CZ-16610 Prague 6, Czech Republic, hocek@uochb.cas.cz

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Conflict of interests

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Abstract

7-(2-Thienyl)-7-deazaadenosine (AB61) showed nanomolar cytotoxic activities against various cancer cell lines but only mild (micromolar) activities against normal fibroblasts. The selectivity of AB61 was found to be due to inefficient phosphorylation of AB61 in normal fibroblasts. The phosphorylation of AB61 in leukemic CCRF-CEM cell line proceeds well and it was shown that AB61 is incorporated into both DNA and RNA, preferentially as a ribonucleotide. It was further confirmed that a triphosphate of AB61 is a substrate for both RNA and DNA polymerases in enzymatic assays. Gene expression analysis suggests that
AB61 affects DNA damage pathways and protein translation/folding machinery. Indeed, formation of large 53BP1 foci was observed in nuclei of AB61-treated U2OS-GFP-53BP1 cells indicating DNA damage. Random incorporation of AB61 into RNA blocked its translation in an in vitro assay and reduction of reporter protein expression was also observed in mice after 4h treatment with AB61. AB61 also significantly reduced tumor volume in mice bearing SK-OV-3, BT-549 and HT-29 xenografts. The results indicate that AB61 is a promising compound with unique mechanism of action and deserves further development as an anticancer agent.

Introduction

7-Deazapurine (IUPAC name: pyrrolo[2,3-d]pyrimidine) nucleosides have become a subject of research interest because of their powerful antibacterial, antifungal and cytotoxic activities.(1) Mechanisms of action of naturally occurring 7-deazapurine nucleosides tubercidin, toyocamycin and sangivamycin (Figure 1) have been extensively studied. Despite structural similarities, their biochemical and biological properties show different features for each nucleoside. Tubercidin, toyocamycin and sangivamycin are all phosphorylated in cells to their 5'-O-mono-, di- and triphosphates which are subsequently incorporated into nucleic acids.(2,3,4) In addition, tubercidin also inhibits nucleic acid and protein synthesis, interferes with mitochondrial respiration, rRNA processing, de novo purine synthesis(2) and it is a potent inhibitor of S-adenosylhomocysteine hydrolase.(5) Toyocamycin showed inhibition of phosphatidylinositol kinase(6) and an inhibitory effect on rRNA synthesis and maturation.(7,8) On the other hand, sangivamycin acts mainly through potent and selective inhibition of protein kinase C.(9)

In our previous research, nanomolar cytotoxic activities of hetaryl-7-deazapurine ribonucleosides were discovered. 6-Hetaryl-7-deazapurine ribonucleosides 1 showed strong
cytotoxicity against a panel of cancer cell lines similar to that of conventional nucleoside cytostatics (gemcitabine, clofarabine).(10) Similarly profound cytotoxic effects were also found in 7-hetaryl-7-deazaadenosines, i.e. 7-hetaryltubercidins 2.(11) Structure-activity-relationship studies revealed that modifications in the 2’ position of the ribose moiety lead to a decrease of cytotoxicity in 7-hetaryl-7-deazapurine ribonucleosides 2 (12,13) and completely inactive compounds in 6-hetaryl-7-deazapurine ribonucleosides 1.(14,15) By contrast, base-modified 7-hetaryl derivatives bearing modifications in positions 2 and 6 of 7-deazapurine showed submicromolar cytotoxic activities.(16) The most promising derivative among the 7-hetaryl-7-adenosines is a 2-thienyl derivative AB61 (Figure 1), which is not only highly cytotoxic against leukemic and solid-tumor-derived cell lines but also non-cytotoxic to normal human fibroblasts.(11) In vivo antitumor activity of AB61 was demonstrated in a mouse leukemia survival model. The preliminary studies of its mechanism of action in CCRF-CEM lymphoblasts showed fast onset of the RNA synthesis inhibition and also inhibition of DNA synthesis at higher concentrations. AB61 is efficiently phosphorylated to its 5’-O-triphosphate (AB61-TP) but only very mild inhibition of RNA polymerase II by AB61-TP was observed.(11) More recently, AB61 and its related derivatives were found(17) to be weak inhibitors of human adenosine kinase and AB61 itself was found to be a weak substrate for this enzyme (2%) as compared to adenosine.

The aims of this study were to elucidate the detailed mechanism of action of AB61 and understand why its cytotoxicity is selective for cancer cell lines. We hypothesized that AB61-TP may get incorporated into nucleic acids that have impaired biological functions. Therefore, the first goal was to investigate phosphorylation of AB61 in normal and cancer-derived cell lines, incorporation of AB61 into nucleic acids both on enzymatic and cellular levels, and the effect of AB61 on gene transcription, translation and DNA integrity.
Furthermore, we have studied the in vivo antitumor activity of AB61 against xenotransplanted human solid-tumors to translate promising in vitro data into preclinical efficacy.

**Materials and Methods**

**Analytical standards**

Synthesis and characterization data of analytical standards AB61-DP, dAB61-MP, [3H]AB61 and Tub-TP are given in the Supporting Information. Synthesis of AB61, AB61-MP and AB61-TP was published previously.(11)

**Cell lines**

The CCRF-CEM (ATCC© CCL-119), HCT116 (ATCC© CCL-247), K-562 (ATCC© CCL-243), BJ (ATCC© CRL-2522), MRC-5 (ATCC© CC-171), HT-29 (ATCC© HTB-38), SK-OV-3 (ATCC© HTB-77), U2OS (ATCC® HTB-96) and BT-549 (ATCC© HTB-122) cell lines were purchased from the American Tissue Culture Collection (ATCC) in 2010-2014. HCT116p53−/− cell line was purchased from Horizon Discovery, UK in 2011. The daunorubicin resistant subline of CCRF-CEM cells (CEM-DNR-bulk) and paclitaxel resistant subline K-562-tax overexpressing major drug resistance transporters were selected in our laboratory.(18,19) Mouse breast cancer 4T1-luc2 cell line stably transfected with firefly luciferase under cytomegalovirus promoter was a kind gift from Prof. Danuta Radzioch (McGill University, Montreal, Canada) in 2009. Cell line derived from human osteosarcoma U2OS stably transfected with 53BP1-GFP fusion gene (U2OS-53BP1-GFP) was prepared by lipofectamine transfection of 53BP1 plasmid (20) (a kind gift from Prof. Jiri Bartek, Danish Cancer Society Research Center, Copenhagen, Denmark) into parental cells and successful transfectants were selected by neomycine. Cell lines authentication was performed using the Promega CELL ID TM System (8 STR markers + amelogenin) to verify that the genetic
profile of the sample matches the known profile of the cell line. Mycoplasma contamination was tested using 1-My and 2-My primers by qPCR (21) for every batch of the frozen cells. The cell lines were cultured for maximum of 6-10 passages and tested for mycoplasma contamination on a weekly basis.

**Oligonucleotides**

DNA oligonucleotides were purchased from Sigma-Aldrich and Generi Biotech. ON1: 5’-GCTAATACGACTCATATAGGGTGAGGTATTCTTAGTGATT (T7 promoter in italics); ON2: 5’-AATCACTACAAGTACCTCACCTAGTGAGTCGTATTAGC (underlined nucleotides represent 2’-O-Me-RNA); Prim248short: 5’-CATGGGGCGGCATGGG; Prb4basII: 5’-CTAGCATGAGCTCAGTCCCATGCCGCCCATG; OligoAterm: 5’-TCCCATGCCGCCCATG; (bio)-OligoAterm: 5’-biotin-TCCCATGCCGCCCATG.

**MTT assay**

The cells were maintained in Nunc/Corning 80 cm² plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI-1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10% fetal calf serum, and NaHCO₃). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25,000–30,000 cells/well based on cell growth characteristics). MTT assay was performed as described before and the IC₅₀ value, the drug concentration lethal to 50% of the cells, was calculated from appropriate dose-response curves. (18)

**Intracellular phosphorylation**

HCT116 and BJ cells were seeded into 6-well plates at 60% confluence (McCoy's 5A and DMEM medium, respectively, supplemented with 5 g/L glucose, 2 mM glutamine, 100 U/mL
penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and NaHCO₃). Next day, cells were treated with AB61 (10 μM). After 3 h incubation, cultures were washed 2 times with phosphate buffered saline containing 0.5% FBS and 3 times with phosphate buffered saline. The cell pellets were extracted with 70% cold MeOH (1 mL). Supernatants were collected, dried under vacuum, and samples were resuspended in DMSO-water (1:1, 100 μL) for analysis. The samples were analyzed using ultra-performance reversed phase chromatography coupled to triple tandem mass spectrometry UPLC-MS/MS. The UPLC chromatograph used in this study was Accela Thermo Scientific system consisting of gradient quaternary pump, thermostated autosampler, degasser, column oven and triple quadrupole mass spectrometer TSQ Quantum Access (Thermo Scientific). Xcalibur™ data system software was used for an instrument control and data analysis. The C18 column (XBridge™ BEH C18, 2.5 μm, 2.1x50 mm) and ammonium acetate (pH 5.04)/acetonitrile gradient elution were applied for chromatographic separation. The electrospray ionization and negative selected reaction mode MS/MS were used for analyte quantification. Standard curves and quality control samples were generated for all analytes using extracts from untreated cells.

