Received: 12 August 2021 | Revised: 22 November 2021 | Accepted: 29 November 2021

DOI: 10.1002/ijc.33901

MOLECULAR CANCER BIOLOGY

IJC INTERNATIONAL JOURNAL of CANCER

# Adult T-cell leukemia-lymphoma acquires resistance to DNA demethylating agents through dysregulation of enzymes involved in pyrimidine metabolism

Nao Yoshida-Sakai[1,2] | Tatsuro Watanabe[1] | Yuta Yamamoto[1] | Hiroshi Ureshino[1,2] | Kazuharu Kamachi[1,2] | Yuki Kurahashi[1,2,3] | Yuki Fukuda-Kurahashi[1,3] | Shinya Kimura[1,2]

[1]Department of Drug Discovery and Biomedical Sciences, Faculty of Medicine, Saga University, Saga, Japan

[2]Division of Hematology, Respiratory Medicine and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Saga, Japan

[3]OHARA Pharmaceutical Co, Ltd, Tokyo, Japan

**Correspondence**
Tatsuro Watanabe, 5-1-1 Nabeshima, Saga 849-8501, Japan.
Email: sn6538@cc.saga-u.ac.jp

**Funding information**
Japan Society for the Promotion of Science KAKENHI, Grant/Award Numbers: JP17H06956, JP20K07593; Nippon Shinyaku Research Grant; Ohara Pharmaceuticals

## Abstract

Adult T-cell leukemia-lymphoma (ATL) is an aggressive neoplasm derived from T-cells transformed by human T-cell lymphotropic virus-1 (HTLV-1). Recently, we reported that regional DNA hypermethylation in HTLV-1-infected T-cells reflects the disease status of ATL and the anti-ATL effects of DNA demethylating agents, including azacitidine (AZA), decitabine (DAC) and a new DAC prodrug, OR-2100 (OR21), which we developed. Here, to better understand the mechanisms underlying drug resistance, we generated AZA-, DAC- and OR21-resistant (AZA-R, DAC-R and OR21-R, respectively) cells from the ATL cell line TL-Om1 and the HTLV-1-infected cell line MT-2 via long-term drug exposure. The efficacy of OR21 was almost the same as that of DAC, indicating that the pharmacodynamics of OR21 were due to release of DAC from OR21. Resistant cells did not show cellular responses observed in parental cells induced by treatment with drugs, including growth suppression, depletion of DNA methyltransferase DNMT1 and DNA hypomethylation. We also found that reduced expression of deoxycytidine kinase (DCK) correlated with lower susceptibility to DAC/OR21 and that reduced expression of uridine cytidine kinase2 (UCK2) correlated with reduced susceptibility to AZA. DCK and UCK2 catalyze phosphorylation of DAC and AZA, respectively; reconstitution of expression reversed the resistant phenotypes. A large homozygous deletion in *DCK* and a homozygous splice donor site mutation in *UCK2* were identified in DAC-R TL-Om1 and AZA-R TL-Om1, respectively. Both genomic mutations might lead to loss of protein expression. Thus, inactivation of UCK2 and DCK might be a putative cause of phenotypes that are resistant to AZA and DAC/OR21, respectively.

**Abbreviations:** AML, acute myeloid leukemia; ATL, adult T-cell leukemia-lymphoma; AZA, azacitidine; BMMC, bone marrow mononuclear nuclear cell; CCR4, C-C chemokine receptor type 4; CDA, cytidine deaminase; DAC, decitabine; DCK, deoxycytidine kinase; DCTD, deoxycytidylate deaminase; DNMT1, DNA methyltransferase1; EZH, enhancer of zeste homolog; FDA, food and drug administration; HTLV-1, human T-cell lymphotropic virus-1; LINE, long interspersed nuclear element; MDS, myelodysplastic syndrome; PBMC, peripheral blood mononuclear cell; RIPA, radioimmunoprecipitation assay; UCK, uridine cytidine kinase.

KEYWORDS
acquired resistance, adult T-cell leukemia-lymphoma, azacitidine, decitabine, pyrimidine metabolism

**What's new?**

Adult T-cell leukemia-lymphoma (ATL) is an aggressive neoplasm derived from T-cells transformed by human T-cell lymphotropic virus-1 (HTLV-1). Since DNA hypermethylation is linked to leukemogenesis in ATL, DNA hypomethylation by DNA demethylating agents such as azacitidine, decitabine, or OR-2100 (a decitabine prodrug) is a possible therapeutic approach. Here, the authors show *in vitro* that down-regulated expression of the pyrimidine metabolism enzymes UCK2 and DCK correlates with acquired resistance to azacitidine and decitabine/OR-2100, respectively. Loss of DCK and UCK2 expression was caused by a genomic deletion in the *DCK* gene and a splice donor site mutation in the *UCK2* gene.

## 1 | INTRODUCTION

Aberrant DNA methylation is an epigenetic hallmark of cancer; thus DNA hypomethylation chemotherapy using DNA demethylating agents has attracted much attention.[1] Adult T-cell leukemia-lymphoma (ATL) is a highly malignant lymphoma derived from $CD4^+$ T-cells transformed by persistent infection with human T-cell lymphotropic virus-1 (HTLV-1).[2,3] Virus infection occurs via breast feeding or sexual activity; however, it takes several decades for HTLV-1-infected T-cells to transform completely to what is recognized clinically as aggressive ATL.[2,3] Although new molecular targeting drugs such as anti-CCR4 antibodies,[4] immunomodulatory drugs[5] and EZH1/2 inhibitors[6] are in use or under development, the prognosis for patients with ATL remains poor, particularly for those with aggressive disease (the acute and lymphoma subtypes); also, there are no chemopreventive drugs for HTLV-1 carriers. Recently, we reported that aberrant DNA methylation accumulates in HTLV-1-infected T-cells, accompanied by disease development and progression of ATL and that DNA methylation status reflects disease status.[7] These findings encouraged us to ask whether DNA hypomethylating agents suppress tumor cell growth in ATL by inducing DNA demethylation.

