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Alpha anomer of 5-aza-2′-deoxycytidine down-regulates *hTERT* mRNA expression in human leukemia HL-60 cells

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ABSTRACT

DNA methylation inhibitors are being extensively studied as potential anticancer agents. In the present study, we compared the capability of alpha anomer of 5-aza-2′-deoxycytidine (α-5-azadCyd) to induce down-regulation of *hTERT* expression in HL-60 cells with other nucleoside analogs that act as DNA methylation inhibitors: β-5-azadCyd (decitabine), (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA], isobutyl ester of (*R*,*S*)-3-(adenin-9-yl)-2-hydroxypropanoic acid [(*R*,*S*)-AHPA-*ibu*] and prospective DNA methylation inhibitors (*S*)-1-[3-hydroxy-2-(phosphonomethoxy)propyl]-5-azacytosine [(*S*)-HPMPazaC] and 5-fluoro-zebularine (F-PymRf). Exposure to α-5-azadCyd induced the down-regulation of *hTERT* expression in low micromolar concentrations (0.05–50 μM). A more cytotoxic beta anomer caused a transient up-regulation of *hTERT* and a subsequent reduction in *hTERT* mRNA levels at concentrations more than 10 times below its GIC_{50} value. In this respect, (*S*)-DHPA and (*R*,*S*)-AHPA-*ibu* were less efficient, since a similar effect was achieved at concentrations above their GIC_{50}. In contrast, F-PymRf treatment resulted in up to a three-fold induction of *hTERT* expression within a broad range of concentrations. In all cases, the down-regulation of *hTERT* expression was concentration-dependent. The correlation was found between c-*myc* overexpression and transiently elevated *hTERT* expression after treatment with all tested compounds except for α-5-azadCyd and (*S*)-HPMPazaC. Although the primary task of hypomethylating agents in anticancer therapy lies in reactivation of silenced tumour-suppressor genes, the inhibition of *hTERT* expression might also be a fruitful clinical effect of this approach.

1. Introduction

Telomerase is a ribonucleoprotein complex that elongates the protective structures at the ends of eukaryotic chromosomes, called telomeres [1]. Thus, the enzyme counteracts the telomere erosion caused by the end-replication problem [2]. Human telomerase activity is present in a majority of cancer cells [3] and requires the up-regulation of the catalytic protein subunit referred to as *hTERT* [4]. As the *hTERT* promoter is situated in a CpG island, DNA methylation has been suggested

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Abbreviations: GIC_{50}; concentration which causes 50% growth inhibition; *hTERT*; human telomerase reverse transcriptase; CTCF; CCCTC binding factor; 5-azadCyd; 5-aza-2′-deoxycytidine; 5-azaCyd; 5-azacytidine; HAT; histone acetyltransferase; SAH; S-adenosyl-L-homocysteine; SAM; S-adenosyl-L-methionine; GAPDH; glyceraldehyde-3-phosphate-dehydrogenase; RPII; RNA polymerase II; G6PDH; glucose-6-phosphate dehydrogenase; TBP; TATA box-binding protein; PBGD; porphobilinogen deaminase; PLA; phospholipase A2; Act; β-actin.

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to be involved in the *hTERT* transcriptional regulation in normal and cancer cells [5,6].

Several studies reported a hypermethylation of the *hTERT* promoter in telomerase-positive tumours and a hypomethylation in telomerase-negative normal tissues [6–8] suggesting a role for methylation in the blocking of negatively-acting transcription factors. It was shown that CTCF, an ubiquitous methylation-sensitive repressor, binds to GC-rich proximal exonic region of *hTERT* and inhibits *hTERT* gene transcription when the *hTERT* CpG island is not methylated, irrespective of the cell type [9,10]. Hypermethylation of its binding site prevents binding of CTCF and can abolish CTCF repressor activity [11]. Renaud et al. [11] hypothesizes that hypomethylation with β-5-azadCyd allows CTCF to bind to the first exon and inhibits *hTERT* expression. Several other groups also observed that β-5-azadCyd and/or 5-azaCyd treatment of telomerase-positive cells caused a down-regulation of *hTERT* expression in several cancer cell lines [12–14]. This compound was also shown to activate *p16* and other methylated tumor-suppressor genes [15]. Kitagawa et al. [14] indicated that up-regulation of *p16* and subsequent down-regulation of c-*myc* might be another possible pathway for *hTERT* repression by 5-azaCyd. c-Myc protein is one of the transcription factors that activate expression of *hTERT* through binding on enhancer box sequences (E-boxes) and recruiting histone acetyltransferases (HATs) [16,17]. c-*Myc* is a very strong proto-oncogene and it is very often found to be up-regulated in many types of cancers. The first to be discovered was its capability to drive cell proliferation (up-regulates cyclins, down-regulates *p21*) [18], but it also plays a very important role in cell growth regulation (up-regulates ribosomal RNA and proteins) [19], apoptosis, differentiation and stem cell self-renewal [20].

