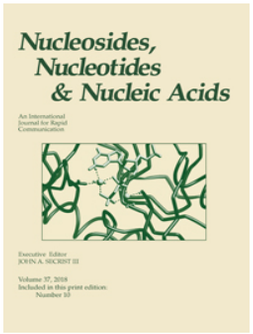

Nucleosides, Nucleotides & Nucleic Acids

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lncn20

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To cite this article: Godefridus J. Peters , Albert Leyva & Gilberto Schwartsmann (2020): Resistance to differentiation affects ribo- and deoxyribonucleotide pools and sensitivity to pyrimidine metabolism antagonists in HL60 cells, Nucleosides, Nucleotides & Nucleic Acids, DOI: 10.1080/15257770.2020.1782933

To link to this article: https://doi.org/10.1080/15257770.2020.1782933

Published online: 30 Jul 2020.

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
https://doi.org/10.1080/15257770.2020.1782933

Resistance to differentiation affects ribo- and deoxyribonucleotide pools and sensitivity to pyrimidine metabolism antagonists in HL60 cells

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ABSTRACT

HL60 myeloid leukemia cells are extensively used as a differentiation model. We investigated a variant of HL60 which is resistant to differentiation induction (HL60-R) by standard differentiation inducers such as retinoic acid and dimethylsulfoxide (DMSO). To find an explanation for this resistance, we examined nucleotide (NTP) and deoxynucleotide (dNTP) pools in HL60-R and its parent cell line, sensitive to differentiation, HL60-S. We also explored whether these differences led to a difference in sensitivity to various antimetabolites. Drug sensitivity was measured with the tetrazolium (MTT) assay, while nucleotides were measured with anion-exchange HPLC. HL60-R cells were between 2- and 5-fold resistant to the antimetabolites 5-fluorouracil, Brequinar, hydroxyurea and N-(phosphonacetyl)-L-aspartate (PALA), but more sensitive to aza-2′-deoxycytidine (DAC), cytarabine and thymidine (5- to 10-fold). The NTP pools in both HL60 variants showed a normal pattern with ATP being the highest (2530–2876 pmol/10^6 cells) and CTP being lowest. However, UTP pools were 2-fold higher in the HL60-S cells ($p < .01$), while CTP and GTP pools were 30% higher ($p < .01$) compared to HL60-R cells. For the dNTP pools, larger differences were observed, with dATP (50 pmol/10^6 cells) being highest in HL60-R cells, but dATP was 4-fold lower in HL60-S cells. In HL-60-R, the triple combination retinoic acid, DMSO and DAC increased all NTPs almost 2-fold in contrast to HL60-S. Uridine increased UTP (1.4-fold), CTP (2-fold) and dCTP (1.4.-fold) pools in both cell lines, but thymidine increased only dTTP pools (4- to 7-fold), with a depletion of dCTP. PALA decreased UTP and CTP in both cell lines, but increased ATP (only in HL60-R). Hydroxyurea decreased dNTP especially in HL60-S cells. In conclusion, the pronounced differences in NTP and dNTP pools between HL60-S and HL60-R possibly play a role in the induction of differentiation and drug sensitivity.

ARTICLE HISTORY
Received 18 February 2020
Accepted 10 June 2020

KEYWORDS
HL60 cells; differentiation; pyrimidine antagonists; ribonucleotide pools; deoxyribonucleotide pools

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1. Introduction

For decades induction of differentiation has been considered a target for cancer therapy. Loss of differentiation may lead to the development of various hematological malignancies.[1] Differentiation is controlled by hypomethylation of DNA, since this will increase the expression of a number of tumor suppressor genes.[2] This (partially) explains the efficacy of several hypomethylating agents (e.g., 5-aza-2′-deoxycytidine, DAC) in the treatment of these malignancies.[3] HL60 myeloid leukemia cells are considered one of the best models to study differentiation,[4,5] which can be induced by several agents, such as retinoic acid (RA) and dimethylsulfoxide (DMSO), which are used as a standard for differentiation induction.[6] RA possibly acts by activation of the nuclear receptor, modifying gene expression, DMSO influences membrane ion (K+) fluxes, and DAC inhibits DNA methyltransferase leading to hypomethylation and induction of gene expression. We earlier studied differentiation induction in two variants of HL60, HL60-S (sensitive to RA) and HL60-R (resistant to RA, DMSO and DAC).[7–9] Interestingly, HL60-R were resistant to each drug when applied alone, and this could not be reversed by a double combination. However, the triple combination completely restored differentiation rate, possibly because they have different mechanisms of action (Table 1).