Azacitidine (AZA) and decitabine (DAC) are analogs of cytidine and deoxycytidine, respectively. Both are metabolized through the pyrimidine metabolism pathway to yield the deoxycytidine triphosphate analog AZA-dCTP, which is incorporated into the growing daughter strand of DNA; this traps and depletes DNA methyltransferase 1 (DNMT1).[8] Since DNMT1 protein catalyzes transfer of a methyl group from S-adenosyl methionine to a cytosine residue to form 5-methylcytosine during DNA replication,[9] treatment with AZA and DAC induces DNA hypomethylation in a cell division-dependent manner.[8]

As expected, treatment of HTLV-1-infected cell lines and ATL cell lines with AZA and DAC suppressed cell growth, with $IC_{50}$ values (the concentration at which activity is inhibited by 50%) at physiologically relevant concentrations that were concomitant with DNA hypomethylation in vitro.[7] Both are clinically used for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[10] The drugs are degraded rapidly by cytidine deaminase (CDA)[11] and administered by injection. So far, several DNA demethylating agents have been developed; indeed, orally bioavailable AZA (CC-486)[12] and DAC (ASTX727)[13] were approved by the FDA in 2020. We developed OR-2100 (OR21), which is a silylated derivative of DAC that releases DAC in a sustained-release manner.[7,14] The log *P* value of OR21 is 2.14, which means that it is better suited to oral administration than DAC (log *P*: −.32).[14] In addition, OR21 is resistant to degradation by CDA. In fact, a pharmacokinetic study of duodenal administration to macaques showed that it reaches blood concentrations 20-fold higher than those of DAC.[7] Although the efficacy of OR21 was almost the same as that of DAC, treatment of ATL-xenograft mice with OR21 suppressed tumor growth with lower hematotoxicity than DAC; in addition, OR21 was safe and stable for administration over the long-term (over 100 days).[7] Based on these results, we believe that DNA hypomethylation chemotherapy is useful for treatment and prevention of ATL.

Primary or secondary resistance to AZA and DAC are often observed in patients, which results in a poor prognosis. Thus, it is important to understand the mechanisms by which tumor cells deal with DNA demethylating agents. Development of improved next generation sequence platforms has made it possible to decipher the molecular mechanisms underlying drug resistance using clinical specimens from patients with several cancers. Although mutation of *TP53* is a predictive biomarker for a favorable clinical response,[15] it is not enough to explain clinical responses to DAC and AZA.

In contrast to clinical studies, characterization of resistant subclones (derived from several cancer cell lines by long-term cultivation with drugs) has identified several resistance mechanisms. One such mechanism is dysregulation of enzymes responsible for pyrimidine metabolism, including deoxycytidine kinase (DCK)[16-18] and uridine cytidine kinase (UCK).[16,19,20] DCK and UCK catalyze monophosphorylation of DAC and AZA, respectively.[18,21] This phosphorylation is the rate-limiting step during generation of AZA-dCTP.[18] There are two *UCK* gene products in humans, *UCK1* and *UCK2*, which show 70% sequence homology.[22] Lower expression of *UCK1* was observed in bone marrow mononuclear cells (BMMCs) from patients with MDS and AML that did

not respond to AZA treatment.[19] Meanwhile, expression of *UCK2* is downregulated in BMMCs at relapse.[16] Knock down of UCK1 by siRNA[19] and forced expression of UCK2 containing inactivating mutations[20] increase resistance to AZA. By contrast, expression of *DCK* is also downregulated in BMMCs at relapse after DAC treatment.[16]

Since DNA demethylating agents show potent efficacy against ATL, we plan to perform a clinical trial using OR21. Therefore, it is important to prepare for the appearance of resistant cells. However, at the present time, no DNA demethylating agents have been approved for treatment of ATL, and we had no clinical specimens. OR21 is the prodrug of a typical DNA demethylating agent, DAC and releases DAC through hydrolysis. Another typical DNA demethylating agent, AZA is also cytidine analog, which is used in Japan. OR21, DAC and AZA share a part in structure and mode of actions; and switching the hypomethylating agents at progression from AZA to DAC or vice versa, is conceivable option that has been described as successful in some cases although extensive data are lacking. Therefore, to better understand and compare the molecular mechanisms underlying resistance and cellular responses to cytidine analogs, we established and analyzed AZA-, DAC- and OR21-resistant HTLV-1-infected cells and ATL cells in vitro.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

MT-2 (RRID:CVCL_2631) cells, derived from umbilical cord blood cells transformed by co-culture with peripheral blood mononuclear cells (PBMCs) from a patient with ATL,[23] were purchased from the JCRB Cell Bank (Osaka, Japan). TL-Om1 (RRID:CVCL_B473) cells, derived from PBMCs from a patient with ATL,[24] were provided by Masao Matsuoka (Kumamoto University). The parental cell lines, as well as the AZA-, DAC- and OR21-resistant subclones, were authenticated recently using short tandem repeat profiling (December 2020 and June 2021) at BEX (Tokyo, Japan). There is no STR profile of the original TL-Om1 cells in the database, and we found no matched STR profiles (Evaluation value [EV] > 0.8) of TL-Om1 cells in the Expasy Profile Database (https://web.expasy.org/cellosaurus-str-search/); this suggests that the TL-Om1 cell line is unique, and not cross-contaminated or misidentified. MT-2 and TL-Om1 cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, Missouri) containing 10% fetal bovine serum. To establish AZA- and DAC-resistant cells, cell lines were cultured in the presence of increasing concentrations of AZA and DAC, respectively. All experiments were performed using mycoplasma-free cells. Resistant cell lines were established by long-term cultivation (for over 200 days) in the presence of incrementally increasing doses of drugs (from 50 nM to 8 μM), as indicated in Figure S1.

### 2.2 | Reagents and oligonucleotides

AZA, DAC and etoposide were purchased from Sigma-Aldrich. Cytarabine (Ara-C) was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). OR-2100 (OR21) was provided by OHARA Pharmaceutical Co. (Shiga, Japan). For gene expression analysis, TaqMan assay was used to amplify *THEMIS* (Hs01041269_m1; Applied Biosystems). The primer sequences are listed in Table S1.

### 2.3 | Cell growth assay

Growth of parental cells and each resistant subclone was measured after 4 days of cultivation in the presence of compounds using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technology, Kumamoto, Japan).

### 2.4 | Cell cycle analysis

Cell cycle status was determined by staining the DNA with propidium iodide as described in detail in Supporting Information Method.

### 2.5 | Western blot analysis

The primary antibodies used in our study were anti-DNMT1 (Abcam, Cambridge, UK), anti-tubulin (Cell Signaling Technology, Danvers, Massachusetts), anti-DCK (Proteintech, Rosemont, Illinois), anti-UCK1 (Proteintech), anti-UCK2 (Proteintech) and anti-DCTD (Proteintech). The secondary antibodies were HRP-conjugated sheep anti-mouse IgG (Cytiva, Sheffield, UK) and HRP-linked donkey anti-rabbit IgG (Cytiva). Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Dallas, Texas) and total protein content was measured in a protein assay (Bio-Rad, Hercules, California). Equal amounts (40 μg) of cell lysate were resolved in Nu-polyacrylamide gels (Invitrogen, Waltham, Massachusetts) and transferred onto nitrocellulose membranes. Proteins were detected using antibodies and ECL reagents (Cytiva).