**Incorporation of modified nucleoside triphosphates into RNA in vitro**

A solution of template oligonucleotides ON1 and ON2 (50 μM each) in annealing buffer [Tris (10 mM), NaCl (50 mM), EDTA (1 mM), pH 7.8] was heated to 95 °C for 5 min and then slowly cooled to 25 °C over a period of 45 min. The resulting dsDNA (50 μM) was used as a template for transcription reactions. *In vitro* transcription reactions (10 µL) (ApliScribe T7-Flash Transcription Kit, Epicentre) were performed in the presence of AB61-TP or tubercidin triphosphate (Tub-TP) (0.45 mM, for synthesis see supporting information), CTP, GTP, UTP (4.5 mM each), DTT (10 mM), ApliScribe T7-Flash 10x Reaction Buffer (2 µL), template (2.5 μM), [α-³²P]GTP (111 TBq/mmol, 370 MBq/mL, 0.4 μL) and ApliScribe T7-Flash Enzyme Solution (2 µL). In the negative control experiment water was used instead of the
solution of the tested compound, in the positive control experiment ATP (0.45 mM) was used instead of the tested compound. The transcription reactions were performed at 37 °C for 2 h. RNA was purified on NucAway Spin Columns (Ambion, elution in DEPC-water). Samples (2 µL) were mixed with RNA loading dye (Fermentas) (2 µL), denatured at 90 °C for 10 min and cooled on ice. The samples were analysed by gel electrophoresis on 12.5% denaturing polyacrylamide gel containing 1× TBE buffer (pH 8) and urea (7M) at 45 mA for 45 min. The gels were dried (85 °C, 75 min), audioradiographed and visualized by phosphorimager (Typhoon 9410, Amersham Biosciences).

**Incorporation of modified nucleoside triphosphates into DNA by DNA polymerases**

The primer extension experiments were performed under following conditions: *Klenow fragment*: The reaction mixture (20 µL) contained DNA polymerase I, large (Klenow) fragment (New England BioLabs, 5 U/µL, 0.04 µL), natural dNTPs (10 mM, 0.4 µL), AB61-TP or Tub-TP (10 mM, 1 µL), primer Prim248short (3 µM, 1 µL), 31-mer template Prb4basII (3 µM, 1 µL) and NEBuffer 2 (2 µL) supplied by the manufacturer. Prim248short was labeled by the use of [γ-32P]ATP according to standard techniques. In the positive and negative control experiments dATP (10 mM, 1 µL) and water, respectively, were used instead of the tested compound. The reaction mixtures were incubated for 15 min at 25°C. *Human DNA polymerase β*: Primer Prim248short was labeled by the use of [γ-32P]ATP and annealed with template Oligo<sup>Aterm</sup> (primer:template ratio 1:1.5) according to standard techniques. The reaction mixture (10 µL) contained human DNA polymerase β (CHIMERx, 5 U/µL, 0.01 µL), AB61-TP (1 mM, 1 µL), primer:template mixture (1 µM primer, 1.5 µM template; 1 µL), BSA (acetylated, 24 mg/ml, 0.167 µL) and glycerol (1.5 µL) and 10× buffer for DNA polymerase β (1 µL) supplied by the manufacturer. In the positive and negative control experiments dATP and water, respectively, were used instead of AB61-TP. The reaction
mixtures were incubated for 3 h at 37°C. **Human DNA polymerase γ**: Primer Prim248short was labeled by the use of [γ-32P]ATP and annealed with template OligoAterm (primer:template ratio 1:1.5) according to standard techniques. The reaction mixture (10 µL) contained human DNA polymerase γ (CHIMERx, 10 U/µL, 0.05 µL), **AB61-TP** (1 mM, 1 µL), primer:template mixture (1 µM primer, 1.5 µM template; 1 µL), BSA (acetylated, 24 mg/ml, 0.25 µL) and 10x MnCl2 solution (1 µL) supplied by the manufacturer and 10× buffer for DNA polymerase γ (1 µL) supplied by the manufacturer. In the positive and negative control experiments dATP and water, respectively, were used instead of **AB61-TP**. The reaction mixtures were incubated for 3 h at 37°C. The reactions were stopped by addition of the same volume of PAGE stop solution (80 % [v/v] formamide, 20 mM EDTA, 0.025 % [w/v] bromophenol blue, 0.025 % [w/v] xylene cyanol) and heated to 95°C for 5 min. Aliquots (2 µl) were analyzed by gel electrophoresis on 12.5% denaturing polyacrylamide gel containing 1× TBE buffer (pH 8) and urea (7M) at 45 mA for 50 min. The gels were dried (85 °C, 75 min), audioradiographed and visualized by phosphorimager (Typhoon 9410, Amersham Biosciences). The incorporation of **AB61-TP** into DNA oligonucleotide was confirmed by MALDI-TOF analysis (see Supplementary information Figure S1).

**Incorporation of [3H]AB61 into RNA or DNA in cells**

CCRF-CEM cells (ATCC: CCL-119) were grown in RPMI-1640 medium (Sigma) supplemented with 10% fetal calf serum, GlutaMAX (Gibco, 10 mL/l), penicillin (100 U/l) and streptomycin (100 mg/l). All experiments were done with exponentially growing cells. CCRF-CEM cells (7.10^5 cells/mL, 10 mL) were incubated overnight in a humidified CO2 incubator at 37 °C. Solution of [3H]AB61 (for synthesis see Supplementary information Synthesis of nucleosides and nucleotides) in water (29 µL, 1 mCi/mL, 11.6 Ci/mmol) was added. The final concentration of [3H]AB61 was 250 nM. Samples of the cell suspension (1.5
mL) were harvested immediately after addition of [³H]AB61 or after incubation at 37 °C (2.5 h). Cells were washed twice with phosphate-buffered saline and then either RNA was isolated using miRNeasy Mini Kit (Qiagen) according to manufacturer’s protocol or DNA was isolated using Dneasy Blood & Tissue Kit (Qiagen) according to manufacturer’s protocol including RNA digestion with RNase A. Concentrations of RNA and DNA samples were measured by NanoDrop 1000 Spectrophotometer. Activity of the RNA (40 μL) or DNA (180 μL) was measured in AquaSafe 500 Plus LSC cocktail (4 mL) on a Liquid Scintillation Analyzer Tri-Carb 2900TR (Perkin Elmer).

**RNA digestion and HPLC-LSC analysis:** RNA was isolated from the CCRF-CEM cells treated by [³H]AB61 (250 nM) for 2.5 h as described above. RNA (150 μL, 240 ng/μL) was digested by Nuclease P1 from *Penicillium citrinum* (Sigma, 1 mg/mL, 1 μL) in a digestion buffer (80 mM Tris-HCl; 10 mM NaCl; 1 mM MgCl₂; 0.2 mM ZnCl₂, pH 5.3). Final volume was 167.7 μL. Digestion was performed at 50 °C for 1 h. Then, ice-cold aqueous solution TCA (10% v/v, 167.7 μL) was added and samples were cooled on ice for 10 min. After centrifugation (14000×g, 5 min, 4 °C) supernatant was transferred into a clear microtube. The supernatant was extracted with 1,1,2-trichlorotrifluoroethane-triethylamine mixture (4:1, 335.4 μL) at 4 °C. The aqueous phase was evaporated using a vacuum concentrator and dissolved in water (90 μL). The sample (50 μL) was analyzed on Waters HPLC system (2996 PDA detector, 616 HPLC pump, 600S controller, PDA software Empower™) with a Supelcosil™ LC-18-T 3μm column (15 cm × 3 mm) with a flow rate of 0.75 mL.min⁻¹. Solution A (50 mM KH₂PO₄, 3 mM tetrabutylammonium hydrogensulfate, pH 3.1) and solution B [50 mM KH₂PO₄, 3 mM tetrabutylammonium hydrogensulfate, pH 3.1, acetonitrile (50 % v/v)] were used as a mobile phase. Elution gradient: 0-5 min: solution A; 5-6 min: linear gradient 0-5 % of solution B in solution A; 6-30 min: linear gradient 5-100 % of solution B in solution A. Fractions were collected for 15 s each. Activity of the fractions was
measured in AquaSafe 500 Plus LSC cocktail on a Liquid Scintillation Analyzer Tri-Carb 2900TR (Perkin Elmer). Nucleotides eluted at the following retention times: \( t = 3.3 \) min (CMP), 8.6 min (AMP), 10.8 min (UMP), 11.3 min (GMP), 13.4 min (AB61), and 13.6 min (AB61-MP).