5-Aza-2′-deoxycytidine (β-5-azadCyd, decitabine) has been recently approved for the treatment of myelodysplastic syndromes. This compound, after activation by cellular kinases, is incorporated into DNA, where it produces an irreversible inactivation of DNA methyltransferase [21]. Less cytotoxic and more stable alpha anomer appeared to hypomethylate genomic DNA to a similar extent as the widely used beta form [22]. α-5-AzadCyd itself is not incorporated into DNA and is not degraded by cytidine deaminase. Its biological activity is based on the spontaneous conversion into the beta anomer that enters the DNA synthesis pathway [23]. These characteristics make alpha anomer a suitable candidate for epigenetic therapy of cancer.

In contrast, both non-specific methylation inhibitors (*R,S*)-AHPA-*ibu* and (S)-DHPA hypomethylate DNA *via* SAH-hydrolase inhibition [24]. SAH-hydrolase is essential to maintain the methylation capacities of the cell. The enzyme eliminates *S*-adenosyl-L-homocysteine (SAH), the product of methyltransferase reactions, which acts as methyltransferase inhibitor. Up to now, structurally related (S)-HPMPazaC and F-PymRf have not yet been studied for their DNA hypomethylating capabilities. Both of them are putative DNA methylation inhibitors.

In this work, we compared the capability of tested nucleoside analogs to down-regulate *hTERT* expression in human leukemia HL-60 cells with a special focus on α-5-azadCyd as a compound with a potent antileukemic activity.

2. Materials and methods

2.1. Cell culture and treatment

The human acute promyelocytic leukemia HL-60 cells (ATCC CCL 240) were cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, antibiotics (200 μg/ml of streptomycin and 200 units/ml of penicillin G),

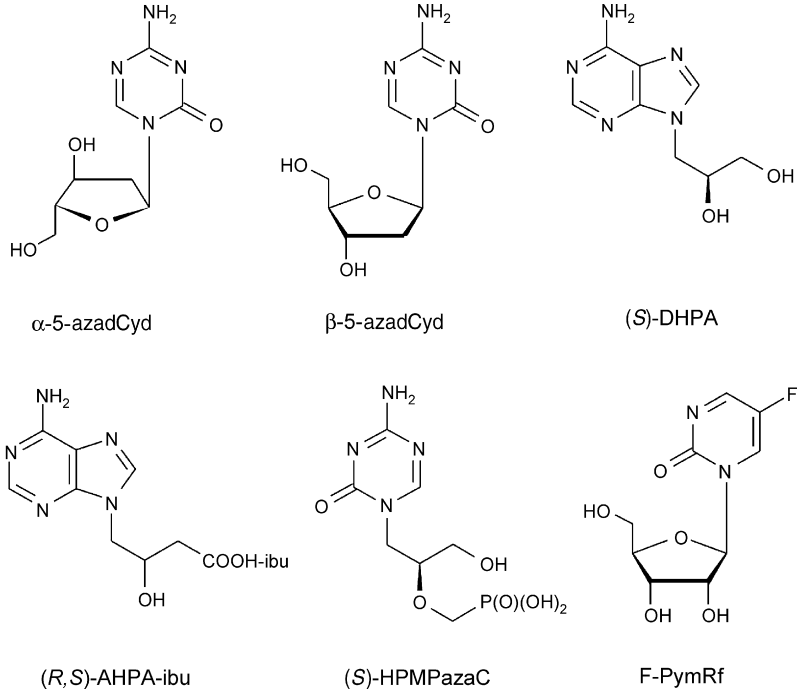

Fig. 1 – Chemical structures of hypomethylating agents.

10 mM β-mercaptoethanol, and vitamin B_{12} at 37 °C in a humidified atmosphere containing 5% CO_2. 24 h after seeding, cells were treated with various concentrations of freshly prepared solutions of hypomethylating agents, α- and β-5-azadCyd, (*S*)-DHPA, (*R,S*)-AHPA-*ibu*, F-PymRf, and (*S*)-HPMPa-zaC. After the 72 h treatment, the cells were pelleted, washed with phosphate-buffered saline (PBS) and collected for RNA extractions (as described below). Three independent treatments with each compound were performed. The synthesis of the tested compounds (Fig. 1) has been described previously [25–29].

All other chemicals and materials were commercial products, e.g. streptomycin, penicillin G, β-mercaptoethanol, propidium iodide, Triton X®-100, RNase A, PBS and RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA), fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), vitamin B_{12} (Léčiva a.s., Prague, Czech Republic), Protector RNase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), and primers listed in Table 1 (Invitrogen Ltd., Paisley, United Kingdom).

2.2. Determination of SAH and SAM levels

For determination of S-adenosyl-L-homocysteine (SAH) and S-adenosyl-L-methionine (SAM) levels cells were harvested 72 h after the addition of SAH-hydrolase inhibitors [(*S*)-DHPA, (*R,S*)-AHPA-*ibu*] into the culture medium. The PBS-washed cell biomass (6×10^6 cells) was extracted using 0.25 M perchloric acid at 4 °C and clarified by centrifugation. The acid-soluble extract was analyzed in the Aliance Waters HPLC system (996 PDA Detector, PDA Software Millenium, version 4.0) equipped with 15 cm × 4.6 mm SUPELCO Discovery C8, 5 μm reverse-phase column. A three-step gradient at a flow rate 0.9 ml/min was used. With (1) solvent A, 50 mM sodium phosphate pH 3.2, 10 mM heptanesulfonic acid, 50% acetonitrile; (2) solvent B, 50 mM sodium phosphate pH 3.2, 10 mM heptanesulfonic acid. The program consisted of (1) 9–11% A, 5 min (convex curve no. 3); (2) 11–17% A (concave curve no. 8), 10 min; (3) 100% A, 5 min (curve no. 11, isocratic). Peaks of SAH and SAM were identified (UV-spectra library) and quantified with the aid of external standards. The concentration of both cofactors was determined relative to the protein content.