### 2.6 | Bisulfite pyrosequencing

The methylation status of CpG sites in the promoter proximal regions of *LINE-1* and *THEMIS* was determined using pyrosequencing-based analysis as described previously.[7] Briefly, bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Irvine, California). Bisulfite-treated DNA was amplified by PCR using the following primer sets: P-LINE1-F and P-LINE1-R; and P-THEMIS-F and P-THEMIS-R. Pyrosequencing reactions were performed using the PyroMark Q24 (Qiagen, Hilden, Germany) and PyroMark Gold-Q24 reagent kits (Qiagen) and the sequencing primers, P-LINE1-S and P-THEMIS-S.

### 2.7 | Lentivirus preparation and infection

Lentiviral particles used for transduction of human *DCK* cDNA (VB-200306-1506qrn, pLV[Exp]-Neo-EF1A.hDCK[NM_00788.3]:IRES:

EGFP), human UCK2 cDNA (VB-200416-1344pbw, pLV[Exp]-Neo-EF1A.hUCK2[NM_012474.5]:IRES:EGFP) and control GFP/mCherry (VB160109-10005, pLV[Exp]-EGFP:T2A:Puro-EF1A.mCherry), and those used for transduction of shRNA targeting human *DCTD* (VB200627-1068wet, pLV[shRNA]-EGFPU6>hDCTD[shRNA#1] and hDCTD[shRNA#2]) and scramble control shRNA (VB151023-10034, pLV[shRNA]-EGFP/Puro-U6>Scramble_shRNA) were prepared by VectorBuilder (Chicago, Illinois). Cells were infected with lentiviral particles using RetroNectin (Takara) and used for further experiments. In the case of low transduction efficiency, GFP-positive cells were isolated on a FACSAriaII (BD Biosciences) and used for subsequent experiments.

### 2.8 | Gene expression microarray analyses

Total RNA was extracted from resistant cells (which were exposed to each drug over 250 days) using the Direct-zol RNA MiniPrep Kit (Zymo Research). The amount and quality of RNA were determined using an Agilent 4200 TapeStation (Agilent Technologies, Santa Clara, California) and a NanoDrop ND-2000 (Thermo Fisher Scientific). RNA samples were probed using the Clariom S assay (Thermo Fisher Scientific); >20 800 genes were quantified. All procedures were performed at RIKEN Genesis (Osaka, Japan).

### 2.9 | Quantitative real-time PCR

Cells were lysed in TRIzol (Invitrogen), and total RNA was isolated using Direct-zol RNA MiniPrep Kits (Zymo Research). Complementary DNA (cDNA) was produced using ReverTra Ace (TOYOBO, Osaka, Japan), and quantitative real-time PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems) for *THEMIS*. Expression of *ACTB*, *DCK* and *UCK2* was determined using THUNDERBIRD SYBR qPCR Mix (TOYOBO) and the following primer sets: ACTB_F and ACTB-R; DCK-F and DCK-R and UCK2-F and UCK2-R. Expression of *ACTB* was used as an internal control.

### 2.10 | Whole genome sequencing

Genomic DNA was extracted from resistant cells (which were exposed to each drug >250 days) using a QIAamp DNA Mini Kit (Qiagen) and then subjected to whole genome sequencing (WGS) analysis at RIKEN GENESIS (Tokyo, Japan). Briefly, sequencing libraries were constructed using a TrueSeq Nano DNA Library Prep Kit (Illumina, San Diego, California) and sequenced on NovaSeq6000 (Illumina) with $2 \times 150$ bp paired reads. Adapter sequences were removed by cutadapt (ver.1.2.1), and reads were mapped to the reference human genome (hg19) using BWA (ver.0.7.10). Mapping results were corrected using Picard (ver.1.73) to remove duplicates, and GATK (ver.1.6-13) was used for local alignment and quality score recalibration. SNV and Indel calls were performed with multisample calling using GATK, and filtered to coordinates with VQSR passed and a variant call quality score ≥30. SV calls were performed using BreakDancer (ver.1.4.5) and Pindel (ver.0.2.5b8). Annotations of variants were based on dbSNP151, CCDS (NCBI, Release 15), RefSeq (UCSC Genome Browser, February 2018), Gencode (UCSC Genome Browser, ver.19) and 1000Genomes (phase3 release ver.5). The sequencing coverage and quality statistics for each sample are summarized in Table S2.

### 2.11 | Validation of the deletion mutation in *DCK*

The heterozygous deletion mutation in the *DCK* gene identified through WGS was validated by genomic PCR using primers (G-DCK-F and G-DCK-R) flanking the deletion breakpoints. PCR products were resolved in agarose gels and detected by staining with ethidium bromide.

### 2.12 | Validation of the donor splice site mutation in *UCK2*

The aberrant splicing caused by a homozygous point mutation at the donor splice site of intron 2 of *UCK2* was confirmed by PCR using cDNA as a template and primers (C-UCK2-F: 5′-GGGCAGAATGAGGTGGACTA-3′ and C-UCK2-R: 5′-GGGAATGGGAGACAAAGTCA-3′) flanking exon 2 and exon 3. PCR products were resolved in agarose gels and detected by staining with ethidium bromide. The sequence of PCR products was determined by direct Sanger sequencing using the same primers. Sanger sequencing was performed at Eurofin Genomics (Ebersberg, Germany).

## 3 | RESULTS

### 3.1 | Generation and cellular phenotype of AZA, DAC and OR21-resistant ATL cell lines

We used three different DNA demethylating agents: AZA, DAC and OR21. AZA and DAC are metabolized into AZA-dCTP, which is incorporated into DNA. OR21 is a prodrug of DAC that releases DAC through hydrolysis (Figure 1A). We previously reported that DNA hypomethylating agents, including AZA, DAC and OR21, suppressed tumor cell growth with DNA hypomethylation in HTLV-1-infected cell lines and ATL cell lines. The potencies of agents did not reflect cellular phenotypes such as origin of cells (established from clinical specimens or in vitro transformation), expression of onco-protein Tax, dependency on IL-2 and DNA methylation status.[7] Therefore, in this experiment, to clearly show the resistant phenotypes, we simply chose most susceptible cell lines to establish resistant cells. MT-2 cells were the most susceptible to AZA (IC50: 0.5 μM), and TL-Om1 cells to DAC (IC50: 0.04 μM), among the six ATL and two HTLV-1-infected cell lines (IC50 of AZA: 0.5-2.39 μM; DAC: 0.04-0.26 μM) tested,[7] we