**DNA digestion and HPLC-LSC analysis:** DNA was isolated from the CCRF-CEM cells treated by \([3H]AB61\) (250 nM) for 2.5 h as described above. DNA (80 µL, 500 ng/µL) was denatured at 100 °C for 10 min and cooled on ice. DNA was digested by Nuclease P1 from *Penicillium citrinum* (Sigma, 1 mg/mL, 10 µL) in 10x digestion buffer (15 µL; 800 mM Tris-HCl, pH 5.3; 100 mM NaCl; 10 mM MgCl₂; 2 mM ZnCl₂). Final volume was 150 µL. Digestion was performed at 50 °C for 16 h. Then, ice-cold aqueous TCA solution (10% v/v, 150 µL) was added. Samples were cooled on ice for 10 min. After centrifugation (14,000×g, 5 min, 4 °C), supernatant was transferred into a clear microtube. The solution was extracted with 1,1,2-trichlorotrifluoroethane-trioctylamine mixture (4:1, 300 µL) at 4 °C. The aqueous phase was evaporated under reduced pressure and dissolved in water (150 µL). The sample (50 µL) was analyzed on Waters HPLC system as described for HPLC analysis after RNA digestion. Nucleotides eluted at the following retention times: \( t = 4.2 \) min (dCMP), 11.2 min (dAMP), 12.3 min (dGMP), 13.1 min (dTMP), 13.8 min (AB61-MP), and 14.8 min (dAB61-MP). (For synthesis of dAB61-MP see Supplementary information Synthesis of nucleosides and nucleotides.)

**Authentication of AB61 metabolites incorporated into RNA and DNA:** RNA and DNA were isolated from the CCRF-CEM cells treated by AB61 (250 nM) for 2.5 h and digested by Nuclease P1 as described above. To detect individual AB61 metabolites in digested samples of DNA or RNA, respectively, individual samples were subjected to LC/MS analysis using chromatograph Ultimate 3000 (Dionex) connected to mass spectrometer QTrap 5500 (AB Sciex). LC conditions were defined as: C18 column (Kinetex 2.6 µ, 100 x 3, Phenomenex),
flow rate 400 μl/min, column oven temperature: 30 °C, separation was performed in a gradient mode of mobile phase A (10 mM ammonium acetate, pH = 5.02) and B phase (100% acetonitrile): 0 min B 10 %, 4 min 50 % B, 4.4 min 50 % B, 4.6 min 10 % B, 6 min 10 % B. Mass spectrometric analysis was performed in positive ESI mode with following conditions: G1 gas 60 psi, G2 gas 60 psi, ion source temperature 600 °C, curtain gas 20 psi, ionization voltage 5.5 kV. MRM mode was used for detection and quantification of AB61 metabolites. For AB61-MP MRM transition 451-330.9 and for dAB61-MP 435-81 were applied. Estimated concentrations of individual compounds were expressed in fmoles per 1 μg of DNA and RNA, respectively.

Metabolic labeling of nucleic acids by AB61 analogue PHN404 (incorporation, click-staining and effect of inhibition of replicative polymerases)

U2OS cells were incubated on coverslips overnight in McCoy medium (with 10% FCSI, 1.5% of L-glutamine, 100 μg/ml streptomycin and 100 U/ml of penicillin) at 37°C and 5% CO2. The next day, cells were pre-treated with medium or inhibitor of replicative DNA polymerases aphidicolin(22) (10 μM) for 1 hour. Afterwards, cells were pulsed with 10 μM of PHN404(11) or medium for 3 hours. Coverslips with cells were washed in PBS, fixed in 4% formaldehyde and permeabilized in 0.25% Triton X-100 in PBS for 15 minutes. Incorporated PHN404 was visualized via click chemistry using Click-iT Cell Reaction Buffer Kit (Thermo Fischer Scientific, cat.: C10269) and Azide-Fluor 568 fluorescent dye (Jena Bioscience, cat.: CLK-AZ106-5) as recommended by manufacturer. Nuclear DNA was counterstained with Hoechst 33342. Red fluorescence of incorporated PHN404 coupled to Azide-Fluor 568 and blue counterstained DNA were analyzed using confocal microscopy (Cell Observer SD, Zeiss, Germany).
**In vitro transcription of EGFP-luciferase DNA template**

In order to evaluate functionality of RNA with incorporated AB61-TP or Tub-TP, we first PCR amplified template DNA coding EGFP-luciferase under T7 promoter. (See Supplementary information PCR amplification of EGFP-luciferase template for T7 polymerase transcription.) MEGAscript T7 kit (Ambion) was used for *in vitro* transcription of EGFP-luciferase template in the presence of hetaryl-7-deazapurine ribonucleoside triphosphates. *In vitro* transcription reactions were performed according to manufacturer’s protocol. Each transcription reaction (20 µL) contained of EGFP-luciferase DNA template (2751 bp, 100 ng), ATP, GTP, CTP and UTP (7.5 mM each), Enzyme Mix (2 µL) and AB61-TP or Tub-TP (final concentration: 1 mM). In the control experiment water was used instead of the solution of the tested compound. The transcription reactions were performed at 37 °C for 2 h. Then, DNase I (2 U/µL, 1 µL) was added and the mixtures were incubated at 37 °C for 15 min. RNA transcripts were purified on NucAway Spin Columns (Ambion). The quantity and purity of RNA transcripts was determined on Agilent 2100 Bioanalyzer (RNA 600 Nano Total RNA kit, Agilent) and by NanoDrop 1000 Spectrophotometer.

**In vitro translation efficacy of EGFP-luciferase RNA transcripts with incorporated AB61-TP or Tub-TP**

The EGFP-luciferase RNA transcripts with or without incorporated AB61-TP or Tub-TP were used as templates for in vitro translation using reticulocyte lysate system (Retic Lysate IVT Kit, Ambion) according to manufacturer’s protocol. The *in vitro* translation mixture (25 µl) contained EGFP-luciferase RNA transcript (500 ng), Low Salt Mix-Met (1 µL), High Salt Mix-Met (0.25 µL), methionine (0.83 mM, 1.5 µL) and the reticulocyte lysate (17 µL). In the
positive control experiment unmodified EGFP-luciferase RNA transcript was used, in the negative control experiment no RNA template was added. The translation reactions were incubated in water bath at 30 °C for 1.5 h. Efficacy of in vitro translation was evaluated via measurement of luciferase enzymatic activity (Luciferase Assay System, Promega) on EnVision Multilabel Reader (PerkinElmer) and by Western blot analysis using primary goat anti-Luciferase antibody (1:1000, Promega) and secondary peroxidase-conjugated rabbit anti-goat IgG (1:10000, Sigma-Aldrich). Blot was visualized by chemiluminescence (ECL Western Blotting Detection Reagent, Amersham).

Gene expression analysis

CCRF-CEM cells (1.10^6 cells/mL, 3 mL) were incubated for 3 h in a humidified CO₂ incubator at 37 °C with or without one of the following compounds at equitoxic concentrations: AB61 (250 nM), Tub (550 nM) or actinomycin D (1 nM). Total RNA was purified from treated cell lines using TRI Reagent (Molecular Research Centre) according to the manufacturer’s instructions. The concentration and purity of RNA was assessed by Nanodrop ND 1000 (ThermoScientific). RNA quality was measured using Agilent RNA 6000 Nano Kit (Agilent Technologies). Microarray analysis using the GeneChip Human Exon 1.0 ST Array and GeneChip Scanner 3000 7G (Affymetrix) was performed. The data were analysed using R/Bioconductor freeware and additional Bioconductor packages. All arrays were preprocessed by RMA method (robust multi-array average) on the gene level using the oligo package. The processed gene expression levels were analyzed for their differential expression between the AB61-treated and control cells. Initially, the raw p values of moderated t-test were calculated by the limma package, and the data were cut at p=0.01 significance level which passed 732 genes. Genetic pathways and processes were evaluated
using the MetaCore Analytical Suite v. 6.18 (GeneGo, Thomson Reuters). Enrichment analysis consisted of mapping gene IDs of the dataset onto IDs in entities of built-in functional ontologies represented in MetaCore by pathway maps and networks prioritized according to their statistical significance. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (23) and are accessible through GEO Series accession number GSE62593 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62593).

