Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.10, No.6, 241-247, 2023



The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

*Letter*

# Effects of expression of the 10-formyltetrahydrofolate metabolizing enzyme ALDH1L1 on pyrimidine nucleotide synthesis and the salvage pathway

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(Received September 22, 2023; Accepted September 29, 2023)

**ABSTRACT** — Proliferating cells, such as tumor cells, require nucleotides for DNA replication. Mammalian cells are equipped with *de novo* purine and pyrimidine nucleotide biosynthesis pathways, and a salvage pathway that recycles purine bases, to supply nucleotides for the same. To avoid imbalance in intracellular nucleotide levels, *de novo* nucleotide biosynthesis pathway is regulated by feedback mechanisms, such as synthase inhibition by nucleotide products. Recently, we reported that the aldehyde dehydrogenase 1 family member L1 (ALDH1L1) consumes 10-formyltetrahydrofolate (10-fTHF), which is utilized by the *de novo* purine nucleotide synthesis, and results in the accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP) during purine biosynthesis. Given that ZMP inhibits pyrimidine nucleotide synthesis, in the present study, we examined the effects of ZMP using brequinar, a dihydroorotate dehydrogenase inhibitor. ALDH1L1-mediated ZMP accumulation was unaffected by brequinar, and no effect was observed when brequinar was combined with 5-aminoimidazole-4-carboxamide riboside (AICAr), a nucleoside of ZMP. Furthermore, we examined involvement in the salvage pathway, because attenuation of *de novo* purine nucleotide synthesis may require a supply of purine nucleotides from the salvage pathway. The guanine analog, 6-thioguanine, and the 2'-deoxycytidine analog, cytarabine, were used in the assessment of dependency on the salvage pathway. We found that neither ALDH1L1-mediated ZMP accumulation nor the presence of AICAr affected the salvage pathway. Collectively, these results suggest that these purine and pyrimidine analogs can be useful for the treatment of tumor cells, regardless of *ALDH1L1* expression.

**Key words:** Nucleotide synthesis, Salvage pathway, One-carbon metabolism, ALDH1L1, ZMP/AICAR

## INTRODUCTION

Intracellular nucleotide levels are generally regulated tightly (Lane and Fan, 2015). In pyrimidine nucleotide biosynthesis in mammalian cells, uridine 5′-monophosphate is synthesized from glutamine, $HCO_3^-$, and aspartate through a series of six reactions. On the other hand, in purine nucleotide biosynthesis, glycine, glutamine, aspartate, and formate (supplied by 10-formyltetrahydrofolate [10-fTHF]) are required for the synthesis of inosine 5′-monophosphate from a series of 10 different reactions involving 6 different enzymes. Additionally, purine nucleotides are regenerated from purine bases (adenine, guanine, and hypoxanthine) through the salvage pathway. The *de novo* synthesis of purine and pyrimidine nucleotides is regulated by the generated nucleotide products to avoid an imbalance in the nucleotide, whereas nucleotide production through the salvage pathway is generally not

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controlled in this manner.

Aldehyde dehydrogenase 1 family member L1 (ALDH1L1) catalyzes the conversion of 10-fTHF to tetrahydrofolate (THF) in the cytosol, and this is accompanied by $NADP^+$ reduction. *ALDH1L1* is mainly expressed in the liver, and its expression is attenuated or diminished in hepatocellular carcinoma cells. Patients with hepatocellular carcinoma with low *ALDH1L1* expression exhibit worse survival (Kondo *et al.*, 2009; Chen *et al.*, 2012; Liao *et al.*, 2016; Zhu *et al.*, 2017). Furthermore, *Aldh1l1* knockout mice exhibit enhanced susceptibility for liver cancer (Krupenko *et al.*, 2021). These observations suggest that *ALDH1L1* is a candidate tumor suppressor gene, specifically in the pathogenesis of liver cancer. 10-fTHF is used as a donor during the formylation of glycinamide ribonucleotide and 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP, also known as AICAR). Hence, sufficient intracellular levels of 10-fTHF are required for *de novo* purine nucleotide synthesis. Consistently, it is speculated that the loss of *ALDH1L1* expression increases the supply of 10-fTHF for *de novo* purine nucleotide synthesis in tumor cells to provide adequate nucleotides for DNA replication. In fact, ZMP reportedly accumulates in the hepatocellular carcinoma cell line HuH-7, which has enhanced *ALDH1L1* expression (Sasaki *et al.*, 2023). This suggests that the one-carbon metabolism mediated by ALDH1L1 is involved in the regulation of *de novo* purine nucleotide synthesis. However, it is unknown whether ZMP accumulation as a consequence of changes in ALDH1L1 expression affects pyrimidine nucleotide synthesis and the salvage pathway. Hence, in the present study, we investigated the role of ZMP accumulation and ALDH1L1 expression in nucleotide synthesis by monitoring cellular activity following treatment with pyrimidine biosynthesis inhibitors and nucleotide analogs.