2.3. Flow cytometric analysis of DNA content

HL-60 cells were plated in 25 cm^2 flasks at a density of 1×10^5 cells per mililiter of media. 24 h after seeding, cells were treated with various concentrations of nucleoside analogs and incubated for additional 72 h. They were then collected by centrifugation, and resuspended with PBS. Cells were centrifuged at 1000 × *g* for 5 min, washed again with PBS, and then fixed with 70% ice-cold ethanol for 30 min. To stain with propidium iodide, fixed cells were sedimented by centrifugation, washed in PBS and treated with RNase A (500 μg/ml) at 37 °C for 30 min, and finally incubated for 1 h with a staining solution containing 0.1% Triton X®-100 and propidium iodide (100 μg/ml) in PBS at a final cell concentration of 1×10^6 cells/ml. Cellular DNA content was determined using a flow cytometer FACSAria (BD Biosciences, San Jose, CA, USA). At least 30,000 cells were used for each analysis, and the results were displayed as histograms. Cell cycle distribution was analyzed using ModFit LT 3.0 program (Verity Software House, Topsham, ME, USA).

2.4. RNA extraction

Total cellular RNAs were extracted from 1–1.5 millions of control (untreated) and treated HL-60 cells using the RNeasy Mini isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Genomic DNA was eliminated by RNase-free DNase I digestion (Qiagen) during the isolation procedure. The concentration and purity of the RNA samples was assessed by measurement of the UV absorption at 260 nm and by the absorption ratio of 260 to 280 nm, respectively. The exact quantification of the RNA was carried out in triplicate using the Quant-iT™ RiboGreen® RNA assay kit (Invitrogen, Eugene, Oregon, USA) as described by the manufacturer. RNA samples were stored at −70 °C.

2.5. One-step real-time qRT-PCR

This assay was based on TaqMan methodology. Primers (Invitrogen) and probe (Generi-Biotech, Hradec Králové, Czech Republic) nucleotide sequences for *hTERT* (GenBank accession number AF015950) were: forward primer 5′-CACGC-GAAAACCTTCCTCA-3′ (placed in exon 10; nt 2690–2708), reverse primer 5′-CAAGTTCACCACGCAGCC-3′, and TaqMan probe 5′(FAM)-CTCAGGGACACCTCGGACCAGGGT-(BHQ1)3′ (both placed in exon 11; nt 2755–2738 and 2734–2711, respectively) [30]. SuperScript™ III Platinum® One-Step Quantitative RT-PCR system (Invitrogen) was used to amplify the *hTERT* mRNA according to the manufacturer's protocol. PCR was performed in a total volume of 50 μL containing 1× TaqMan

Table 1 – Sequences of PCR primers in two-step real-time qRT-PCR

Gene	Sequence 5′-3′	
	Sense	Antisense
GAPDH	gAAggTgAAggTCggAgTC	gAAgATggTgATgggATTTC
RPII	gCACCACgTCCAATgACAT	gTgCggCTgCTTCCATAA
TBP	TTCggAgAgTTCTgggATTgTA	TggACTgTTCTTCACTCTTggC
G6PDH	ATCgACCACTACCTgggCAA	TTCTgCATCACgTCCCggA
PBGD	ggCTgCAACggCggAA	CCTgTggTggACATAgCAATgATT
PLA	AAgTTCTTgATCCCCAATgCTT	gTCTgATAggATgTgTTggTTgC
β-actin	TCCTTCCTgggCATggAg	AggAggAgCAATgATCTTgATCTT
hTERT	TgACACCTCACCTCACCCAC	CACTgTCTTCCgCAAgTTCAC

buffer, 4 mM $MgSO_4$, 200 nM each primer, 100 nM probe, 40 ng of total RNA, 20 U of Protector RNase inhibitor. The thermal cycling conditions included 15 min at 50 °C and 2 min at 95 °C for the reverse transcription step, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All samples were amplified in triplicate using a DNA Engine Opticon® 2 (Bio-Rad, Hercules, CA, USA) and the mean Ct value was obtained for further calculations. Normalization of *hTERT* mRNA levels was performed against total RNA in the reaction mixture.

2.6. cDNA synthesis

Total RNA (500 ng) was reverse transcribed using SuperScript™ II Reverse Transciptase kit (Invitrogen) according to the manufacturer's instructions in a total volume of 20 μL containing 0.5 μg oligo(dT)$_{12–18}$ primer, 0.5 mM deoxynucleotides, 10 mM DTT, and 40 U of Protector RNase inhibitor. Tubes were heated to 65 °C for 5 min to denature the secondary RNA structure. The RT reaction was completed by adding 200 U SuperScript™ II Reverse Transciptase before incubation at 42 °C for 50 min and then 70 °C for 15 min. cDNA samples were stored at −70 °C.