DNA damage

A sensoric cell line derived from human osteosarcoma U2OS stable transfected with 53BP1-GFP fusion gene (U2OS-53BP1-GFP) was used to visualize DNA damage foci in the nucleus of treated/control cells. U2OS-53BP1 cells were grown in McCoy medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin at 37 °C and 5% CO₂. Cells were plated in density 10^5 cells per well into 96 well microplate in final volume of 150 µL. Following 24h incubation etoposide, irinotecan and AB61 were added to wells in final concentration of 1 µM. After drug was added cultivation of cells was continued in Operetta automated microscope (PerkinElmer) equipped with an incubation chamber. Using Operetta imaging system 21 fields were acquired from each well with conditions of 475 nm excitation and 525 nm emission main wavelength, 40x objectives and 200 ms exposure time. Images of cells were taken with the time-lapse fluorescence at 1h intervals for 12 hours. Number of 53BP1 foci was quantified using Columbus 2.4 imaging analysis system (PerkinElmer).

The validation of the DNA damage effects demonstrated using 53BP1-GFP sensoric cell line was performed on U2OS cells grown and treated under identical conditions, but fixed after 12 hours with 4% paraformaldehyde and stained for reference DNA damage marker, the
phospho-γH2AX(24), antibody: Anti-phospho–Histone H2A.X (Ser139), clone JBW301, Millipore (cat. no. 05-638), and evaluated for nuclear foci formation.

**Inhibition of protein synthesis in vivo.**

4T1-luc2 cells were expanded in vitro in DMEM medium supplemented with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and NaHCO₃. 10⁵ cells were orthoptically transplanted into right inferior mouse breast pad of syngeneic Balb/c mice (Charles Rivers). Tumor bearing mice with palpable 3-5 mm tumors were injected intraperitoneally with single dose of D-luciferin (150 mg/kg) and baseline tumor luminescence corresponding to firefly luciferase protein expression was recorded 10 minutes later using Photon Imager (Biospace lab). Then, the animals were injected intraperitoneally with vehicle or AB61 at dose corresponding to the maximum tolerated dose (MTD, 60 mg/kg). Luciferase activity was again recorded 4 hour later after 30 minute pretreatment with D-luciferin (150 mg/kg, i.p.). Cumulative luminescence [cpm.mm⁻²] was used as a surrogate indicator of luciferase protein expression in tumors.

**In vitro pharmacology**

Pharmacological properties of AB61 and AB61-MP, eg. stability in plasma, microsomes and penetration through artificial membrane, were tested under in vitro conditions.

**In vitro stability in human plasma:** Tested compound was added to 1.3 ml of preheated human plasma (Transfusion Department, University Hospital Olomouc) to yield final concentration of 3.3 μM, DMSO did not exceed 0.1%. Procaine was used as a positive control of the assay. The assay was performed at 37°C. Plasma aliquots (75 μL) were sampled at 0, 15, 30, 60 and 120 minutes and the reactions were terminated adding 150 μL of acetonitrile-methanol mixture (2:1), centrifuged at 4°C for 10 min at 4,000 rpm. Supernatant was lyophilized, then
dissolved in the 200 μL of the mobile phase and analyzed by the RapidFire RF300 system mass spectrometry (RF-MS). *In vitro* plasma half-life \((t_{1/2})\) was calculated using the equation \(t_{1/2} = 0.693/k\), where \(k\) is the slope of the natural logarithm of the percent compound remaining vs time curve. (25)

**Parallel artificial membrane permeability assay (PAMPA):** The PAMPA assay was performed with the Millipore MultiScreen filter MultiScreen-IP Durapore 0.45μm plates and receiver plates (Merck Millipore). The assay was performed according to the manufacturer’s protocol (PC040EN00). Compounds were tested at the concentration of 20 μM. Briefly, filters in each well were wetted with 10% lecithin (Sigma Aldrich) in dodecane mixture to create the artificial layer. The filter plate was placed on the top of the receiver plate, which was pre-filled with donor solutions (tested compounds dissolved in PBS at final concentration 20 μM). After incubation, 120 μL aliquots of acceptor and donor solutions were removed to the 96-well plate, lyophilized and residues were dissolved in 200 μL of the mobile phase prior the RF-MS analysis. The relative permeability \(\log P_e\) was calculated with equation: \(\log P_e = \log \{C \times \ln(1 - \text{drug}_A/\text{drug}_E)\}\); where \(C = (V_A \times V_D)/(V_D + V_A) \times A \times T\). \(V_D\) and \(V_A\) are the volumes of the donor and acceptor solutions, \(A\) is the active surface area in cm², \(T\) is time of the incubation in seconds, \(\text{drug}_A\) and \(\text{drug}_E\) is the mass of the compound in the acceptor and in the solution in theoretical equilibrium (as if the donor and acceptor were combined), respectively.

**Microsomal stability assay:** Reaction mixtures included tested compound (5 μM), human liver microsomes (ThermoFisher Scientific, 0.5 mg/ml), NADPH generating system consisting of NADP⁺, isocitrate dehydrogenase, isocitric acid and MgSO₄ in 0.1 M K₃PO₄ buffer according to the protocol. (26,27) Assay was performed at 0, 15, 30 and 60 min. Reactions were terminated using acetonitrile-methanol (2:1) mixture. Prototype stable versus non-stable drugs (propranolol and verapamil, respectively) were used as a reference. (28)
Bioanalytics - RF-MS System setup: The RapidFire RF300 system (Agilent Technologies) was interfaced with QTRAP 5500 (AB Sciex) mass spectrometer. For detailed description see (27). Samples were aspirated directly from 96-well plates into a 10-uL sample loop and passed through a C4 cartridge (Agilent Technologies) with solvent A (95% water/5% acetonitrile/0.1% formic acid) at a flow rate of 1.5 mL/min for 3 seconds. After the desalting step, analyte retained on the cartridge was eluted with solvent B (95% acetonitrile/5% water/0.1% formic acid) to the mass spectrometer at a flow rate of 0.4 ml/min for 7 seconds. The intrinsic clearance was calculated using the formula: \( CL_{int} = V \times (0.693/t_{1/2}) \), where \( V \) is the volume of incubation in μL related to the weight of microsomal protein in mg per reaction. Half-life values (\( t_{1/2} \)) were calculated using the equation \( t_{1/2} = 0.693/k \), where \( k \) is the slope of the ln of the percent compound remaining vs time curve.

Anticancer activity in human xenografts

Human colorectal cancer cell line HT-29, ovarian cancer cells SK-OV-3 and breast tumors BT-549, respectively, were grown under in vitro conditions, harvested and xenotransplanted (5.10⁶ cells/mice) subcutaneously on right flank of immunodeficient NOD SCID mice (NOD.CB17-Prkdcscid/NcrCrl, Charles Rivers). Tumor bearing mice (200-300 mm³) were treated with AB61 at dose corresponding to half maximum tolerated dose (MTD ½, 30 mg/kg) (11) for 3 or 5 consequent days per week in two to four cycles. Tumor length versus width was measured by caliperation three times per week and tumor volume was calculated. Animals were housed in Specific Pathogen Free conditions, 12 hour light/night regime, clinically examined on daily basis, water and food ad libitum. Experiments were approved by Animal Ethics Committee of the Faculty of Medicine and Dentistry, Palacký University.

Results
Synthesis of nucleosides and nucleotide analytical standards

In order to obtain analytical standards of AB61-derived metabolites, the nucleotides dAB61-MP and AB61-DP were prepared under standard conditions for Suzuki cross-coupling reaction(11,29,30) of the corresponding nucleotide or by phosphorylation of the nucleoside, respectively. [3H]-Labelled AB61 ([3H]AB61) was synthesized by Suzuki cross-coupling of 5-iodotubercidin with 5-chlorothiophene-2-boronic acid reaction followed by tritiation (Figure 2). For detailed information on synthesis see Supplementary information Synthesis of nucleosides and nucleotides.

AB61 Shows Differential In Vitro Cytotoxic Activity Against Cancer Cell Lines And Normal Fibroblasts

Cytotoxic activity of AB61 was determined using MTT assay following a 3-day incubation (Table 1). AB61 showed nanomolar IC_{50} values against cancer cell lines (A549, CCRF-CEM, HCT 116 and K-562). A strong cytotoxic effect of AB61 was also observed against paclitaxel- and daunorubicin-resistant cancer cell lines overexpressing multidrug resistance transporters (K-562-tax, CEM-DNR-bulk) and a colorectal cancer cell line with inactivated p53 gene (HCT116p53/-/). IC_{50} values of AB61 against normal lung and foreskin fibroblasts (MRC-5, BJ) were 2- to 5-orders of magnitude higher (micromolar) than the IC_{50} values against the cancer cell lines. On the other hand, cytotoxic activities of AB61-5’-O-monophosphate (AB61-MP) against cancer cell lines were always at least one order of magnitude lower than those of AB61. Interestingly, AB61-MP also showed poor selectivity towards malignant versus non-malignant cells. Its IC_{50} values against normal fibroblasts were similar to those against cancer cell lines.