## MATERIALS AND METHODS

### Reagents

5-Aminoimidazole-4-carboxamide riboside (AICAr) was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Cytarabine, 6-thioguanine, and brequinar were purchased from Tokyo Chemical Industry (Tokyo, Japan).

### Cell culture

*ALDH1L1*-expressing lentivirus infected HuH-7 cells (H7-1L1) and empty lentivirus infected HuH-7 cells (H7-emp), which were established previously (Sasaki *et al.*, 2023), were used in the present study. HuH-7 cells were cultured at 37°C in a 5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 5% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA), and 1 × antibiotic-antimycotic mix solution (Nacalai Tesque).

### Cellular activity assay

Cell activity was assessed using the colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay, using the CellTiter96$AQ_{ueous}$NonRadioactiveCellProliferationAssay (Promega, Madison, WI, USA). HuH-7 cells were seeded into 96-well plates at a density of 3,000 per well. To prepare MTS/phenazine methosulfate (PMS) solution, 3.15 mM PMS (Nacalai Tesque) and 2 mg/L MTS were mixed in a 1:20,000 (v/v) ratio. MTS/PMS solution was diluted in the cell culture medium to prepare a 15% MTS/PMS media, and 100 µL of 15% MTS/PMS medium was added to each well. Plates were incubated at 37°C for 1–3 hr until the absorbance at 490 nm was approximately 1. Absorbance was measured using an SH-1300Lab microplate reader (Hitachi High-Tech, Tokyo, Japan). Absorbance was normalized to and expressed as the relative absorbance of vehicle-treated H7-emp or H7-1L1 cells.

### Statistical Analysis

The relative absorbance values of H7-emp and H7-1L1 cells were compared using an unpaired Student's *t*-test. A $P$-value $\leq 0.03$ or less was considered to be significant.

## RESULTS AND DISCUSSION

We previously showed that ALDH1L1 consumes 10-fTHF, leading to the accumulation of ZMP, and that ALDH1L1 expressing cells exhibited resistance to AICAr by maintaining mitochondrial respiratory activity (Sasaki *et al.*, 2023). Previous studies indicated that *de novo* pyrimidine synthesis is inhibited by ZMP (Paglia *et al.*, 2012; Bardeleben *et al.*, 2013) (Fig. 1). To determine whether ALDH1L1 induced ZMP accumulation affects *de novo* pyrimidine synthesis, both ALDH1L1-expressing HuH-7 cells (H7-1L1) and control empty vector-transfected cells (H7-emp) were treated with brequinar (Brq), a dihydroorotate dehydrogenase inhibitor, in the presence or absence of AICAr (Fig. 2). When cellular activity was evaluated using mitochondrial MTS reductase activity, Brq was found to inhibit cellular activity in H7-emp cells in a dose-dependent manner following 48 and 72 hr of treatment, but not after 24 hr of treatment. Compared with H7-emp cells, H7-1L1 cells showed slightly higher