2.7. Two-step real-time qRT-PCR

Quantification of c-*myc* and *hTERT* mRNA expression was performed by qRT-PCR using the DNA Engine Opticon® 2 and DyNAmo™ SYBR® Green qPCR kit (Finnzymes Oy, Espoo, Finland). Reactions were done in triplicate, each 20-μL PCR reaction mixture contained 4 μL (<10 ng/μl) of cDNA template, 6 pmol of each primer (primer sets listed in Table 1 [30], and 10 μl of DyNAmo™ SYBR® Green qPCR mix supplemented with the kit. The thermocycling program included: an initial denaturation at 95 °C for 15 min to ensure a complete reactivation of the hot start DNA polymerase; 40 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The specifity of an amplified product was checked by melting curve analysis. The melting protocol called for heating from 65 to 90 °C, holding for 10 s at each temperature, with increases of 0.5 °C per step. *hTERT* and c-*myc* expression levels were normalized by dividing the raw *hTERT* and c-*myc* quantities for each sample by the appropriate normalization factor calculated as the geometric mean of the four most stable housekeeping genes (GAPDH, RPII, TBP and PLA) selected using a geNorm Visual Basic Application (VBA) for Microsoft Excel (freely

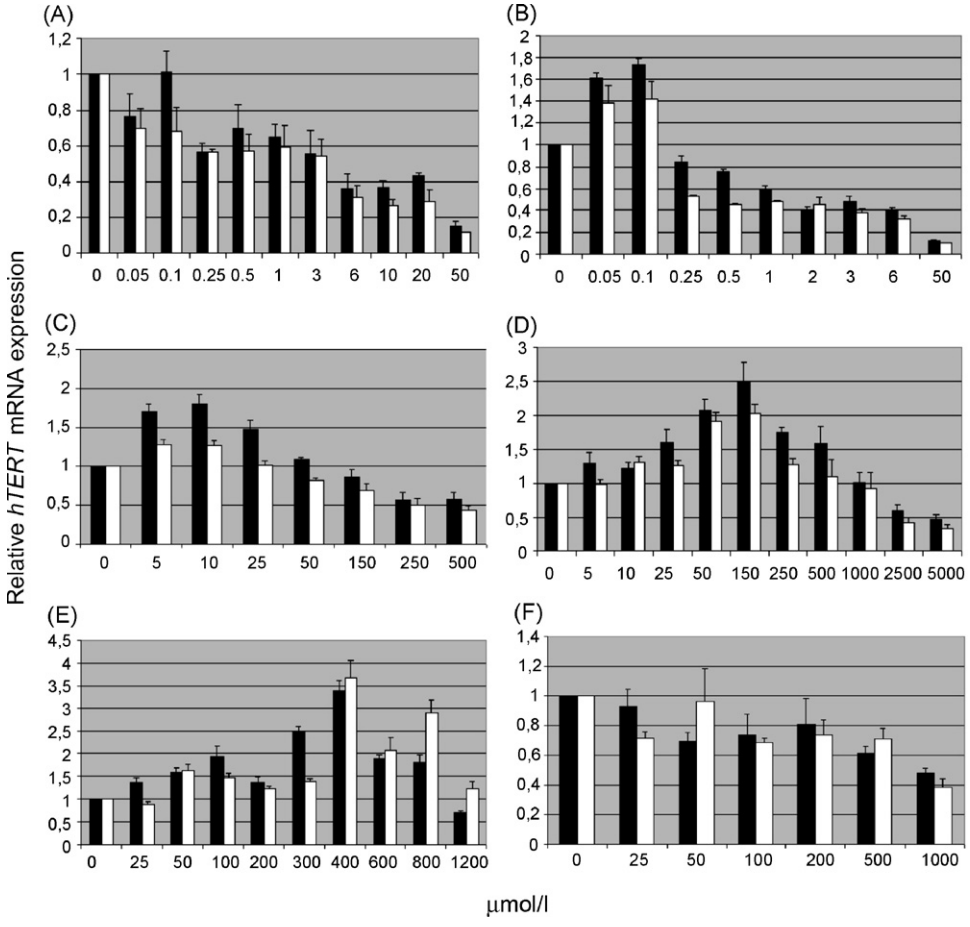

Fig. 2 – Relative *hTERT* gene expression after 72 h treatment with α-5-azadCyd (A), β-5-azadCyd (B), (*R*,*S*)-AHPA-*ibu* (C), (*S*)-DHPA (D), F-PymRf (E), (*S*)-HPMPazaC (F) as measured by real-time qRT-PCR using SYBR® Green I dye (black columns, normalization to housekeeping genes) and TaqMan probe (white columns, normalization to total RNA). The gene expression levels in each sample are compared to the expression levels in untreated controls, which are set to 1. Graphs show the mean values of three independent experiments in duplicate. Error bars represent standard deviation (S.D.).

available at http://medgen.ugent.be/jvdesomp/genorm/). The underlying principles and calculations are described in Vandesompele et al. [31].

3. Results

hTERT expression was quantified by both one-step quantitative reverse transcription-PCR (qRT-PCR) based on TaqMan methodology and two-step qRT-PCR using SYBR® Green I. To obtain reliable results we applied two different strategies for normalization of real-time PCR data. In case of one-step qRT-PCR, *hTERT* expression was normalized against total RNA in the reaction mixture and in the latter case, normalization was performed to a set of housekeeping genes that exhibited the most stable expression in tested samples. Advantages and pitfalls of both approaches have been discussed previously [32].