Intracellular Phosphorylation of AB61 Is Limited In Normal Fibroblasts

Intracellular phosphorylation is often a key step in activation of nucleoside drugs. Efficient phosphorylation of AB61 was previously shown in a Du145 prostate cancer cell line.(11)
Intracellular levels of AB61, its nucleoside monophosphate (AB61-MP), diphosphate (AB61-DP) and triphosphate (AB61-TP) were determined by HPLC analysis after 1h and 3h treatment with AB61 (1 μM or 10 μM) in colon cancer cell line (HCT116) and in normal foreskin BJ fibroblasts (Table 2). High levels of AB61-MP were observed in HCT116 cells after 3h treatment, whereas after 1h treatment no or only a minor amount of AB61-MP was detected after treatment with 1 or 10 μM concentration of AB61, respectively. BJ fibroblasts showed good uptake of AB61 but no AB61-MP was detected, thus indicating ineffective intracellular phosphorylation of AB61 in BJ fibroblasts. AB61-DP and AB61-TP were not detected in any cell line, presumably due to short treatment time, fast incorporation into macromolecules and/or concentrations under the detection limit. Both AB61 and AB61-MP were shown to cross artificial cellular membrane in the PAMPA assay, however the membrane permeability for AB61-MP was one order of magnitude lower than that of AB61 (Table 3). Furthermore, AB61 is stable in plasma and human microsomes, whereas AB61-MP is slowly degraded with plasma with half time around 52 minutes, presumably to the parent nucleoside AB61 due to serum phosphatase activity. (Table 3)

**AB61-TP Is Incorporated into RNA and DNA by T7 RNA Polymerase and by Klenow Fragment of DNA Polymerase I and DNA Polymerase γ, Respectively**

Enzymatic incorporation of AB61-TP into RNA was tested using viral T7 RNA polymerase. The 40-mer dsDNA template contained two 2’-OMe-RNA nucleotides at the 5’-end of the noncoding strand to avoid n+1 activity of the T7 RNA polymerase.(31) The template is transcribed into a 21 bp RNA transcript containing four ATP insertions. The transcription reactions performed in presence of either ATP, AB61-TP or tubercidin 5’-O-triphosphate (Tub-TP) and three natural NTPs (GTP, UTP and CTP) afforded a full length transcript (Figure 3A). The results show that both AB61-TP and Tub-TP are substrates of T7 RNA polymerase and are able to substitute for ATP during transcription and to be incorporated into
RNA. Furthermore, these experiments provided evidence that T7 RNA polymerase is able to further elongate the transcript after incorporation of AB61-TP. Our studies also focused on enzymatic incorporation of AB61-TP into DNA in a primer extension experiment with bacterial DNA polymerase I (Klenow fragment) and a 31-bp DNA template allowing incorporation of four dATPs. A primer extension reaction with AB61-TP instead of dATP led to formation of a full length product, confirming that AB61-TP was successfully incorporated into DNA without causing the polymerase to pause. In contrast, Tub-TP was not incorporated by Klenow fragment (Figure 3B). Incorporation of AB61-TP by commercially available human DNA polymerases were also tested using a primer extension experiment. While AB61-TP is not a substrate for DNA polymerase β (Figure 3C), AB61-TP was successfully incorporated by DNA polymerase γ (Figure 3D). Incorporation of AB61-TP by DNA polymerase γ was also confirmed by MALDI-TOF analysis (See Supplementary information Figure S1).

**AB61 Is Incorporated into RNA and DNA in cancer cell lines**

Further studies focused on incorporation of AB61 into RNA and DNA in living cells. CCRF-CEM cells were incubated with 3H-labeled AB61 ([3H]AB61) (250 nM), cellular RNA and DNA were isolated and activities of the RNA and DNA samples were measured using liquid scintillation counting (LSC) (Table 4). Significant increases in activities were observed for both RNA and DNA samples after 2.5h treatment. In order to further confirm the incorporation of AB61, RNA and DNA samples from cells treated with AB61 or [3H]AB61 (250 nM) were digested with nuclease P1 affording nucleoside monophosphates. The hydrolyzed RNA and DNA samples were analyzed by HPLC-MS (Table 5) and HPLC with UV/VIS detection and radioactivity of HPLC fractions was determined by LSC (Figure 4). Figures 4A,D show authentic analytical standards of ribo- or deoxyribonucleos(t)ides, respectively. After digestion of RNA, peaks of all natural NMPs were identified in the HPLC
chromatogram (Figure 4B). Three peaks were observed in HPLC-LSC analysis (Figure 4C). One of the peaks corresponds with standard of AB61-MP confirming the incorporation of [3H]AB61 into RNA, while the two other peaks are presumably related to short [3H]AB61-containing oligonucleotides that were formed due to incomplete digestion of the RNA sample. The presence of AB61-MP was also confirmed by LC-MS. Peaks of all natural dNMPs were found in the HPLC chromatogram of the digested DNA sample (Figure 4E). A single peak was observed after digestion of DNA sample by the HPLC-LSC analysis (Figure 4F). This peak clearly corresponds to ribonucleotide AB61-MP. In the LC-MS analysis of DNA isolated from AB61-treated cells, AB61-MP was detected as a major component, but a minor presence of the corresponding 2’-deoxyribonucleotide (dAB61-MP) was also observed. The molar ratio of AB61-MP and dAB61-MP was approx. 30:1 (Table 5). The results show that AB61 is mainly incorporated into DNA as a ribonucleotide although 2’-deoxyribonucleotide is also present in residual quantities. On the contrary, only AB61-MP was detected in the RNA of the treated cells. In order to localize incorporation of AB61 in cells and to further investigate the mechanisms of incorporation into the DNA, we have employed structural analogue of AB61, compound PNH404 (Figure 1) (11), which contains alkyne group available for azide-alkyne CuAAC cycloaddition of fluorescent dye Azide-Fluor 568 (“click chemistry”).(32) In U2OS cells, PNH404 was incorporated predominantly into nuclei of treated cells with residual staining in the cytoplasm. Interestingly, the incorporation of the compound was not significantly inhibited by inhibitor of replicative DNA polymerases aphidicolin(22) (Figure 5).

**Gene expression analysis suggests AB61 effects DNA damage pathways and protein translation/folding machinery**

To evaluate the biological activity of the AB61 more broadly we performed transcriptional microarray profiling coupled to the pathway analysis. Based on the differentially expressed
genes the “DNA Damage_DBS Repair” network process was identified as the most significant process network following the AB61 treatment. Furthermore, other networks indicating significantly affected protein folding and translation, cell cycle and angiogenesis were top ranked. Upregulation of BRIP1, RPA4, FANCF, TOP1 and downregulation of TdT, FANCG, LIG4, LIG3, NRB54, Chk2, MGMT, RAD51B, Histone H4, Ubiquitin and C1D genes which are well known to be involved in DNA damage processes was observed. This corresponds well to the cell biology data (vide infra). Reference compounds, tubercidin (Tub) and actinomycin D (ActD), with structural or mechanistic similarity to AB61, were used. The networks involved in „development_regulation of angiogenesis“ and „development&blood vessel morphogenesis“ were similarly affected, however, the DNA damage, cell cycle alteration and protein translation and folding were not among top listed networks in cells treated with ActD and Tub (Table 6).