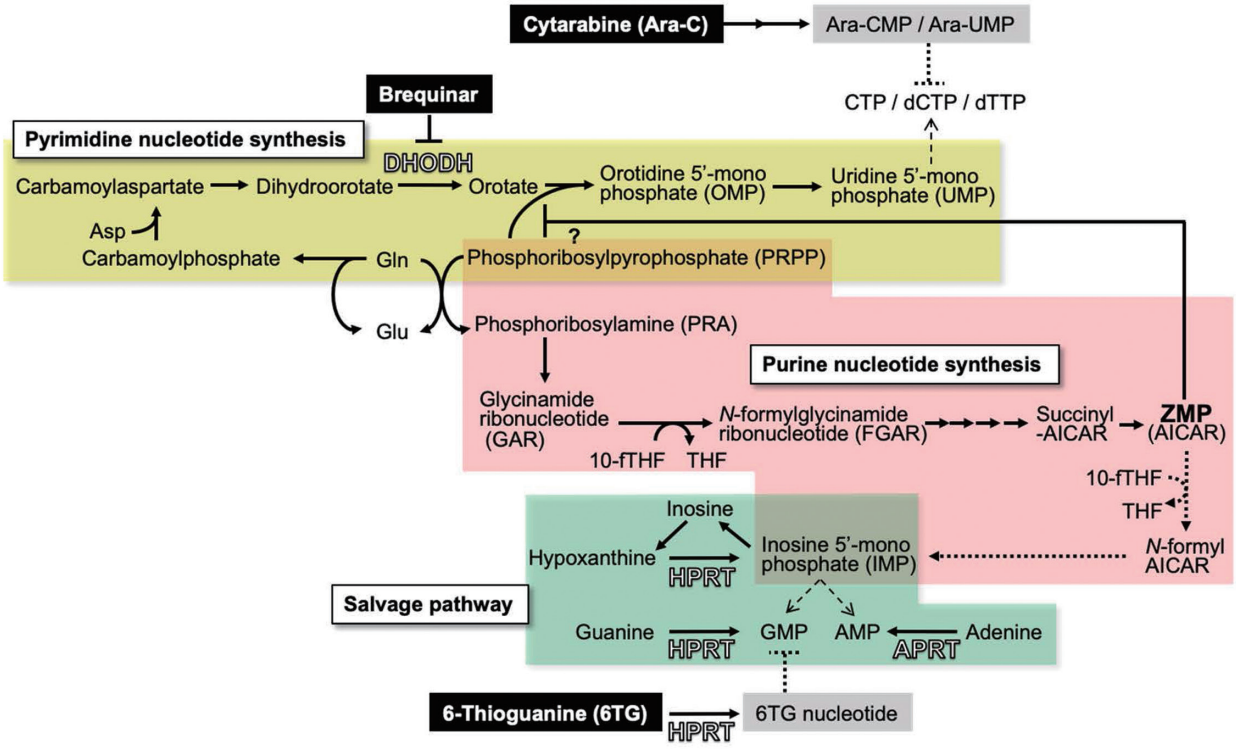


**Fig. 1**. Schematic representation of the *de novo* purine nucleotide synthesis, pyrimidine nucleotide synthesis, and salvage pathways. 10-fTHF, 10-formyltetrahydrofolate; AICAR, 5-aminoimidazole-4-carboxamide riboside; Ara-C, 2′-deoxycytidine analog cytarabine; AMP, adenosine monophosphate; APRT, adenine phosphoribosyltransferase; Asp, aspartate; CMP, cytosine monophosphate; CTP, cytidine triphosphate; DHODH, dihydroorotate dehydrogenase; Gln, glutamine; Glu, glutamic acid; GMP, guanosine monophosphate; HPRT, hypoxanthine phosphoribosyltransferase; THF, tetrahydrofolate; TTP, thymidine triphosphate; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide.

cellular activity at 48 hr. As reported previously, H7-1L1 cells possess higher mitochondrial respiratory activity, and the results might reflect the same (Sasaki *et al.*, 2023). Consistent with these observations, H7-1L1 cells showed higher cellular activity than H7-emp cells when treated with AICAr in the absence of Brq for 48 and 72 hr. Importantly, Brq did not show an additive effect when combined with AICAr, even at 100 μM. These results suggest that ZMP does not affect pyrimidine synthesis in H7-1L1 cells. Alternatively, the effect of ZMP could have been so strong that the effect of Brq was not apparent, because Brq inhibits pyrimidine synthesis upstream of ZMP-mediated inhibition.