Differentiation is a tightly regulated process which is affected by DNA methylation and cellular signals which are controlled by normal metabolism, including adequate nucleotide pools.[10] To better understand the role of these nucleotides, we measured cellular ribonucleotide (NTP: ATP, GTP, CTP and UTP) and deoxyribonucleotide (dNTP: dATP, dGTP, dCTP and dTTP) pools and the response to several inhibitors of pyrimidine metabolism was compared in differentiation sensitive- versus resistant-HL60 cells.

Table 1. Differentiation rate of HL60-S and HL60-R cells.

	HL60-S	HL60-R	
Drugs	% NBT positive cells		*p*
None	7 ± 4	5 ± 3	.03
1 μM retinoic acid (RA)	89 ± 5	12 ± 3	.0001
1 % dimethylsulfoxde (DMSO)	46 ± 5	11 ± 2	.0001
1 μM DAC	29 ± 6	9 ± 2	.001
RA + DMSO	95 ± 4	18 ± 6	.0001
RA + DAC	94 ± 3	13 ± 2	.0001
DMSO + DAC	72 ± 3	12 ± 3	.0001
RA + DMSO + DAC	96 ± 3	89 ± 7	n.s.

Cells were exposed to the drug(s) for 4 days, and differentiation induction was measured by the ability of cells to reduce nitro blue tetrazolium (NBT, from 7, 8), as a result of superoxide anions produced during phorbol-ester induced respiration burst. Cell viability (as determined by trypan blue exclusion) decreased to 85% after exposure to a single agent and to 74% after exposure to double and triple agents. n.s.; not significant ($p > .05$).

2. Materials and methods

2.1. Cell culture

The origin of the HL60-S and HL-60-S cell lines has been described previously.[7,8] The cells are routinely cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C under a 5% CO_2 atmosphere and grown as a suspension. All cells were regularly checked for mycoplasma. Drug sensitivity was determined with the tetrazolium (MTT) assay[11,12] using 96-well round bottom plates. Briefly, 30,000 cells were plated in each well, and after one day, drugs were added in a concentration range below and above the expected IC50 values. After 72-h exposure, the number of cells was determined by the MTT assay, which is based on the ability of living cells to convert yellowish tetrazolium salt MTT into a purple formazan, which can be solubilized to determine the absorbance. The IC50 is defined as the concentration which results in 50% growth inhibition

2.2. Nucleotide measurements

To measure the concentration of all nucleotides, cells were cultured in 25-cm^2 culture flasks in order to harvest $1–10 \times 10^6$ cells in an exponential growth phase. After harvesting, the cells were counted, washed, centrifuged and precipitated with ice-cold trichloroacetic acid followed by neutralization with a 1:4 mixture of tri-N-octylamine and 1,1,2, trichlorotrifluoroethane (alamine-freon). After centrifugation, the supernatant was pipetted off and stored at −20 °C until analysis. The concentrations of NTPs were measured using a validated anion exchange HPLC assay with a Partisil-SAX column with a gradient of increasing NaCl and phosphate concentration.[13] For the measurement of the dNTP, the ribonucleotides were degraded with periodate. NTP and dNTP concentrations were given as pmol/10^6 cells. The effect of drugs was determined in cells exposed to the drug at the concentrations specified in the figures and harvested as described above.

2.3. Statistics

To evaluate whether the differences between HL60-S and HL60-R were significant ($p \leq .05$), we used Student's *t*-test for unpaired samples. The effects of drug exposure were evaluated for significance (cutoff $p \leq .05$) using a paired Student's *t*-test.

3. Results

3.1. Nucleotide pools in HL60-S and HL60-R cell lines and effect of differentiation agents

Both cell lines showed a relatively normal pattern of nucleotide pools (Table 2), with ATP showing the highest levels of all nucleotides in both cell lines, but ATP was higher in HL6-R cells. In contrast, GTP was higher in the HL60-S compared to HL60-R cells, but these differences were not significant. However, the levels of both UTP and CTP were significantly higher in HL60-S cells. Regarding the dNTPs, considerable differences were observed, where all dNTP pools were higher in HL60-R cells. In HL60-S cells, dTTP pools were the greatest of the dNTP pools, but in HL60-R cells, dATP levels were the highest. Also, dGTP levels were higher in HL60-R cells (3-fold) compared to HL60-S cells.