**Induction of DNA damage in AB61 treated cells**

To confirm AB61-induced DNA damage by an independent method, the U2OS human osteosarcoma cell line was stably transfected with the 53BP1-GFP(20) fusion gene. 53BP1 protein accumulates at DNA lesions, preferably at DNA double strand breaks, thus its GFP-conjugate enables visualization of DNA-damage sites in nucleus. Indeed, U2OS-53BP1-GFP cells treated with AB61 or control DNA-damaging agents like topoisomerase I or II inhibitors (irinotecan and etoposide, respectively) showed time-dependent accumulation of 53BP1 foci in the nuclei of treated cells (Figure 6). Although the absolute number of 53BP1 foci in AB61-treated cells (Figure 6B) was lower than in irinotecan- and etoposide-exposed cells (Figure 6C-E), the foci were markedly larger compared to both irinotecan- and etoposide-treated versus untreated control cells (Figure 6A). These results were independently validated using phospho-γH2AX staining in U2OS cells treated with AB61, etoposide or irinotecan, respectively (Figure 6F-I).
Incorporation of AB61-TP into mRNA Blocks Its Translation In Vitro

Following the evidence of AB61-TP and Tub-TP incorporation into mRNA, we wanted to evaluate functional properties of the RNA harboring ribonucleoside analogues in terms of translational efficacy. A template with a model EGFP-luciferase fusion gene under T7 promoter was transcribed into RNA. The transcription experiments were performed in the presence of all four natural NTPs with/without AB61-TP or Tub-TP so that AB61-TP or Tub-TP could be randomly incorporated into the RNA transcripts. After the in vitro transcription, the template DNA was digested by DNase I and the RNA transcripts were purified. In the next step the RNA transcripts were used as templates for in vitro translation using a commercial reticulocyte lysate in vitro translation system. The resulting EGFP-luciferase protein in the translation reactions was detected based on its enzymatic luciferase activity and western blotting (Figure 7). The results showed that the translation reactions with unmodified RNA template and RNA template produced in the presence of Tub-TP afforded similar amounts of EGFP-luciferase. In contrast, synthesis of EGFP-luciferase protein from RNA template produced in the presence of AB61-TP was completely blocked, as evidenced from both the measurement of luciferase enzymatic activity and protein content (Figure 7). This result shows that AB61-TP can be incorporated into RNA by T7 RNA polymerase in the presence of ATP. Furthermore, the presence of AB61 in the RNA hampers its function as a template for the translation. This effect was not observed for Tub-TP and to our knowledge it is the first description of such an effect in the class of cytostatic nucleosides.

AB61 inhibits protein expression and tumor growth in vivo

To evaluate the effect of AB61 on luciferase activity/expression in vivo, Balb/c mice were transplanted with the 4T1-Luc2 breast cancer cells stably transfected with luciferase under strong CMV promoter and bioluminescence imaging technique was employed to visualize and quantify the luciferase (Figure 8). Indeed, the tumor bearing mice treated with AB61 (MTD,
60 mg/kg) intraperitoneally demonstrated 4 hours later significant decrease of luciferase activity in vivo as compared to vehicle treated mice. In vivo anticancer activity of AB61 was determined using human ovarian (SK-OV-3), breast (BT-549) and colorectal (HT-29) tumors xenografted to the NOD SCID mice (Figure 9). Animals were treated with MTD½ (30 mg/kg) of AB61 in 3 (low-intensity) or 5 consequent days per week (high-intensity scheme) in two to four cycles. In all cancer models employed, highly significant reduction of tumor volume was observed in treated mice. However, significant reduction of tumor volume in ovarian cancer model SK-OV-3 was only observed with high intensity scheme suggesting dose dependent effect. AB61 also significantly prolonged survivals in BT-549 and HT-29 models, but not in the SK-OV-3 due to no efficacy in the low-intensity administration schedule and toxicity in the high-intensity regimen.

**Discussion**

In this study we focused on elucidation of the mechanism of action of a novel 7-deazaadenine analogue, AB61. Despite the very strong cytotoxic effect of AB61 against cancer cell lines we found out that cytotoxic activity of AB61-MP against cancer cell lines was always lower (typically by 2 orders of magnitude) than that of AB61. The main reason is probably in lower (by one order of magnitude) permeability of the monophosphate AB61-MP through cell membrane compared to nucleoside AB61 (as confirmed by PAMPA assay) but we can also hypothesize on involvement of membrane efflux and/or complex phosphorylation/dephosphorylation dynamics. On the other hand, AB61-MP showed higher cytotoxicity against normal fibroblasts compared with AB61, which was comparable to cytotoxicity in cancer cell lines. The PAMPA test confirmed that AB61-MP can still relatively well penetrate through membrane (though less efficiently than nucleoside AB61) causing the non-specific cytotoxicity. Apparently, intracellular availability of AB61-MP
increases the cytotoxic effect. Our further results demonstrated that AB61 is phosphorylated in HCT116 colon cancer cell line (cytotoxicity IC_{50} = 1.9 nM) but the phosphorylation does not proceed in normal BJ fibroblasts (cytotoxicity IC_{50} = 8 \mu M). This data is in accordance with the fact that expression of adenosine kinase, the enzyme that might be involved in phosphorylation of AB61 is increased in colorectal tumors compared to the normal tissue.\textsuperscript{(33,34,35)} These findings reveal the importance of intracellular phosphorylation for both the efficiency and cancer selectivity of AB61 and suggests for potentially synergistic combinations with therapies increasing nucleoside phosphorylation rates in tumors, e.g. radiation\textsuperscript{(36)}. Further we showed that AB61-TP is effectively incorporated into RNA by T7 RNA polymerase in an enzymatic assay. Although AB61-TP is not accepted as a substrate by human DNA polymerase α(12b) and β, both human DNA polymerase γ and Klenow fragment successfully incorporated AB61-TP into DNA, even though it is not a 2'-deoxyribonucleotide but a ribonucleotide. However, our experimental design for AB61-TP incorporation by DNA polymerase β did not contain a single nucleotide gap substrate, which is a typical site of polymerase β action\textsuperscript{(37)}, nor accessory proteins involved with polymerase β activity (PARP, XRCC1)\textsuperscript{(38)}, and therefore the incorporation of AB61-TP might not have been a suitable substrate in the cell free system we have used. In contrary, Tub-TP is accepted neither by the human DNA polymerase α (12b) nor the Klenow fragment. Both DNA polymerase γ and Klenow fragment represent A family DNA polymerases. Despite the fact that DNA polymerase γ is a mitochondrial polymerase and Klenow fragment is a bacterial enzyme, we hypothesize that AB61-TP might be also incorporated by nuclear DNA polymerases from the A family, i.e. DNA polymerase θ and DNA polymerase ν which are both involved in DNA repair processes and are known for lower fidelity.\textsuperscript{(39)} Also treatment of CCRF-CEM cells with AB61 or [\textsuperscript{3}H]AB61 resulted in its incorporation into both RNA and DNA. HPLC-MS analysis of DNA lysates revealed that AB61 is incorporated into DNA predominantly as a
ribonucleotide and only rarely as a 2’-deoxyribonucleotide (30:1 ratio) which means that AB61-TP is a substrate for human DNA polymerases. Despite this finding might seem surprising, ribonucleotide incorporation into cellular DNA is quite common. (40,41,42) By contrast, tubercidin (2) and toyocamycin (3) are incorporated into DNA only after reduction by ribonucleotide reductase as 2’-deoxyribonucleotides. Consistently with mass-spectrometry data, PNH404, the ethynyl-analogue of AB61, was detected in nuclei and cytoplasm of treated U2OS cells, which is in agreement with incorporation of AB61 into DNA and RNA, respectively. Interestingly, the incorporation of AB61 analogue into nuclei was not affected by the inhibitor of replicative DNA polymerases aphidicolin, indicating (in accord with previous data) that incorporation is rather dependent on non-replicative, e.g. reparative DNA polymerases.(39) To see a broader picture of the AB61 induced changes, we performed microarray gene expression profiling, which, in principle, suggested DNA damage repair networks and translational or folding machinery are primarily affected in cellular response to the AB61. Several genes involved in DNA damage and/or repair mechanisms were altered. However, the affected networks are only partially overlapping with reference compounds tubercidin and actinomycin D, suggesting a different mechanism of action. In order to validate DNA repair pathway at the cellular level, we used the 53BP1-GFP reporter U2OS cell line. Protein 53BP1 is known to rapidly accumulate at DNA double strand breaks leading to activation of DNA repair mechanisms.(43) After treatment with AB61 formation of large 53BP1-GFP foci was observed in a time dependent manner indicating that AB61 induces DNA damage. However, the pattern of 53BP1-GFP nuclear foci is different from the DNA-damage patterns induced by topoisomerase inhibitors irinotecan, etoposide or p53 activator 7-iodotubercidin (44) which suggests that DNA damage caused by AB61 is achieved, maintained or (un)repaired via different mechanisms. In addition to the 53BP1-GFP accumulation in the DNA damage sites, similar staining pattern was independently confirmed
for phospho-γH2AX, one of the most robust surrogate markers of DNA double strand breaks. Since i) **AB61-TP** is not a substrate for human polymerase α(12b) or polymerase β, ii) incorporation of **AB61** analogue into cell nuclei was not decreased upon inhibition of replicative but not reparative DNA polymerases by aphidicolin, iii) gene expression study revealed activation of DNA damage pathway, and vi) formation of unusually large 53BP1 and/or phospho-γH2AX DNA damage foci in treated tumor cells was observed, we hypothesize that **AB61** is incorporated into DNA by polymerases involved in the repair mechanisms, thus further aggravating the local DNA damage in a positive back-loop manner and thereby inducing stronger signal for 53BP1 and/or phospho-γH2AX to accumulate.