Because of relatively small differences in *hTERT* mRNA levels between treated samples and untreated controls, the requirement for a proper reference (housekeeping) gene for normalization was stringent. As numerous studies reported that the housekeeping gene expression can vary considerably [33,34] we decided to use multiple housekeeping genes rather than a single one to ensure accurate and reliable normalization of gene expression data. We investigated the expression stability of seven housekeeping genes from different abundance and functional classes [30] potentially useful as reference genes to study changes in *hTERT* and c-*myc* expression in HL-60 cells after treatment with hypomethylating agents. Validation of their expression stability was performed by the geNorm VBA applet using data from three independent treatments with each nucleoside analog. The program ranked the tested candidate genes from most stable to least stable as follows: GAPDH, RPII, TBP, PLA, PBGD, Act, G6PDH. The combination of 4 reference genes (GAPDH, RPII, TBP and PLA) was identified as being most suitable for normalization of samples obtained after treatment with all tested compounds. Their mean expression was used for normalization factor calculation and subsequent normalization of *hTERT* and c-*myc* mRNA levels. A guidance for determination of the optimal number of housekeeping genes for normalization factor calculation is described by Vandesompele et al. [31].

Both α-5-azadCyd and β-5-azadCyd down-regulated *hTERT* expression, however, treatment with these compounds induced a distinct pattern of *hTERT* expression in HL-60 cells. α-5-AzadCyd inhibited *hTERT* expression in the whole range of tested concentrations (Fig. 2A) whereas the beta anomer (decitabine) caused a transient elevation of *hTERT* mRNA at low micromolar concentrations followed by subsequent *hTERT* down-regulation at higher concentrations of β-5-azadCyd (Fig. 2B). The increase of *hTERT* expression correlated with up-regulation of c-*myc* and was obvious even at a concentration as low as 0.05 μM β-5-azadCyd, which is about 70 times below its GIC_{50} value (Table 2). Under these conditions c-*myc* mRNA levels were increased three-fold while *hTERT* expression increased for about 50% compared with control. However, the subsequent decrease in *hTERT* expression seems to be independent of c-*myc* expression since c-*myc* remained up-regulated to some extent even at higher concentrations of β-5-azadCyd.

Table 2 – Cytotoxicity of tested compounds on HL-60 cell line after 72 h of exposure

Compound	GIC_{50} (μmol/l)*
α-5-azadCyd	12.5 ± 2.1
β-5-azadCyd	3.4 ± 1.3
(*R,S*)-AHPA-*ibu*	174 ± 32
(*S*)-DHPA	650 ± 108
(S)-HPMPazaC	1050 ± 107
F-PymRf	520 ± 85

* Values are means ± S.D. of the three independent determinations.

After treatment with α-5-azadCyd c-*myc* mRNA levels slightly decreased, however, no dose-dependent manner was observed as it was for *hTERT* (Table 3). *hTERT* expression has been shown to decrease gradually within the whole range of concentrations and at 10 μM α-5-azadCyd reached approximately 30% of control (Fig. 2A). The real-time qRT-PCR results on *hTERT* expression after normalization to reference genes were in accordance with those after normalization against total RNA. As shown in Table 4, α-5-azadCyd exerts similar effect on cell cycle as the beta anomer. Both compounds caused an inhibition of cell cycle progression, resulting in an increase of the percentage of cells in the G_0/G_1 phase.

Table 3 – Comparison of relative *hTERT* and c-*myc* expression after 72 h treatment

	μmol/l	c-Myc*	*hTERT**
α-5-azadCyd	0	1	1
	0.1	0.78 ± 0.05	1.02 ± 0.11
	0.25	0.70 ± 0.06	0.56 ± 0.05
	1	0.80 ± 0.06	0.65 ± 0.08
	10	0.97 ± 0.29	0.37 ± 0.04
β-5-azadCyd	0.05	2.80 ± 0.13	1.60 ± 0.05
	0.1	3.05 ± 0.25	1.73 ± 0.06
	1	1.70 ± 0.08	0.59 ± 0.03
	6	1.11 ± 0.02	0.39 ± 0.03
(*R,S*)-AHPA-*ibu*	5	4.19 ± 0.19	1.70 ± 0.11
	10	4.84 ± 0.27	1.81 ± 0.12
	50	2.05 ± 0.07	1.09 ± 0.02
	250	3.89 ± 0.45	0.56 ± 0.10
(*S*)-DHPA	5	1.30 ± 0.17	1.30 ± 0.16
	50	3.81 ± 0.41	2.07 ± 0.17
	250	5.14 ± 0.44	1.76 ± 0.06
	1000	3.50 ± 0.47	1.01 ± 0.14
(S)-HPMPazaC	50	0.73 ± 0.11	0.69 ± 0.06
	200	0.61 ± 0.09	0.81 ± 0.18
	500	0.67 ± 0.10	0.61 ± 0.05
	1000	0.34 ± 0.05	0.48 ± 0.04
F-PymRf	300	6.21 ± 0.40	2.49 ± 0.11
	400	5.13 ± 0.62	3.40 ± 0.21
	600	4.16 ± 0.97	1.90 ± 0.08
	800	3.88 ± 0.47	1.81 ± 0.16

Data obtained by two-step qRT-PCR – normalization to reference genes. The gene expression levels in each sample are compared to the expression levels in controls.
* Values are presented as the mean ± S.D. for at least three independent experiments in duplicate.