Under conditions that induce differentiation in HL60-S cells RA reduced the CTP pool in HL60-S cells but slightly increased it in HL60-R cells (Figure 1). However, ATP and UTP pools were increased in HL60-S cells. Still, in the triple combination, which induces differentiation in both cells lines, all nucleotides pools increased 1.6- to 2-fold in the HL60-R cells, but in HL60-S cells, only ATP increased almost 2-fold, and UTP slightly.

3.2. Effect of normal nucleosides and pyrimidine analogs on NTP and dNTP pools

Since changes in the NTP and dNTP pools seem to play a role in differentiation, we also investigated the effect of modulators of NTP and dNTP pools. Modulation of uridine pools is a registered treatment (as triacetyluridine) for protection against lethal (hematological and gastrointestinal) toxicity of 5FU, as was extensively investigated by us (reviewed[14]), while thymidine protects against methotrexate (hematological) toxicity but may have an antitumor effect by itself.[15] Therefore, we investigated the effects

Table 2. Nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) concentrations in HL60-S and HL60-R cell lines.

Nucleotide	HL60-S	HL60-R	Ratio R/S	*p*
ATP	2530 ± 286	2876 ± 264	1.14	n.s.
GTP	1111 ± 122	813 ± 75	0.73	n.s.
UTP	1377 ± 97	787 ± 30	0.52	<.01
CTP	460 ± 17	307 ± 13	0.67	<.01
dATP	12.8 ± 1.2	50.1 ± 2.7	3.62	<.0001
dGTP	8.5 ± 0.89	21.0 ± 1.5	2.47	<.0001
dTTP	27.5 ± 2.0	34.2 ± 2.3	1.36	<.05
dCTP	13.2 ± 1.2	15.4 ± 1.1	1.2	n.s.

NTP and dNTP concentrations (in pmol/10^6 cells) were measured in exponentially growing cells and are means ± SE of 4 (NTP) or 7-12 (dNTP) separate measurements. *p* values were calculated using the *t*-test for non-paired samples. n.s.; not significant ($p > .05$).

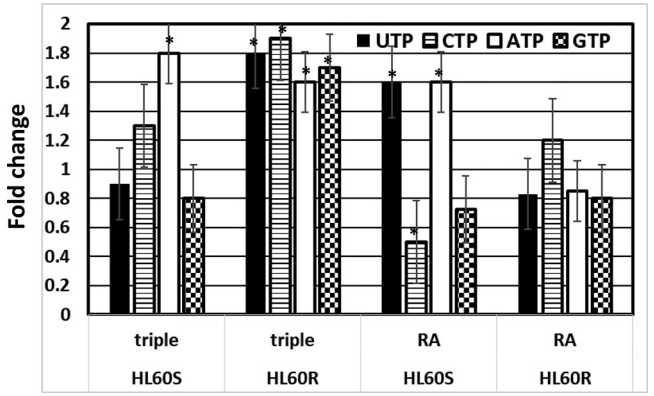

Figure 1. Effect of differentiation-inducing agents on NTP pool of HL60-S and HL60-R cells. Values (means ± SEM of three separate experiments) are given as ratios between exposed and control cells. Drugs were applied at the concentrations given in Table 1, and NTPs were measured 72 h after exposure. RA retinoic acid; triple, RA + DMSO + DAC. *, significantly different from controls at a level of $p < .01$ (paired test).

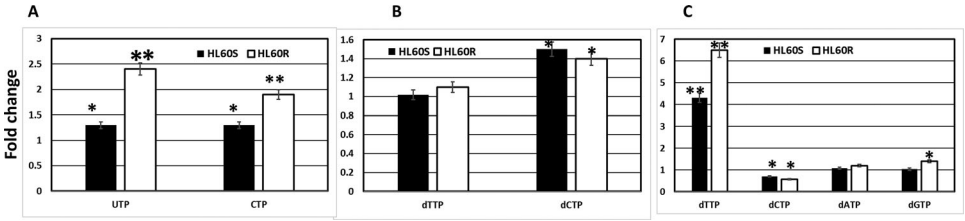

Figure 2. Effect of short-term 3-h exposure to 100 μM uridine (A, B) or 3 h with 100 μM thymidine (C) on NTP and dNTP pools of HL60-S and HL60-R cells. Values are ratios of (d)NTP concentrations in exposed and control cells and means ± SEM of three separate experiments for NTPs, three separate experiments for the thymidine effects on dNTPs and 10–12 separate experiments for uridine effects on dNTPs. Significantly different from controls at a level of *, $p < .05$, and **, $p < .01$ (paired *t*-test).