Purine and pyrimidine analogs are generally converted via the salvage pathway to generate nucleotide analogs. Nucleotide analogs are incorporated into DNA or RNA, usually resulting in the inhibition of replication, transcription, and translation. Because ZMP accumulation in H7-1L1 cells suggests attenuation of *de novo* purine nucleotide synthesis, we hypothesized that the number of nucleotides supplied by the salvage pathway in H7-1L1 cells is greater than that in H7-emp cells. To test this hypothesis, we utilized the guanine analog 6-thioguanine (6TG) and the 2′-deoxycytidine analog cytarabine (Ara-C) and compared their cellular activities in H7-emp and H7-1L1 cells. If H7-1L1 cells incorporate more 6TG and Ara-C, cellular activity is expected to decrease. Although 6TG attenuated cellular activity in a dose-dependent manner, no difference was observed between H7-emp and H7-1L1 cells. When the cells were co-treated with AICAr and 6TG, the activity of H7-1L1 cells was not affected by 6TG treatment. In contrast, in H7-emp cells, 6TG increased cellular activity in a dose-dependent manner at 48 and 72 hr (Fig. 3). AICAr may be phosphorylated by adenosine kinase to generate ZMP, because 10-fTHF is not consumed by ALDH1L1 in H7-emp cells.

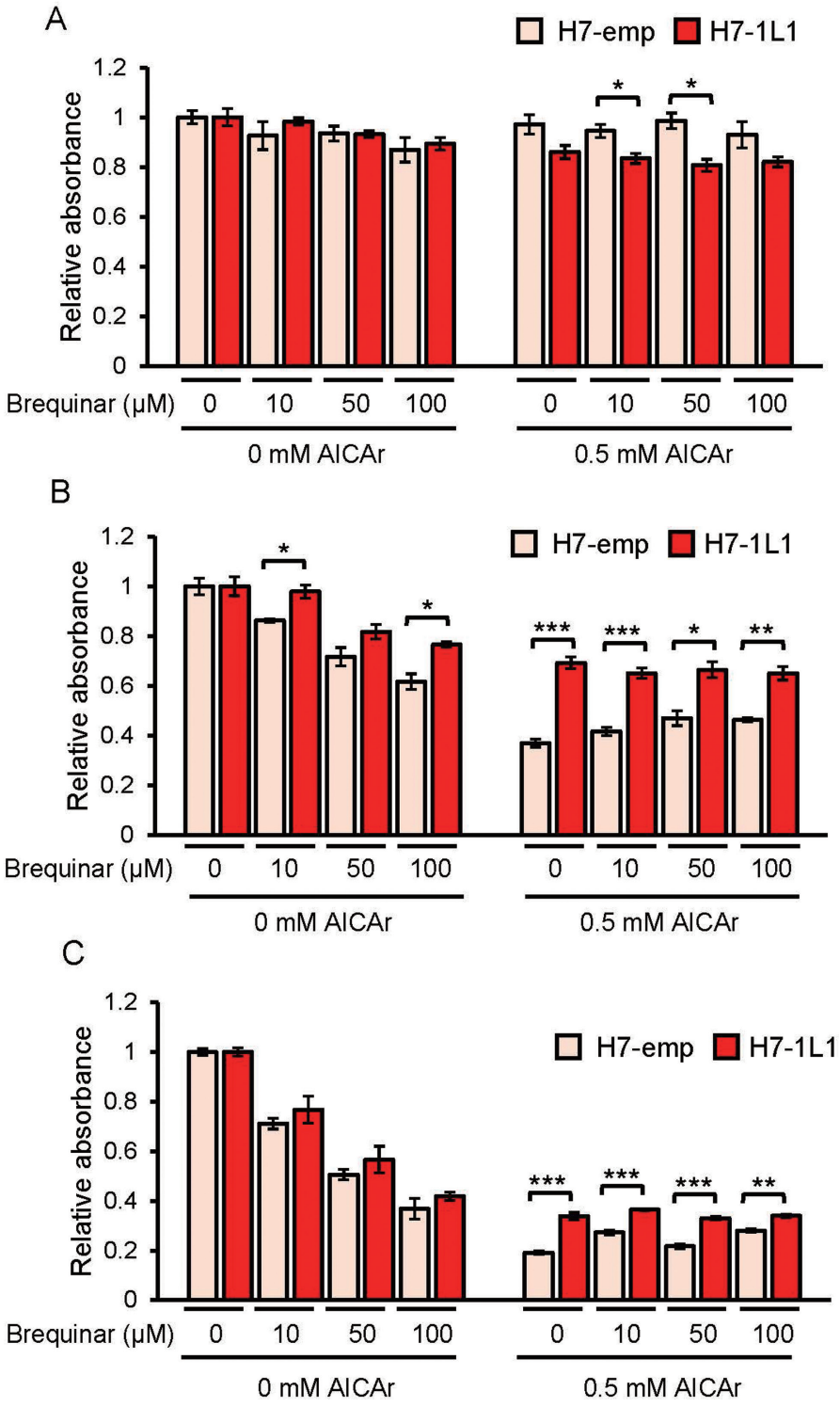


**Fig. 2**. Cellular activity of empty lentivirus infected HuH-7 (H7-emp) and *ALDH1L1*-expressing lentivirus infected HuH-7 (H7-1L1) cells cotreated with brequinar and AICAr for 24 hr (A), 48 hr (B), and 72 hr (C). Results are presented as the mean ± standard error of mean (SEM); *$P < 0.03$, **$P < 0.01$, ***$P < 0.001$ vs. H7-emp cells, unpaired Student's *t*-test.