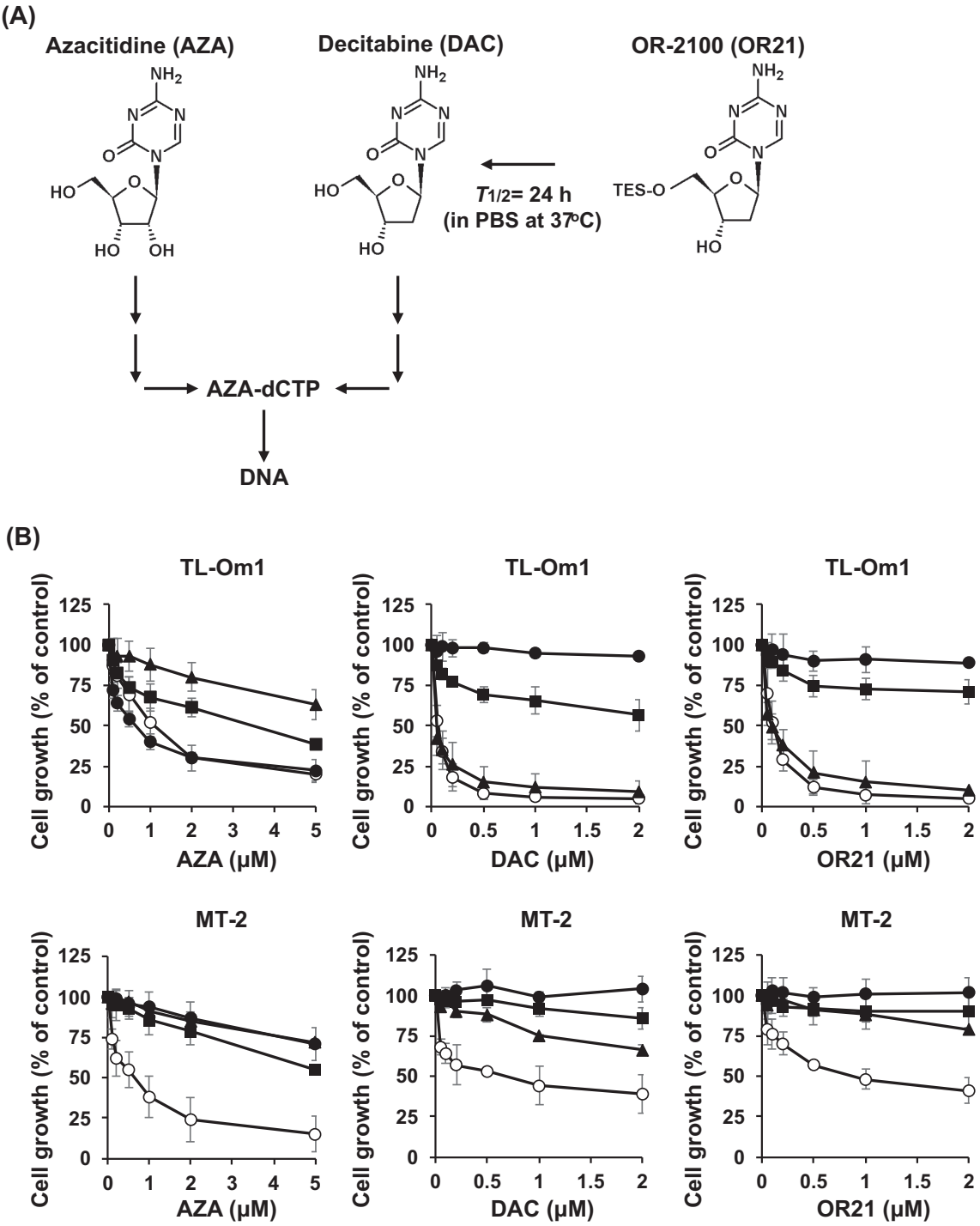


**FIGURE 1** Susceptibility of each established drug-resistant cell line and parental cell line to AZA, DAC and OR21. (A) Structure and metabolism of pyrimidine analogs. (B) DAC-R (●), AZA-R (▲) and OR21-R (■) cells derived from TL-Om1 and MT-2 cells and each parental cell line (○) were treated with different concentrations of AZA, DAC and OR21. Cell growth was assessed using CCK-8 reagent, as described in the Section 2. The absorbance of nontreated cells was defined as 100%. The results are expressed as the mean of three independent experiments ± SD

generated AZA-, DAC- and OR21-resistant cells from both MT-2 and TL-Om1 cells. After long-term cultivation in the presence of incrementally increased doses of drugs, each cell line grew in the presence of these compounds (Figure S1). AZA-treated cells are named AZA-R, DAC-treated cells are named DAC-R and OR21-treated cells are named OR21-R. Next, we compared the effects of AZA, DAC and OR21 on AZA-R, DAC-R and OR21-R cells, and on parental cells. AZA-R cells derived from TL-Om1 were sensitive to DAC and DAC-R cells were sensitive to AZA (Figure 1B). However, DAC-R cells derived from MT-2 were resistant to AZA and AZA-R cells exhibited reduced sensitivity to DAC (Figure 1B). OR21-R cells derived from both MT-2 and TL-Om1 were less susceptible to AZA and DAC (Figure 1B). The

growth inhibitory effects of OR21 in each cell line were almost the same as those of DAC (Figure 1B).

AZA, DAC and OR21 are processed into AZA-dCTP, which is incorporated into DNA. DNMT1 protein is trapped at the AZA-dCTP sites and is then degraded by the ubiquitin-proteasome pathway.[8] To better understand whether these cellular responses are intact in each resistant cell line, we measured the amount of DNMT1 protein. Each compound depleted DNMT1 protein from parental cells; however, although treatment of DAC-R TL-Om1 cells with AZA decreased the amount of DNMT1 protein, treatment with DAC did not. By contrast,