We had previously reported poor inhibition of RNA polymerase II by **AB61-TP**.(11) Having the evidence that **AB61** is incorporated into RNA of exposed cells, the RNA polymerase II is not inhibited, but the RNA synthesis is down regulated, we focused on the possible consequences of **AB61** incorporation into mRNA. We studied in vitro translation of RNA templates containing randomly incorporated **AB61**. We discovered that the incorporation of **AB61** into mRNA template completely prevents formation of a protein. This result was further confirmed in mice by the observation of significant and rapid inhibition of luciferase protein expression in **AB61**-treated 4T1-luc tumors. By contrast, even though **Tub-TP** is incorporated into RNA by T7 RNA polymerase, no effect on in vitro translation was observed. In vivo studies of **AB61** in human colorectal (HT-29), ovarian (SK-OV-3) and breast (BT-549) cancer xenografts revealed highly significant reduction of tumor mass in treated animals, which was reflected with increased survival preferentially in low-density regimen. Despite better tumor control of the high-intensity schedule, it was not always translated into increased survival due to cumulative therapeutic toxicity with leading symptoms of body weight lost and anorexia. Interestingly, the highest in vivo activity of the compound was demonstrated in slowly growing HT-29 tumors, suggesting preferential
anticancer activity in slowly proliferating tumor cells. Therefore, the in vivo anti-cancer effect of AB61 is very promising and warrants further studies.

Most of the nucleosides used in cancer therapy are incorporated into DNA leading to termination of nascent DNA strand extension and DNA damage response activation.(45) In this point of view the mechanism of action of AB61 is more complex. The study clearly showed that the cytotoxic effect of AB61 is tightly linked with its intracellular phosphorylation and the inefficient phosphorylation in non-malignant cells is a key factor of AB61 selectivity towards cancer cell lines. AB61 is incorporated both into DNA and RNA as a ribonucleotide (and only in trace amounts as a 2’-deoxyribonucleotide into DNA) where it leads to DNA damage and protein translation arrest, respectively. Rapid inhibition of translational machinery may also explain previously observed down-regulation of RNA and DNA synthesis as a secondary effect.(11) In spite of the structural similarity of AB61 with tubercidin, its mode of action seems to be unique and different from the one of gemcitabine, the only clinically used nucleoside cytostatic for treatment of solid tumors.(46) The mechanism targeting at the same time DNA damage/repair and inhibition of protein translation machinery might be highly relevant for elimination of slowly proliferating, dormant and senescent tumor cells, which are not cycling enough thus highly resistant to conventional chemotherapy and radiation. In summary, we showed that AB61 is a potent and selective cytostatic with exceptional mechanisms of action. Further pharmacological studies of AB61 are currently under way.

Acknowledgement
We would like to thank Lenka Radova and Josef Srovnal for the help with the microarray experiments and data analysis and Natalie Taborska for the help with in situ metabolic labelling.

References


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Tables

Table 1: Cytotoxic activity of AB61 and its 5'-monophosphate AB61-MP

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AB61</th>
<th>IC₅₀ (μM)</th>
<th>AB61-MP</th>
<th>IC₅₀ (μM)</th>
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<tr>
<td>A549</td>
<td>0.010</td>
<td></td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>0.00036</td>
<td></td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>CEM-DNR-bulk</td>
<td>0.043</td>
<td></td>
<td>0.45</td>
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<td>HCT 116</td>
<td>0.0019</td>
<td></td>
<td>0.18</td>
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<td>0.067</td>
<td></td>
<td>0.83</td>
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<td></td>
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<td>MRC-5</td>
<td>11.4</td>
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<td>7.84</td>
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*a The data presented here are from fresh retesting

Table 2: Intracellular phosphorylation of AB61 at various concentrations

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<tr>
<th>Cell line</th>
<th>AB61</th>
<th>AB61-MP</th>
<th>AB61</th>
<th>AB61-MP</th>
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<tr>
<td></td>
<td>(pmol/5·10⁵ cells)</td>
<td>(pmol/5·10⁵ cells)</td>
<td>(pmol/5·10⁵ cells)</td>
<td>(pmol/5·10⁵ cells)</td>
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<tr>
<td>Compound</td>
<td>In vitro t1/2 (min)</td>
<td>% compound remaining</td>
<td>PAMPA</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>-------</td>
<td></td>
</tr>
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<td></td>
<td>15 min</td>
<td>30 min</td>
<td>60 min</td>
<td>120 min</td>
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<td>no</td>
<td></td>
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<tr>
<td>AB61</td>
<td>degradation observed</td>
<td>98.6±11.7</td>
<td>102.8±5.7</td>
<td>104.5±13.6</td>
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<tr>
<td>AB61-MP</td>
<td>51.9±3.9</td>
<td>76.6±8.4</td>
<td>51.4±5.2</td>
<td>25.4±1.3</td>
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Table 3: Plasma and microsomal stability and parallel artificial membrane permeability assay (PAMPA) for AB61 and its 5’-monophosphate AB61-MP.

<table>
<thead>
<tr>
<th>Compound</th>
<th>In vitro CLint (μL/min/mg protein)</th>
<th>Clearance categoryb</th>
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<tbody>
<tr>
<td>AB61</td>
<td>6.6±1.7</td>
<td>low</td>
</tr>
</tbody>
</table>
AB61-MP 9.4±7.1 medium

\(^a\) According to the logPe obtained from the reference drugs, compounds with logPe > -7 were categorized as highly permeable, while those with logPe < -7 were considered as poorly permeable.

\(^b\) Reference (28)

Table 4: Incorporation of \[^{3}H\]AB61 into RNA and DNA of living cells (Data from 3 independent experiments)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>RNA</th>
<th>DNA</th>
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<tr>
<td>0</td>
<td>0.5 ± 0.06</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>2.5</td>
<td>25.6 ± 1.6</td>
<td>80.9 ± 2.3</td>
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</table>

Table 5: Incorporation of AB61 into RNA and DNA samples from CCRF-CEM cells after 2.5h treatment and subsequent digestion with nuclease P1 (Data from 3 independent experiments)

<table>
<thead>
<tr>
<th>Sample</th>
<th>AB61-MP [fmol/μg of nucleic acid]</th>
<th>dAB61-MP [fmol/μg of nucleic acid]</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>1.68±0.5</td>
<td>n.d.(^a)</td>
</tr>
<tr>
<td>DNA</td>
<td>0.667±0.28</td>
<td>0.021±0.0103</td>
</tr>
</tbody>
</table>