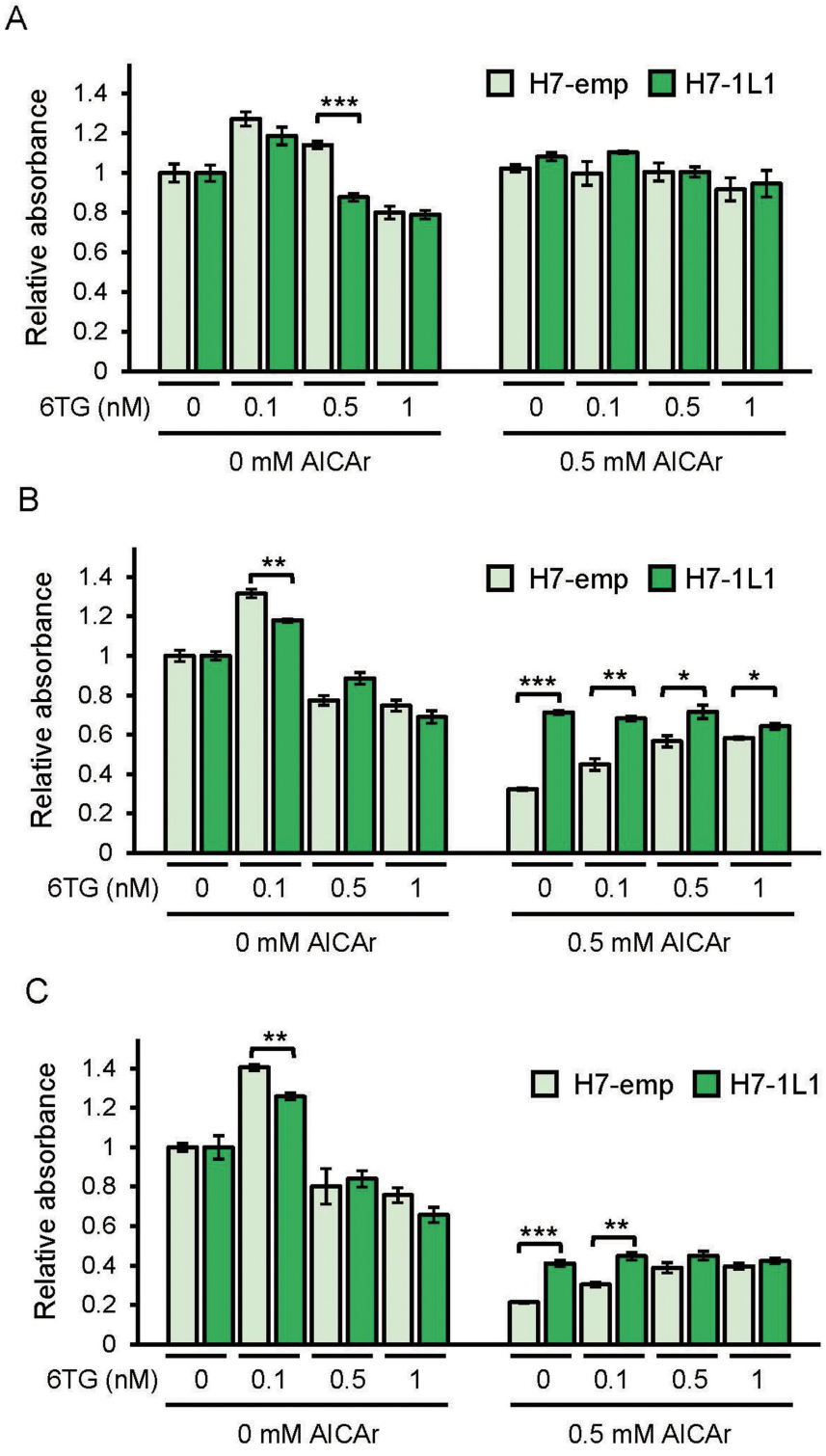


**Fig. 3**. Cellular activity of H7-emp and H7-1L1 cells cotreated with 6-thioguanine and AICAr for 24 hr (A), 48 hr (B), and 72 hr (C). Results are presented as the mean ± SEM; *$P < 0.03$, **$P < 0.01$, ***$P < 0.001$ vs. H7-emp cells, unpaired Student's *t*-test.