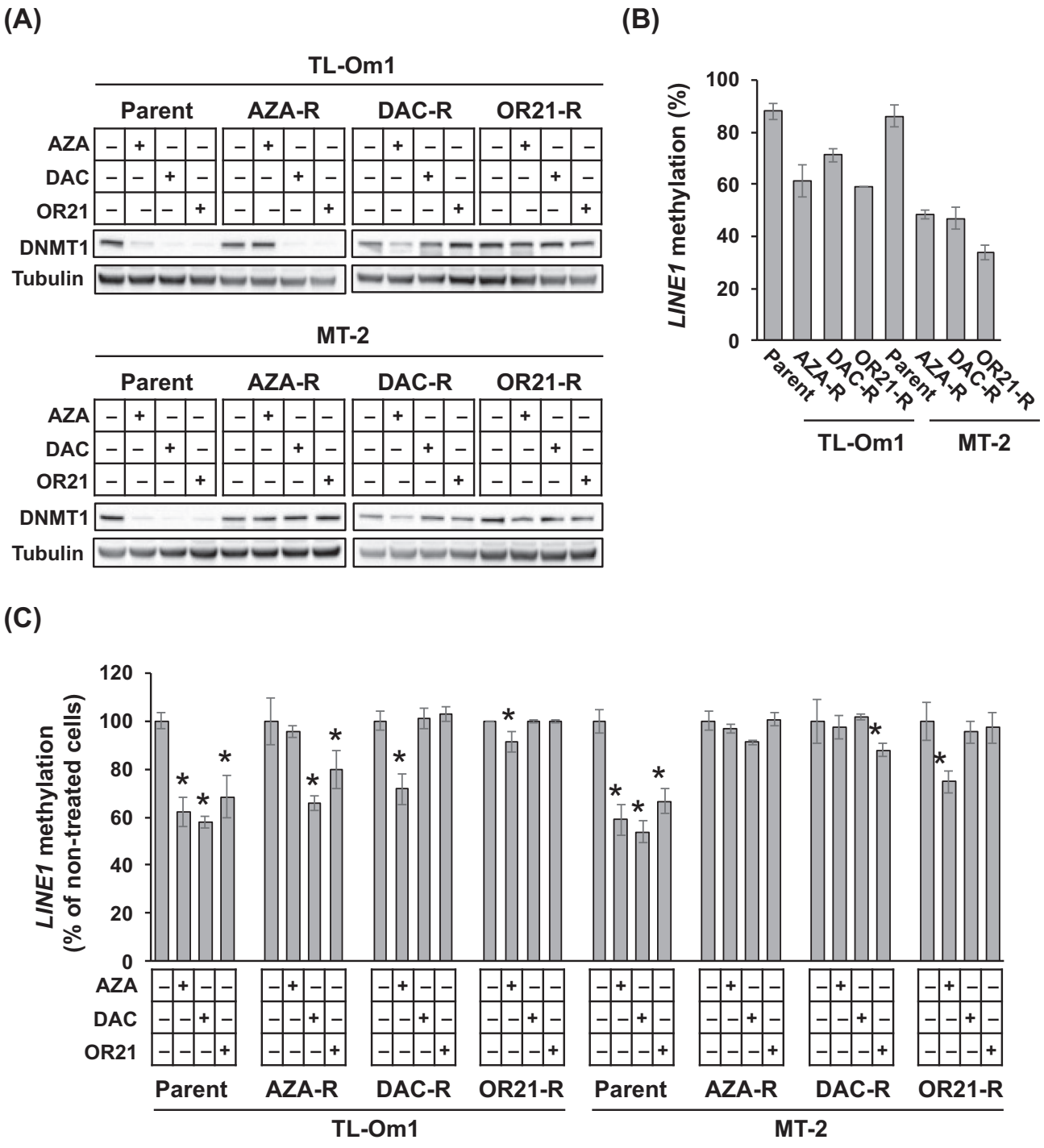


**FIGURE 2** Cellular responses to AZA, DAC and OR21 in each established drug-resistant cell line and parental cell. (A) Immunoblots showing the amounts of DNMT1 protein in each cell line after treatment with 100 nM AZA, 50 nM DAC or 50 nM OR21 for 4 days. (B) DNA methylation levels in the *LINE-1* regions, as determined by bisulfite pyrosequencing of each cell line. Data are expressed as the means of three independent experiments ± SD. (C) *LINE-1* DNA methylation in each cell line treated with the indicated compounds for 2 days. The methylation level of nontreated cells was defined as 100%. The results are expressed as the mean of three to five independent experiments ± SD

the amount of DNMT1 protein in AZA-R TL-Om1 cells decreased after treatment with DAC but not after treatment with AZA (Figure 2A). Treatment of DAC-R MT-2 cells with AZA partially reduced the amounts of DNMT1 protein, but DAC did not. AZA-R MT-2 cells, neither AZA nor DAC decreased DNMT1 levels (Figure 2A). Treatment with AZA decreased DNMT1 levels slightly in OR21-R cells derived from TL-Om1 and MT-2. Treatment with OR21 induced cellular responses similar to those induced by DAC (Figure 2A), as shown by the growth inhibitory effects. To determine DNA methylation status, we measured DNA methylation at long interspersed nuclear element-1 (LINE-1), a surrogate marker for global DNA methylation.[25] The basal methylation status of all resistant cell lines was lower than that of the respective parental cells (Figure 2B). Treatment with AZA, DAC and OR21 reduced LINE-1 methylation in both of TL-Om1 and MT-2 parental cells by about 60%. Treatment with AZA and DAC decreased *LINE-1* methylation in DAC-R and