\(^a\): not detected
Table 6: Transcriptomic microarray analysis of AB61, tubercidin (Tub) and actinomycin D (Act D) cellular effects.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Process networks</th>
<th>log(p-value)</th>
<th>Networks objects</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB61</td>
<td>DNA damage_DBS repair</td>
<td>-4.81</td>
<td>↑ BRIP1, RPA4, FANCE, TOP1;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ TdT, FANCG, LIG4, LIG3, NRB54,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chk2, MGMT, RAD51B, Histone H4,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ubiquitin, C1D</td>
</tr>
<tr>
<td></td>
<td>Protein folding_Protein folding nucleus</td>
<td>-4.46</td>
<td>↑ PSMD4; ↓ RBBP7, SFRS4, HSP90,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>DJ-1, YY1, OSP94, NPM1, FKBPA,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AUF1</td>
</tr>
<tr>
<td></td>
<td>Cell cycle_S phase</td>
<td>-3.05</td>
<td>↑ BRIP1, TOP1, PLK1; ↓ SMC1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ORC6L, LIG4, HSP90, Chk2, UHRF2,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Geminin, Histones (H1, H1.5, H4),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ubiquitin</td>
</tr>
<tr>
<td></td>
<td>Development_regulation of angiogenesis</td>
<td>-2.19</td>
<td>↑ S2P, CEACAM1; ↓ PKC, Cathepsin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B, ITGB1, HB-EGF, HMDH, ID1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>VEGF-A, Ephrin-B, Ephrin-B2,</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Calnexin, CXCR4, XCR1, CCR10,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AKT</td>
</tr>
<tr>
<td></td>
<td>Translation_Translation initiation</td>
<td>-2.07</td>
<td>↑ eIF4G2, RPS27A, eIF5; ↓ RPS2,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPS24, RPS10, RPS25, PAIP2, eI3S7,</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>RPL21, RPS3A, RPS30, RPL17</td>
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<tr>
<td>Tub</td>
<td>Development_Blood vessel morphogenesis</td>
<td>-4.51</td>
<td>↑ PDE7A, PDE, CCR10, ANG1, Elk-3,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A2aR; ↓ TERT, KLF5, HB-EGF, AKT,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CXCR4, XCR1, S1PR1, S1PR3,</td>
</tr>
<tr>
<td>Pathway</td>
<td>Signature</td>
<td>Upregulated Genes</td>
<td>Downregulated Genes</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------------------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Apoptosis_Endoplasmic stress pathway</td>
<td>-2.89</td>
<td>↑ CREB-H, S2P, eIF5; ↓ Bim, XBP1</td>
<td>eIF3S7, HERP, NUR77</td>
</tr>
<tr>
<td>Signal Transduction_BMP and GDF signaling</td>
<td>-2.83</td>
<td>↑ CD44, CREB1, SMURF2, RUNX2</td>
<td>↓ YY1, ID1, AKT, PP2A</td>
</tr>
<tr>
<td>Proliferation_Negative regulation of cell</td>
<td>-2.81</td>
<td>↑ CCR10, Prohibitin, WARS; ↓ GAB1</td>
<td>Mxi1, RBBP7, IGF1R, MNT, TOB2</td>
</tr>
<tr>
<td>Development_regulation of angiogenesis</td>
<td>-2.57</td>
<td>↑ CCR10, ANG1, S2P, CREB1</td>
<td>↓ PKC, HB-EGF, ID1, AKT, CXCR4, XCR1, VEGF-A, Ephrin-B, Ephrin-B2</td>
</tr>
<tr>
<td>Proteolysis_Ubiquitin-proteasomal proteolysis</td>
<td>-3.41</td>
<td>↑ PSMA2, Rnf14, PSMB2, SENP1</td>
<td>SMURF2, SMURF, LNX2, PSME3</td>
</tr>
<tr>
<td>Proteolysis_Ubiquitin-proteasomal proteolysis</td>
<td></td>
<td>↓ BLMH, TRC8, Ubiquitin, BAG-1</td>
<td>NEDD4L</td>
</tr>
<tr>
<td>Signal Transduction_BMP and GDF signaling</td>
<td>-3.23</td>
<td>↑ RUNX2, CD44, SMURF2, SMAD1</td>
<td>↓ ATF-2, PP2A, YY1, ID1, AKT</td>
</tr>
<tr>
<td>ActD</td>
<td></td>
<td>↑ s2P; ↓ PKCA, PKC, Cathepsin B</td>
<td>ID1, Ephrin-B, Ephrin-B2, AKT</td>
</tr>
<tr>
<td>Development_regulation of angiogenesis</td>
<td>-2.26</td>
<td>ID1, Ephrin-B, Ephrin-B2, AKT</td>
<td>PLAUR, CXCR4, XCR1, CCR10, VEGF-A</td>
</tr>
<tr>
<td>Development_Blood vessel morphogenesis</td>
<td>-2.18</td>
<td>↑ PDE, PDE7A, Elk-3; ↓ PKCA</td>
<td>S1PR1, S1PR3, AKT, CCR10, AREG</td>
</tr>
</tbody>
</table>
Neuro physiological process_Croticoliberin signaling

-2.06 ↓ cPKC, PKCA, NUR77, EGR1, NURR1

*: Only the most affected networks from the pathway analysis are listed; ↑: upregulated genes; ↓: downregulated genes

**Figure captions:**

Figure 1: Structures of 7-deazapurine nucleosides.

Figure 2: Structures of nucleosides and nucleotides used in this study.

Figure 3: Enzymatic incorporation of AB61-TP and Tub-TP into RNA and DNA. A) Denaturing PAGE analysis of in vitro transcription reactions with T7 RNA polymerase. Key: +, all natural NTPs; A-: GTP, CTP, UTP; AB61: \textbf{AB61-TP}, GTP, CTP, UTP; Tub: \textbf{Tub-TP}, GTP, CTP, UTP. B) Primer extension experiment with Klenow fragment. Key: p: $^{32}$P-labelled primer; +: dATP, dCTP, dTTP, dGTP; A-: dCTP, dTTP, dGTP; AB61: \textbf{AB61-TP}, dCTP, dTTP, dGTP; Tub: \textbf{Tub-TP}, dCTP, dTTP, dGTP; C: Primer extension experiment with human DNA polymerase β. Key: p: $^{32}$P-labelled primer; +: dATP; A-: without dATP; AB61: \textbf{AB61-TP}; D: Primer extension experiment with human DNA polymerase γ. Key: p: $^{32}$P-labelled primer; +: dATP; A-: without dATP; AB61: \textbf{AB61-TP}.

Figure 4: Incorporation of $[^3]$HAB61 into RNA or DNA in CCRF-CEM cells exposed to the drug for 2.5 hrs. A) HPLC analysis of NMP standards. B) HPLC analysis of nuclease-P1-digested RNA. C) HPLC-LSC analysis of nuclease-P1-digested RNA sample after 2.5h

Figure 5: Visualization of incorporation of AB61 analogue (PNH404) in situ by clicky staining with Azide-Fluor 568 fluorescent day (red fluorescence) in treated U2OS cells (250 nM, 2.5 hours) with DNA counterstained with Hoechst 33342 (blue fluorescence) compared to untreated cells (control). Confocal microscopy demonstrates specific and preferential accumulation of PNH404 in nuclei and partially in the cytoplasm of the cells. Inhibition of replicative DNA polymerases by aphidicolin (10 μM) did not decrease neither incorporation rate nor subcellular localization of the AB61 analogue.

Figure 6: 53BP1 foci in U2OS-53BP1-GFP cells exposed to vehicle (A), AB61 (B), irinotecan (C) or etoposide (D) for 12h. E) Time-dependence of 53BP1 foci formation during treatment with AB61, irinotecan and etoposide. Phospho-histone γH2AX foci in U2OS cells exposed to vehicle (F), AB61 (G), irinotecan (H) or etoposide (I) for 12h. All compounds were tested at 1 μM concentration. Nuclei contours are highlighted by dashed line for convenience.

Figure 7: In vitro translation reactions with different RNA templates for EGFP-luciferase reporter gene. A) luciferase activity, data represent averages of two independent experiments; B) western blot analysis using anti-luciferase antibody. Key: +: unmodified RNA template; -: no template; AB61: RNA template produced in the presence of AB61-TP; Tub: RNA template produced in the presence of Tub-TP.
Figure 8: Tumor luciferase activity in mice bearing 4T1-luc2 tumors 0h and 4h after treatment with AB61. A) Mice treated by vehicle at time 0 h and B) 4 hours later; C) mice treated by AB61 (60 mg/kg), time 0 h and D) 4 hours after the administration. E) Graph representation of tumor luminescence before (0 h) or after (4 h) timepoints of treatment by AB61. Whiskers represent lowest and highest data within 1.5 interquartile range. Outliers are not shown. **: p<0.01 Data was analyzed by paired t-test.

Figure 9: In vivo antitumor activity of AB61. Mice were treated daily with AB61 (30 mg/kg). Tumor volume of human ovarian SK-OV-3 (A), breast BT-549 (B) and colorectal HT-29 (C) xenografts in SCID mice, dosing in three consecutive days. Survival of mice with SK-OV-3 (D), breast BT-549 (E) and colorectal HT-29 (F) xenografts, dosing in three consecutive days. Tumor volume of human ovarian SK-OV-3 (G), breast BT-549 (H) and colorectal HT-29 (I) xenografts in mice, treatment regimen for five consecutive days. Survival of mice with SK-OV-3 (J), breast BT-549 (K) and colorectal HT-29 (L) xenografts, treatment regimen for five consecutive days. Dashed line: vehicle; solid line: AB61 (30 mg/kg). Bold lines indicate therapeutic regimen and individual cycles.
Figure 1

Tubercidin, $R = H$
Toyocamycin, $R = CN$
Sangivamycin, $R = \text{CONH}_2$

2, $R = \text{hetaryl}$

$1$, $R = \text{het}$

AB61, $R = 2$-thienyl
PNH404, $R = \text{ethynyl}$
Figure 2

AB61-MP

AB61-DP

AB61-TP

dAB61-MP

Tub-TP

$[^3]H$AB61
Figure 3

A) 21-mer

[alpha-\textsuperscript{32}P]GTP

+ A- AB61 Tub

B) GATCGTACTCGAGTCT

p + A- AB61 Tub

C) D)
Figure 4
Figure 5

<table>
<thead>
<tr>
<th>Azide-fluor 568</th>
<th>Hoechst 33342</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNH404</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNH404 aphidicolin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

A) Luminescence [cpm]

B) + - AB61 Tub

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Figure 8
Figure 9
Molecular Cancer Therapeutics

7-(2-Thienyl)-7-deazaadenosine (AB61), a new potent nucleoside cytostatic with a complex mode of action

Pavla Perlíková, Gabriela Rylová, Petr Naus, et al.

Mol Cancer Ther Published OnlineFirst January 27, 2016.

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