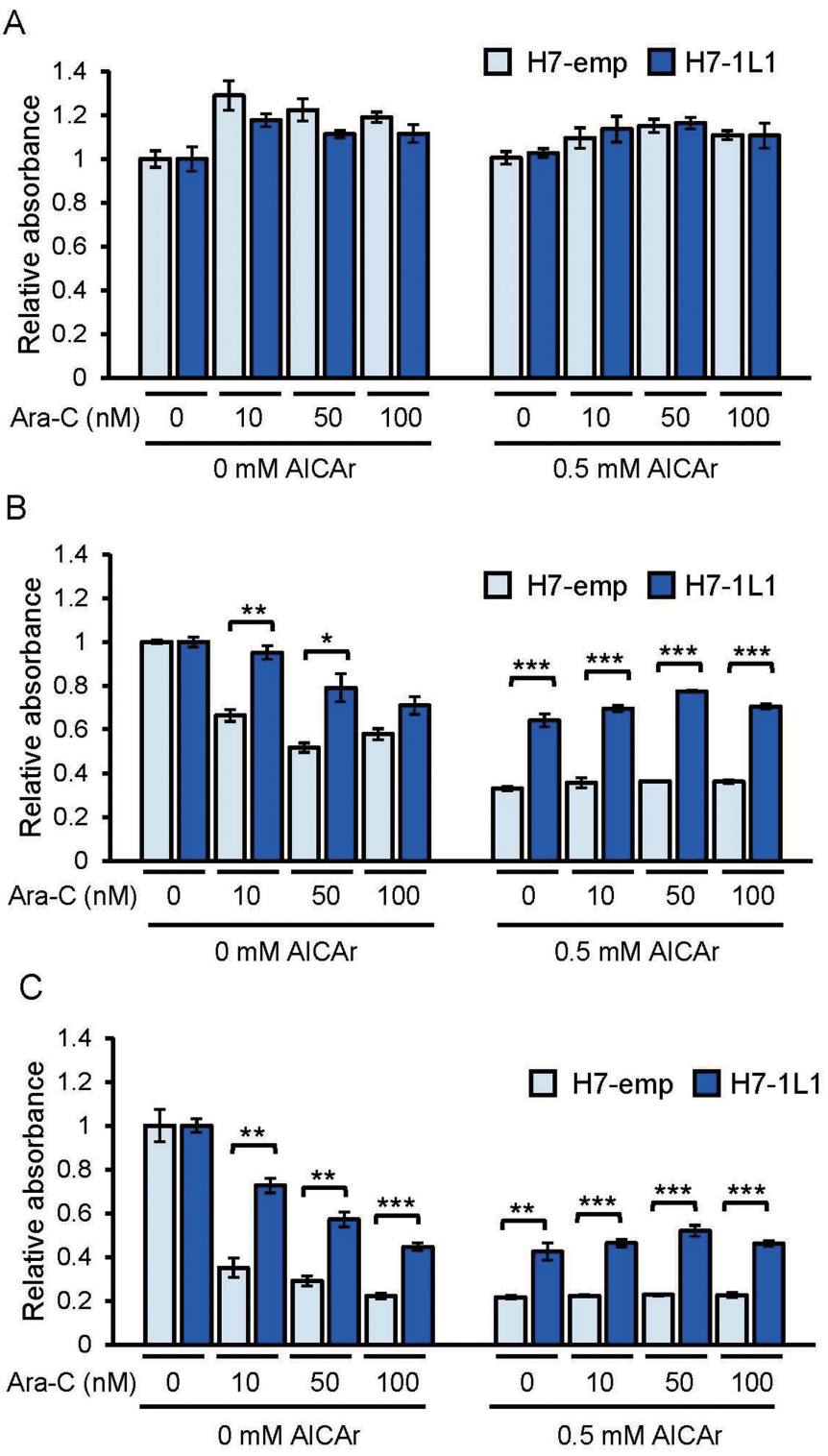


**Fig. 4**. Cellular activity of H7-emp and H7-1L1 cells cotreated with 2′-deoxycytidine analog cytarabine and AICAr for 24 hr (A), 48 hr (B), and 72 hr (C). Results are presented as the mean ± SEM; *$P < 0.03$, **$P < 0.01$, ***$P < 0.001$ compared with H7-emp cells, unpaired Student's *t*-test.

Therefore, these findings in H7-emp cells may reflect the interaction of AICAr with the *de novo* purine synthesis pathway, in contrast to that noted in H7-1L1 cells. Similar to the response with 6TG, Ara-C suppressed cellular activity in both H7-emp and H7-1L1 cells in a dose-dependent manner at 48 and 72 hr. However, H7-1L1 cells exhibited higher cellular activity than H7-emp cells (Fig. 4). With co-treatment of Ara-C and AICAr, the effect of Ara-C was not noted; however, cellular activity of H7-1L1 cells was higher than that of H7-emp cells. The higher cellular activity of H7-1L1 cells after 48 and 72 hr of Ara-C and AICAr co-treatment indicated that ALDH1L1 expression may have acquired Ara-C resistance, but in any case, Ara-C did not show additive effects with AICAr. Further validation by cell counting would be needed to clarify this possibility. Collectively, these finding highlight that 6TG and Ara-C sensitivity was not enhanced in H7-1L1 cells and hence, H7-1L1 cells do not rely on the salvage pathway.

Unlike the *de novo* purine nucleotide synthesis pathway, the activity of enzymes involved in the salvage pathway, namely hypoxanthine phosphoribosyltransferase and adenine phosphoribosyltransferase, is not known feedback inhibition. Therefore, it is assumed that the salvage pathway is not affected, even when *de novo* purine nucleotide synthesis is suppressed by a decrease in intracellular 10-fTHF levels. Moreover, in the present study, 6TG and Ara-C did not exhibit additive effects with AICAr. Further, we previously reported that AICAr arrested the cell cycle in the S-phase (Sasaki *et al.*, 2023). Thus, 6TG and Ara-C may not have been effective. These findings are promising as they suggest that purine and pyrimidine analogs can be useful in the treatment of hepatocellular carcinoma regardless of *ALDH1L1* expression status.

## ACKNOWLEDGMENTS

This work was supported by JSPS KAKENHI Grant Number JP20K05731. We would like to thank Editage (www.editage.jp) for English language editing.

**Conflict of interest----** The authors declare that there is no conflict of interest.

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