Table 4 – The cell cycle distribution after 72 h treatment with concentrations corresponding to GIC_{50} values on HL-60 cells for each studied compound

Compound	Concentration (μmol/l)	G_0/G_1	S (%)	G_2/M
Control	Not added	46.5	44.2	9.3
α-5-azadCyd	12.5	58.0	31.0	11.0
β-5-azadCyd	3.4	56.3	32.4	11.3
(*R,S*)-AHPA-*ibu*	174	48.9	42.3	8.8
(*S*)-DHPA	650	50.4	42.6	7.0
(*S*)-HPMPazaC	1050	13.5	76.5	10.0
F-PymRf	520	39.5	52.4	8.1

We also intended to evaluate the effect of (*R,S*)-AHPA-*ibu* and (*S*)-DHPA on *hTERT* expression. We examined the levels of methylation intermediates SAM and SAH after 72 h treatment with (*R,S*)-AHPA-*ibu* and (*S*)-DHPA. The results (Table 5) show a significant and concentration dependent increase in SAH/SAM ratio for both compounds, which is caused predominantly by an increase in SAH. The character of the increase in SAH/SAM ratio predicts a reduced methylation capacity of HL-60 cells after treatment with both (*R,S*)-AHPA-*ibu* and (*S*)-DHPA.

The prevalent effect of reversible SAH-hydrolase inhibitor (*S*)-DHPA is the up-regulation of *hTERT* within a broad range of concentrations up to 1000 μM. The increase in *hTERT* mRNA levels is obvious even at a concentration as low as 5 μM (*S*)-DHPA and reaches its peak at 100 μM when *hTERT* expression is elevated more than two-fold (Fig. 2D). c-Myc expression is significantly elevated at all tested concentrations (Table 3). In accordance with the higher hypomethylation capacity, the irreversible SAH-hydrolase inhibitor (*R,S*)-AHPA-*ibu* exhibited stronger potency to inhibit *hTERT* expression when compared to (*S*)-DHPA. In contrast to (*S*)-DHPA, we observed a significant decrease in *hTERT* mRNA levels (compared to control) from concentration corresponding to its GIC_{50} value (174 μM, Table 2, Fig. 2C). Again, there is an up-regulation of *hTERT* expression at lower concentrations of (*R,S*)-AHPA-*ibu* and c-*myc* remains overexpressed within the whole range of tested concentrations. Similar to β-5-azadCyd and (*S*)-DHPA, the down-regulation of *hTERT* seems to be independent of c-*myc* expression.

(*S*)-HPMPazaC and 5-fluoro-zebularine (F-PymRf) exert a distinct effect on cell cycle distribution. In contrast to the other compounds studied, these antimetabolites most probably interfere with DNA replication and cause S-phase arrest (Table 4). Treatment with (*S*)-HPMPazaC, compound with a potent and selective activity against several DNA viruses, results in the decrease of *hTERT* mRNA levels (Fig. 2F). The effect of (*S*)-HPMPazaC on expression of *hTERT* and c-*myc* is similar to that of α-5-azadCyd, however, at considerably higher concentrations. The down-regulation of *hTERT* expression is apparent already at 25 μM (*S*)-HPMPazaC and the subsequent decrease of *hTERT* mRNA levels is slow within the broad range of concentrations up to 1000 μM (*S*)-HPMPazaC. No transient elevation of *hTERT* mRNA levels and no c-*myc* overexpression is observed.

F-PymRf significantly up-regulates *hTERT* and c-*myc* (Table 3, Fig. 2E) from concentrations well below its GIC_{50} value (520 μM, Table 2). From the studied compounds, F-PymRf was shown to have the highest potency to increase c-*myc* and *hTERT* mRNA levels (Fig. 2E).

4. Discussion

Real-time PCR data normalization is an important step in gene expression studies. It is now generally accepted that gene expression levels should be normalized to an invariably expressed housekeeping gene that reflects differences in cellular input, RNA quality, and RT efficiency. Many other factors in real-time PCR may affect the results, including the selection of the reference genes. A number of studies have shown that their transcription levels can be affected by the experimental treatment and vary significantly between different individuals, different cell types, and different developmental stages as well. Therefore, thorough validation of candidate housekeeping genes is critical for accurate analysis of gene expression. We suggest using the mean expression of multiple housekeeping genes rather than a single one for normalization.