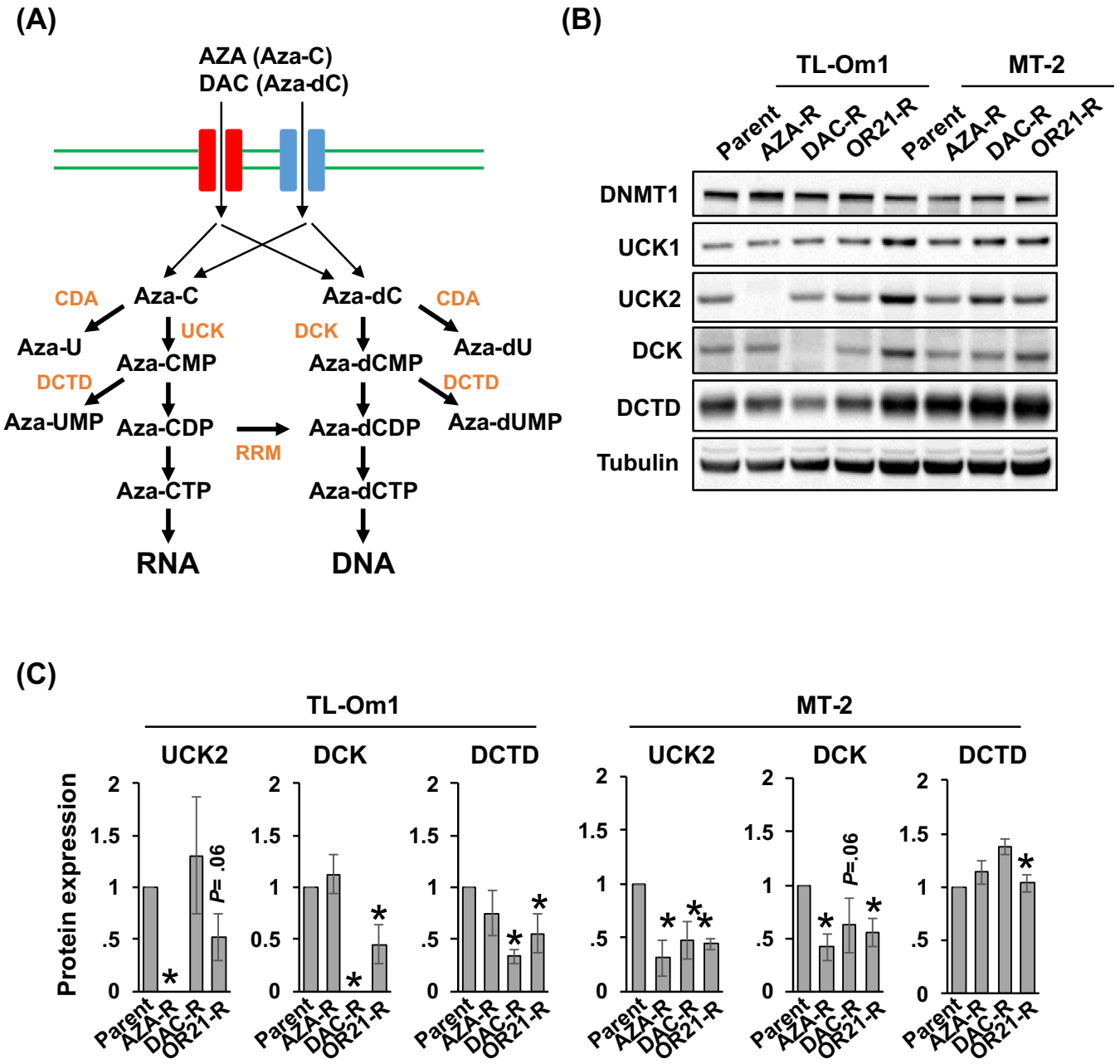


**FIGURE 3** Dysregulation of enzymes regulating pyrimidine metabolism in drug-resistant cells. (A) Metabolic pathway of DAC and AZA. AZA and DAC undergo multistep phosphorylation, and are incorporated into DNA and RNA. (B) Amounts of enzymes for pyrimidine metabolism enzymes (DCK, UCK2, UCK1 and DCTD) in each drug-resistant cell line and parental cell line were determined by immunoblotting. (C) Signal intensity of UCK2, DCK and DCTD on western blots. Graphs show relative expression levels (compared to each parental cell line), after normalization to tubulin expression

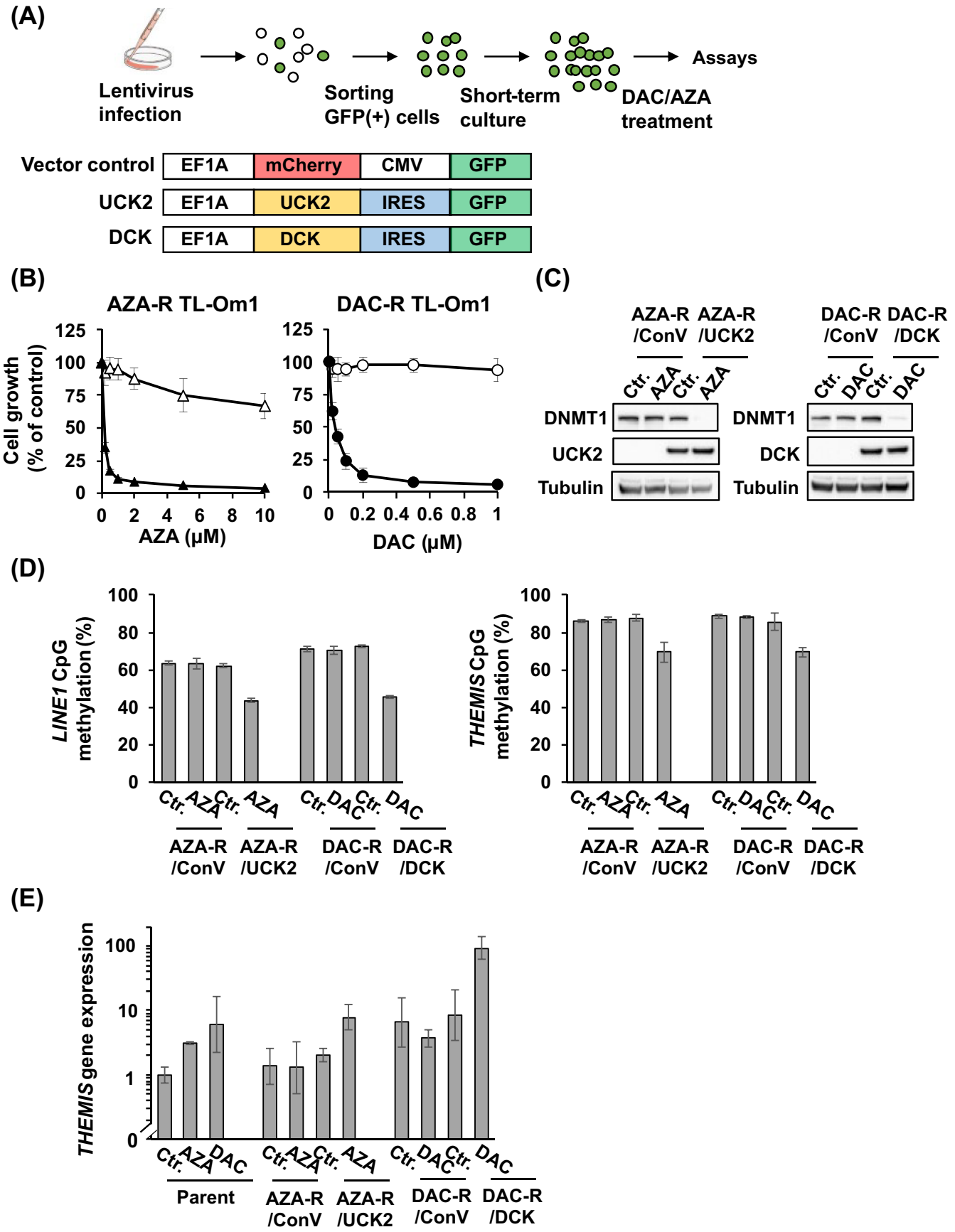


FIGURE 4 Legend on next page.

AZA-R TL-Om1 cells, respectively (Figure 2C). By contrast, there was almost no reduction in both AZA-R and DAC-R MT-2 cells; DAC induced only a slight decrease in AZA-R cells (Figure 2C). Treatment with AZA decreased methylation levels slightly in OR21-R cells derived from TL-Om1 and MT-2 (Figure 2C). Again, treatment with OR21 induced similar cellular responses to DAC (Figure 2C). Taken together, the data suggest that DNA demethylation was induced in the same manner as DNMT1 depletion.

High doses of DAC and AZA induced cell cycle arrest at $G_2$/M phase, along with cytotoxic effects. As expected, DAC-R and AZA-R TL-Om1 cells treated with AZA and DAC, respectively, arrested at $G_2$/M phase (Figure S2). The effects of DAC and AZA on the cell cycle were much weaker in MT-2 cells than in TL-Om1 cells, and neither DAC nor AZA induced significant changes in resistant MT-2 cells (Figure S2).