of these drugs. Exposure to 100 μM uridine increased the pools of both UTP and CTP but did not affect ATP and GTP pools (Figure 2). Uridine increased dCTP pools in both cell lines; the effects on the other dNTP pools were marginal, except for dGTP, which were increased in HL60-R cells by 32%, while the effects on dNTP in HL60S cells were marginal. However, in HL60-R cells, all dNTP pools decreased by about 25%. As expected, 100 μM thymidine increased dTTP pools in both cell lines, to 430 and 650%, while dCTP was decreased in both cell lines by 30%–44%.

Since the major differences between HL60-S and HL60-R cells were found in the UTP and CTP pools, we investigated the effects of a pyrimidine de novo synthesis inhibitor (Figure 3). N-(Phosphonacetyl)-L-aspartate (PALA) is a potent inhibitor of aspartate transcarbamylase (ATC) and decreased UTP and CTP pools in both cell lines, although the effect was

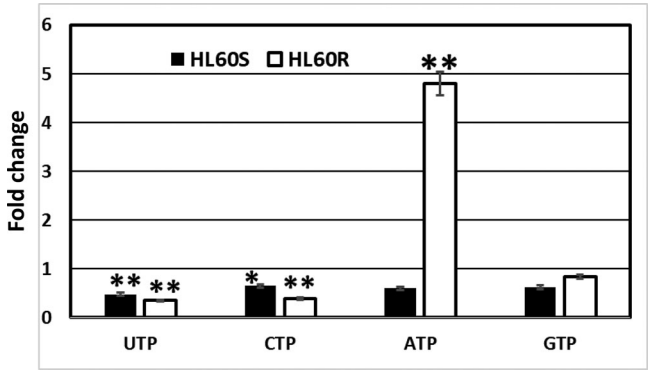

Figure 3. Effect of 24-h exposure to 100 μM PALA on NTP pools of HL60-S and HL60-R cells. Values are ratios of exposed and control cells and are means ± SEM of three separate experiments. Significantly different from controls at a level of: *, $p < .05$, and **, $p < .01$ (paired *t*-test).

more pronounced in HL60-R cells (>60%). Interestingly, PALA increased ATP pools in these cells almost 5-fold.

For hydroxyurea, we only investigated the effects on dNTPs. A 3-h exposure to 500 μM reduced the dTTP pools by 50% in HL60-S cells ($p < .05$), but only 10% in the more resistant HL60-R cells (not significant); for dCTP, this was 70% and 50%, respectively. For the purine dNTP, which are both much higher (3–4-fold) in HL60-R cells, hydroxyurea exposure resulted in a comparable effect for dATP (62% and 68% decrease in HL60-S and HL60-R cells, respectively), but somewhat higher for dGTP in HL60-S cells (69% decrease compared to 56% decrease in HL60-R cells). This means that the actual dNTP pools in HL-60-R cells remain higher than in HL-60-S cells.

3.3. Drug sensitivity

NTP pools were originally investigated to get insight into the possible role of these pools in the differentiation process. Considering the pronounced differences in these pools and the different effects on modulators, we also investigated whether there were differences in the sensitivity to various inhibitors of pyrimidine metabolism (Figure 4). HL60-R cells were more sensitive to the deoxycytidine analogs ara-C and DAC as well as thymidine. The latter natural deoxynucleoside has been investigated at a high dose as a potential anticancer drug. The other compounds either had a marginal effect (methotrexate) or HL-60R cells were 2.5- to 5-fold more resistant (the ribonucleotide reductase inhibitor hydroxyurea), with a similar effect of the pyrimidine de novo inhibitors PALA and Brequinar.