Normalization to total RNA ensures the same reverse transcriptase input, but does not correct for differences in reverse transcriptase and PCR efficiencies between samples [32]. It is also assumed that rRNA:mRNA ratio does not change between samples. However, this assumption can be wrong since we found an increased c-*myc* mRNA levels in treated samples. The effect on rDNA transcription is not clear, but we

Table 5 – Intracellular changes of SAH and SAM concentrations in (*S*)-DHPA and (*R,S*)-AHPA-*ibu* treated human leukemia HL-60 cells

	μmol/l	SAH (pmol/mg)*	SAM (pmol/mg)*	SAH/SAM
(*S*)-DHPA	Not added	18.68	696.86	0.027
	50	238.47	877.70	0.272
	250	730.17	1048.64	0.696
	500	853.85	830.81	1.028
	5000	1817.44	706.33	2.573
(*R,S*)-AHPA-*ibu*	Not added	1.88	79.17	0.024
	25	30.07	84.92	0.354
	50	40.14	78.85	0.509
	100	53.37	56.17	0.950
	250	97.66	60.35	1.618

* Per mg of cellular protein.

can not rule out the possibility that rRNA:mRNA ratio is increased in treated samples with upregulated c-*myc* expression [19]. Furthermore, it has been shown that even a particular cell type may contain different quantities of total RNA and/or mRNA under various physiological conditions [35,36]. The above mentioned considerations on normalization strategies led us to involve both commonly used methods of normalization to obtain reliable *hTERT* expression data.

Both anomers of 5-azadCyd are chemically unstable in aqueous solution. HPLC analysis of chemical stability revealed that the half-life of alpha and beta anomer in RPMI 1640 media (pH 7.2) supplemented with 10% fetal calf serum at 37 °C is 12 and 3.5 h, respectively. Under these conditions, a relatively slow spontaneous conversion of alpha anomer to beta anomer occured in the media, reaching a maximum concentration of β-5-azadCyd after 6 h incubation (initial velocity v_0 ~4 pmol/min at 2 mM α-5-azadCyd). Surprisingly, treatment with α-5-azadCyd causes a distinct expression pattern of c-*myc* and *hTERT* than treatment with β-5-azadCyd. α-5-AzadCyd does not induce c-*myc* overexpression and transient *hTERT* up-regulation as it is observed for beta anomer. The reason is unclear because α-5-azadCyd is supposed to be a prodrug of beta anomer [23]. With regard to relatively short half-life of alpha anomer in the media (12 h) we might expect that after 72 h treatment all α-5-azadCyd is converted into beta anomer which is supposed to exert its biological activity. However, more stable alpha anomer most likely exhibits a prolonged action compared to beta anomer.

The mechanism of action of (*S*)-HPMPazaC remains to be elucidated, however, it could be similar to that of HPMPC [37] and 5-azadCyd. Due to the presence of 5-azacytosine ring, (*S*)-HPMPazaC when incorporated into DNA might probably covalently trap DNA methyltransferase. The lower potency of (*S*)-HPMPazaC to inhibit *hTERT* expression might be explained by weaker ability to incorporate into DNA and/or by lower uptake into the cells compared to 5-azadCyd.

Our results show that all tested compounds [β-5-azadCyd, F-PymRf, (*R*,*S*)-AHPA-*ibu*, (*S*)-DHPA] except for α-5-azadCyd and (*S*)-HPMPazaC cause the increase in c-*myc* expression which is accompanied with transient increase of *hTERT* mRNA levels. The correlation was found between c-*myc* overexpression and transiently elevated *hTERT* expression, indicating that the up-regulation of *hTERT* observed might be conferred through the transactivation of *hTERT* by c-*myc*. Indeed, association between c-*myc* overexpression and induction of telomerase activity has been previously reported [16]. The *hTERT* promoter contains numerous c-myc-binding sites that mediate *hTERT* transcriptional activation. c-Myc-induced *hTERT* expression is rapid and independent of cell proliferation [17]. Similarly, *hTERT* expression has been shown to correlate with c-*myc* overexpression in human prostate cancer [38].

Subsequent decrease in *hTERT* expression at higher concentration of hypomethylating agents seems to be independent of c-*myc* expression. We can speculate that more extensive hypomethylation of DNA caused by treatment with high doses of hypomethylating agents enables binding of CTCF to GC-rich proximal exonic region. Then the effect of transcriptional repressor CTCF might prevail over the activation of *hTERT* expression by c-*myc*. However, the methylation status of the *hTERT* promoter was not examined. This is why we can not state whether inhibition of *hTERT* expression was due to *hTERT* promoter hypomethylation or it originated from altered expression of transcription factors affecting the *hTERT* transcription. Most likely the regulation of *hTERT* expression is a complex process and both aspects contribute to some extent.