### 3.2 | Dysregulation of pyrimidine metabolism enzymes in resistant cells

Although DAC and AZA did not arrest the cell cycle at $G_2$/M phase in resistant subclones, treatment with etoposide suppressed cell growth and induced $G_2$/M cell cycle arrest (Figure S3A,B). We found that DAC-R cells derived from both TL-Om1 and MT-2 showed reduced susceptibility to another pyrimidine analog, Ara-C (Figure S3C). Based on these results, we hypothesized that AZA-CTP derived from AZA, DAC and OR21 is not incorporated into DNA in resistant cells due to dysregulation of the pyrimidine metabolism pathway. Therefore, to identify molecular mechanisms responsible for acquired resistance, we first focused on enzymes involved in pyrimidine metabolism (Figure 3A). Expression of uridine cytidine kinase 2 (UCK2), which phosphorylates uridine and cytidine to yield uridine monophosphate and cytidine monophosphate, respectively, was not detected in AZA-R TL-Om1 cells, and levels were decreased in OR21-R TL-Om1 cells (Figure 3B,C). Levels were also reduced in AZA-R, DAC-R and OR21-R MT-2 cells (Figure 3B,C). By contrast, DAC-R TL-Om1 cells did not express deoxycytidine kinase (DCK), which catalyzes phosphorylation of deoxycytidine, deoxyguanosine and deoxyadenosine to the monophosphate form (Figure 3B,C). It was downregulated in OR21-R TL-Om1 cells and all three resistant cell lines derived from MT-2 (Figure 3B,C). However, there was no significant differences in expression level of UCK1 in each cell line. The amount of deoxycytidylate deaminase (DCTD), which converts deoxycytidine monophosphate to deoxyuridine monophosphate, increased in DAC-R MT-2 cells (Figure 3B,C).

### 3.3 | Reconstitution of UCK2 and DCK expression restores sensitivity to AZA and DAC, respectively

Since enzymes responsible for pyrimidine metabolism are also involved in processing pyrimidine analogs such as AZA and DAC/OR21, we next determined whether inactivation of these enzymes contributes to acquired resistance to AZA and DAC. Since expression of UCK2 and DCK was disappeared completely from AZA-R and DAC-R TL-Om1 cells, respectively (Figure 3B,C), we thought that they were the most suitable for the rescue experiments. We transduced AZA-R and DAC-R TL-Om1 cells with lentiviral vectors expressing the *UCK2* and *DCK* genes, respectively (Figure 4A). AZA-R TL-Om1 cells infected with vector control lentivirus still exhibited strong resistance to AZA, whereas TL-Om1 AZA-R cells exogenously expressing UCK2 protein exhibited strong growth inhibition after treatment with AZA (Figure 4B). Exogenous expression of DCK protein in DAC-R TL-Om1cells also restored susceptibility to DAC (Figure 4B). On the other hands, AZA-R MT-2 cells were less susceptible to AZA and DAC with reduced expression of UCK2 and DCK as shown in Figures 1B and 3B. Exogenous expression of UCK2 and DCK proteins rescued susceptibility to AZA and DAC, respectively (Figure S4A-D).

As expected, DNMT1 depletion and DNA demethylation at the *LINE-1* region induced by AZA and DAC were observed in AZA-R TL-Om1cells exogenously expressing UCK2 protein, and in DAC-R TL-Om1 cells exogenously expressing DCK protein (Figure 4C,D). Recently, we showed that expression of *THEMIS* is downregulated via DNA hypermethylation in HTLV-1-infected cells compared to normal T-cells.[7] Here, we found that DNA hypomethylation and expression of *THEMIS* were also induced in UCK2-rescued AZA-R cells and DCK-rescued DAC-R cells (Figure 4D,E). We speculated that overexpression of DCTD, which was observed in DAC-R MT-2 cells, also contributes to increased resistance to DAC because DCTD inactivates pyrimidine analogs via deamination of substrates. Transduction of shRNA targeting *DCTD* led to marked reduction in the amount of DCTD protein (Figure S5A,B). However, knockdown of DCTD did not affect susceptibility of TL-Om1 DAC-R cells to DAC (Figure S5C).

**FIGURE 4** Reconstitution of UCK2 and DCK expression rescues susceptibility to AZA and DAC, respectively, in drug-resistant cells. (A) Experimental protocol generating drug-resistant TL-Om1 cells exogenously expressing UCK2 or DCK, and subsequent drug treatment. (B) AZA-R exogenously expressing UCK2 (●) or mCherry (vector control) (○) were treated with AZA after GFP-sorting (left). DAC-R exogenously expressing DCK (▲) or mCherry (vector control) (○) were treated with DAC after GFP-sorting (right). Cell growth was accessed using CCK-8 reagent 4 days after treatment. The absorbance of nontreated cells was defined as 100%. The results are expressed as the mean of three independent experiments ± SD. (C) Immunoblots showing the amounts of DNMT1, UCK2 and DCK in cells after treatment with 100 nM AZA or 50 nM DAC for 4 days. (D) DNA methylation levels in the *LINE-1* and *THEMIS* promoter regions, as determined by bisulfite pyrosequencing of each cell line treated with 100 nM AZA or 50 nM DAC for 2 days. Data are expressed as the means of three independent experiments ± SD. (E) *THEMIS* expression in cells treated with 100 nM AZA or 50 nM DAC for 4 days was measured by real-time PCR. Graph shows the mean-fold increase (±SD) in *THEMIS* expression compared to that in untreated parental cells, based on data from three independent experiments