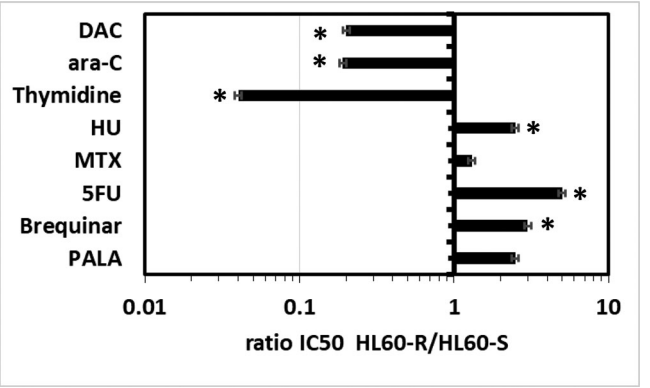

Figure 4. Differential sensitivity of HL60-S and HL60-R cells to several antimetabolites. Values (means ± SEM) are given as ratios of the IC50s (after 72-h drug exposure) in HL60-R compared to HL60-S cells. Ara-C, cytarabine; HU, hydroxyurea; MTX, methotrexate; 5FU, 5-fluorouracil. *, significantly different between HL60-R and HL60-S cells at a level of $p < .01$.

4. Discussion

The differentiation-resistant cell line HL60-R shows considerable differences in the composition of nucleotide pools, especially dNTP pool. Possibly, the larger dNTP pools may play a role in this resistance. Although none of the differentiation-inducing drugs are known to have a direct effect on nucleotide metabolism, RA alone already increased the pools of UTP and ATP 1.6-fold; the effect on ATP was also found in the triple combination. In the HL60-R cells, the triple combination had a pronounced effect on normal NTP pools, which were increased about 2-fold, apparently playing a role in the observed normalization of differentiation. One might speculate that the higher supply of nucleotides enables the cells to enter the process of differentiation, but in the present investigation, no direct interaction in this process was investigated. These data have been obtained in one pair of cell lines, sensitive and resistant to differentiation. Validation of these results in other models for differentiation could not be performed since we did not have resistant variants of other models such as U937 and THP1 cells.

The different response of the two cell lines to modulators of nucleotide metabolism, such as the normal nucleosides uridine and thymidine, led us to speculate that the cells might have different sensitivities to inhibitors of pyrimidine metabolism. High-dose thymidine has been investigated as a potential therapeutic option,[15] while thymidine can also protect against toxicity induced by antifolates such as methotrexate and pemetrexed.[16] The higher accumulation of dTTP in HL60-R cells after thymidine exposure is in line with the higher sensitivity of these cells to thymidine. This effect is possibly mediated by inhibition of ribonucleotide reductase by dTTP,[17] which indeed leads to a depletion of dCTP, which was indeed

more pronounced in HL60-R cells. However, the resistance of HL60-R cells to the ribonucleotide reductase inhibitor hydroxyurea may be related to the higher dNTP pools in these cells. Moreover, the effect of hydroxyurea on dNTP pools was lower in HL60-R cells than in HL60-S cells, especially when focusing on absolute pools.

Apparently, dCTP pools are more sensitive to modulation in HL60-R cells, which might partially explain the higher sensitivity of these cells to both deoxycytidine analogs ara-C and DAC; both drugs have to compete with dCTP for incorporation into DNA.[3] In contrast, the high levels of dNTP pools in HL60-R cells may partially explain the lower sensitivity to 5FU, which acts by inhibition of thymidylate synthase,[14] leading to a decrease in dTTP. Uridine has been developed as a protective agent against 5FU-induced toxicity, by specifically increasing pyrimidine nucleotide pools.[14] Although both pyrimidine de novo inhibitors Brequinar (dihydroorotate dehydrogenase)[18] and PALA (ATC) show a lower sensitivity in HL60-R cells, PALA causes a more pronounced depletion of UTP and CTP, which was shown earlier to be related to PALA sensitivity[19]; however, PALA also results in a substantial increase in ATP, which may lead to a protection against the toxic effects of this drug. Brequinar was recently identified as a differentiation inducer.[20]

In conclusion, resistance to differentiation is associated with pronounced differences in NTP and especially dNTP pools. Normalization of differentiation is also associated with pronounced increase in all NTP pools. The different response of each HL60 variant seems to be related to the different sensitivities to various pyrimidine metabolism antagonists.

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