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REFERENCES

[1] Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell 1989;59:521–9.
[2] Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB. Telomere end-replication problem and cell aging. J Mol Biol 1992;225:951–60.
[3] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
[4] Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 1997;90:785–95.
[5] Horikawa I, Cable PL, Afshari C, Barrett JC. Cloning and characterization of the promoter region of human telomerase reverse transcriptase gene. Cancer Res 1999;59:826–30.
[6] Devereux TR, Horikawa I, Anna CH, Annab LA, Afshari CA, Barrett JC. DNA methylation analysis of the promoter region of the human telomerase reverse transcriptase (hTERT) gene. Cancer Res 1999;59:6087–90.
[7] Nomoto K, Maekawa M, Sugano K, Ushiama M, Fukayama N, Fujita S, et al. Methylation status and expression of human telomerase reverse transcriptase mRNA in relation to hypermethylation of the p16 gene in colorectal cancers as analyzed by bisulfite PCR-SSCP. Jpn J Clin Oncol 2002;32:3–8.
[8] Dessain SK, Yu H, Reddel RR, Beijersbergen RL, Weinberg RA. Methylation of the human telomerase gene CpG island. Cancer Res 2000;60:537–41.
[9] Renaud S, Bosman FT, Benhattar J. Implication of the exon region in the regulation of the human telomerase reverse transcriptase gene promoter. Biochem Biophys Res Commun 2003;300:47–54.
[10] Renaud S, Loukinov D, Bosman FT, Lobanenkov V, Benhattar J. CTCF binds the proximal exonic region of hTERT and inhibits its transcription. Nucl Acids Res 2005;33:6850–60.
[11] Renaud S, Loukinov D, Abdullaev Z, Guilleret I, Bosman FT, Lobanenkov V, et al. Dual role of DNA methylation inside and outside of CTCF-binding regions in the transcriptional regulation of the telomerase hTERT gene. Nucl Acids Res 2007;35:1245–56.
[12] Guilleret I, Benhattar J. Demethylation of the human telomerase catalytic subunit (hTERT) gene promoter reduced hTERT expression and telomerase activity and shortened telomeres. Exp Cell Res 2003;289:326–34.

[13] Kumakura S, Tsutsui TW, Yagisawa J, Barrett JC, Tsutsui T. Reversible conversion of immortal human cells from telomerase-positive to telomerase-negative cells. Cancer Res 2005;65:2778–86.
[14] Kitagawa Y, Kyo S, Takakura M, Kanaya T, Koshida K, Namiki M, et al. Demethylating reagent 5-azacytidine inhibits telomerase activity in human prostate cancer cells through transcriptional repression of hTERT. Clin Cancer Res 2000;6:2868–75.
[15] Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, et al. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med 1995;1:686–92.
[16] Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. Genes Dev 1998;12:1769–74.
[17] Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J, et al. Direct activation of TERT transcription by c-MYC. Nat Genet 1999;21:220–4.
[18] Mukherjee S, Conrad SE. c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem 2005;280:17617–25.
[19] Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, et al. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol 2005;7:311–8.
[20] Eischen CM, Packham G, Nip J, Fee BE, Hiebert SW, Zambetti GP, et al. Bcl-2 is an apoptotic target suppressed by both c-Myc and E2F-1. Oncogene 2001;20:6983–93.
[21] Schermelleh L, Spada F, Easwaran HP, Zolghadr K, Margot JB, Cardoso MC, et al. Trapped in action: direct visualization of DNA methyltransferase activity in living cells. Nat Meth 2005;2:751–6.
[22] Fojtová M, Pískala A, Votruba I, Otmar M, Bártová E, Kovařík A. Efficacy of DNA hypomethylating capacities of 5-aza-2′-deoxycytidine and its alpha anomer. Pharmacol Res 2007;55:16–22.
[23] Veselý J, Pískala A. Effects of the alpha-D-anomer of 5-aza-2′-deoxycytidine on L1210 mouse leukemic cells in vitro and in vivo. Cancer Res 1984;44:5165–8.
[24] Holý A, Votruba I, Merta A, DeClercq E, Jelínek R, Sláma K, et al. Biological consequences of S-adenosyl-L-homocysteinase inhibition by acyclic adenosine analogs. In: Borchardt RT, Creveling CR, Ueland PM, editors. Biological Methylation and Drug Design. Clifton: Humana Press; 1986. p. 397–408.
[25] Cech D, Beerbaum H, Holý A. A simple synthesis of 5-fluoro-2-pyrimidinone and its N^1-substituted derivatives. Collect Czech Chem Commun 1977;42:2694–700.
[26] Pliml J, Šorm F. Synthesis of a deoxy-D-ribofuranosyl-5-cytosine. Collect Czech Chem Commun 1964;29:2576–7.
[27] Krečmerová M, Holý A, Pískala A, Masojídková M, Andrei G, Naesens L, et al. Antiviral activity of triazine analogues of 1-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]cytosine (cidofovir) and related compounds. J Med Chem 2007;50:1069–77.
[28] Holý A. Aliphatic analogues of nucleosides, nucleotides, and oligonucleotides. Collect Czech Chem Commun 1975;40:187–214.
[29] Holý A, Votruba I, De Clercq E. Structure-activity studies on open-chain analogues of nucleosides: Inhibition of S-adenosyl-L-homocysteine hydrolase and antiviral activity. Collect Czech Chem Commun 1985;50:262–79.
[30] Radonić A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004;313:856–62.
[31] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:1–12.
[32] Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 2005;6:279–84.
[33] Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. Housekeeping genes as internal standards: use and limits. J Biotechnol 1999;75:291–5.
[34] Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000;29:332–7.
[35] Boon K, Caron HN, van Asperen R, Valentijn L, Hermus MC, van Sluis P, et al. N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. EMBO J 2001;20:1383–93.
[36] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Meth 2000;46:69–81.
[37] Holý A. Phosphonomethoxyalkyl analogs of nucleotides. Curr Pharm Des 2003;9:2567–92.
[38] Latil A, Vidaud D, Valéri A, Fournier G, Vidaud M, Lidereau R, et al. hTERT expression correlates with MYC overexpression in human prostate cancer. Int J Cancer 2000;89:172–6.