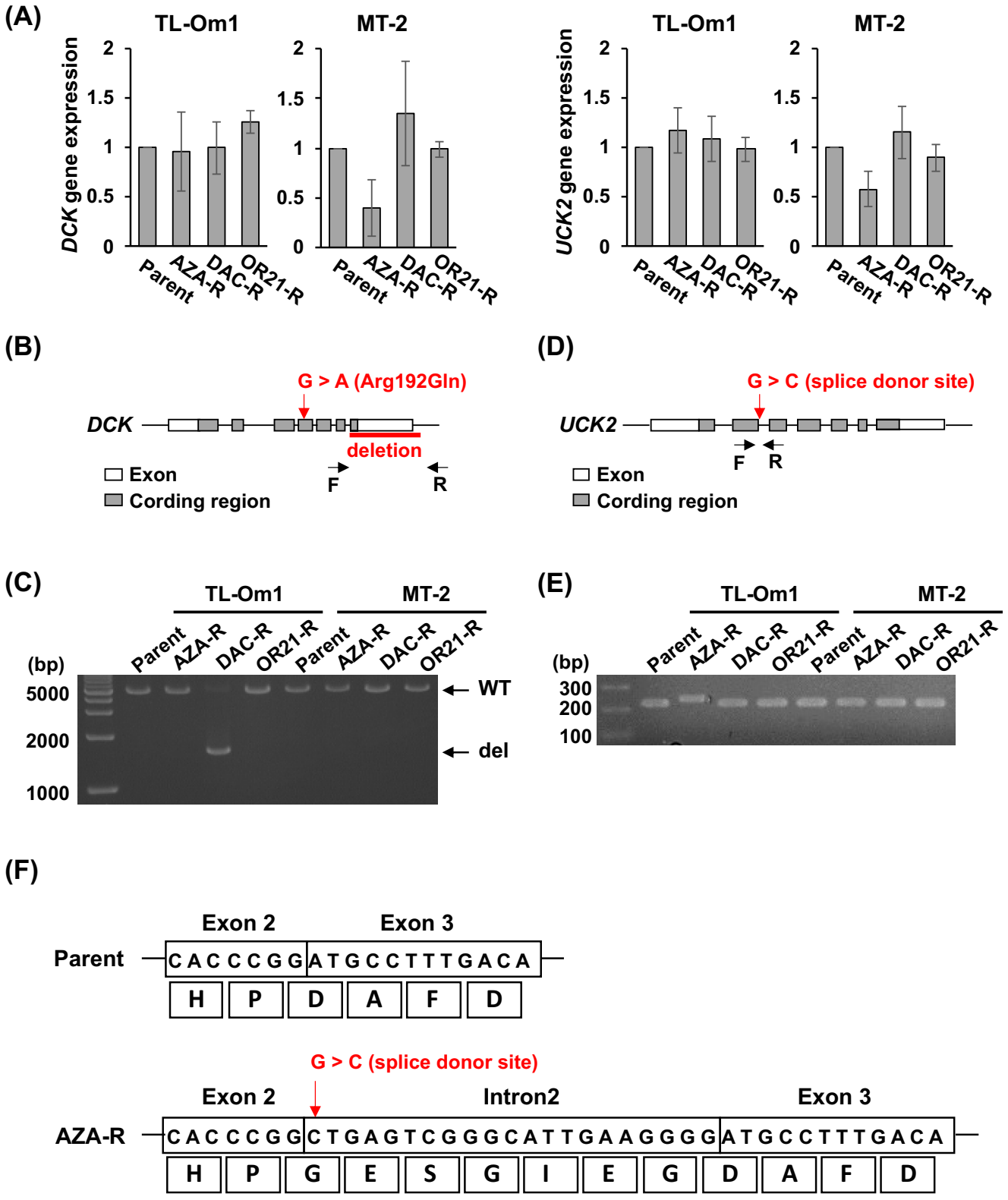


**FIGURE 5** Loss of DCK and UCK2 expression is caused by genetic mutation of DAC-R and AZA-R, respectively, in TL-Om1. (A) Expression of *DCK* and *UCK2* was quantified by real-time PCR. Graphs show relative expression levels (compared to each parental cell line), after normalization to *ACTB* mRNA levels. The results are expressed as the mean of three independent experiments ± SD. (B) Structure of DCK in DAC-R TL-Om1 cells, along with the genetic mutations identified by whole genome sequencing: a heterozygous large deletion mutation that involves exon 7 and the 3′-untranslated region, and a low frequency missense mutation that results in amino acid substitution (Arg192Gln). (C) The deletion mutation in DAC-R TL-Om1 cells was validated by genomic PCR. (D) Structure of *UCK2* in AZA-R TL-Om1 cells, along with the homozygous splice donor site mutation in intron 2 identified by whole genome sequencing. (E) Aberrantly spliced form detected in AZA-R TL-Om1 by PCR using cDNA as a template. (F) Sequence of *UCK2* cDNA in DAC-R TL-Om1 cells and parental TL-Om1 cells, as determined by Sanger sequencing

Thus, we think the cells acquired resistance to AZA and DAC through downregulation of UCK2 and DCK, respectively.

### 3.4 | Mutations in *DCK* and *UCK2* gene are present in DAC-R and AZA-R TL-Om1 cells, respectively

To understand the mechanisms underlying reduced protein expression of DCK and UCK2 in resistant cells, we first examined expression of mRNA encoding *DCK* and *UCK2*. Although reduced expression of UCK2 and DCK protein by AZA-R MT-2 cells was concomitant with a decrease in the gene expression, expression of *UCK2* and *DCK* in other resistant cells was comparable with that in the respective parental cells (Figure 5A). Since UCK2 and DCK were not detected in AZA-R and DAC-R TL-Om1 cells, respectively, we next searched for genetic abnormalities by performing WGS and identified genetic mutations in *DCK* and *UCK2*. A heterozygous large deletion (roughly 3 kbp) involving exon 7 and the 3′-untranslated region, and a low frequency missense mutation, which results in an amino acid substitution (Arg192Gln), were found in *DCK* of DAC-R TL-Om1 cells (Figures 5B and S6A,B). The deletion mutation was confirmed by genomic PCR and was observed only in TL-Om1 DAC-R cells, but in a homozygous manner (Figure 5C). A homozygous point mutation at the splice donor site in *UCK2* was identified in AZA-R TL-Om1 cells (Figures 5D and S6C). As expected, an aberrant splicing form was detected in AZA-R TL-Om1 cells by PCR using cDNA as a template (Figure 5E); the aberrant 5′ splice site was located 21 bp downstream from the authentic junction (Figure S7).

## 4 | DISCUSSION

Here, we established AZA-, DAC- and OR21-resistant cells from HTLV-1-infected cells (MT-2) and the ATL cell line (TL-Om1). Although both AZA and DAC are used clinically as DNA demethylating agents, their metabolic enzymes and pharmacodynamics are slightly different.[8] Therefore, AZA-R TL-Om1 maintained susceptibility to DAC and DAC-R TL-Om1 to AZA (Figure 1B). OR21, a prodrug of DAC, was developed recently.[7,14] Cellular responses, including growth suppression (Figure 1B), DNMT1 depletion (Figure 2A) and DNA demethylation (Figure 2C) induced by OR21 in each cell line, were almost the same as those induced by DAC. Since OR21 is a silylated derivative of DAC and releases DAC through hydrolysis,[7,14] the pharmacodynamics of OR21 are thought to be derived from released DAC. We think that this is the reason why the cellular responses induced by DAC and OR21 were almost the same.

DAC and OR21 decreased in DNMT1 protein expression and induced DNA hypomethylation in AZA-R TL-Om1 cells, but not in other resistant cells. By contrast, AZA affected DAC-R and OR21-R TL-Om1, and OR21-R MT-2, but not other resistant cells. Our western blotting results showed loss or downregulation of UCK2 expression by AZA-R and OR21-R TL-Om1, and by AZA-R, DAC-R and OR21-R MT-2 cells (Figure 3B), all of which showed decreased cellular responses to AZA, such as cell growth inhibition, DNMT1 reduction and DNA demethylation. Recently, we established an AZA-resistant HL-60 cell line and confirmed that UCK2 expression was downregulated significantly compared to that by the parental cells.[26] Similarly, loss or downregulation of DCK expression were observed in DAC-R and OR21-R TL-Om1 cells, and AZA-R, DAC-R and OR21-R MT-2 cells (Figure 3B), which showed decreased cellular responses to DAC/OR21, such as cell growth inhibition, DNMT1 reduction and DNA demethylation. As expected, reconstitution of UCK2 and DCK expression in both of TL-Om1 and MT-2 cells clearly rescued susceptibility to AZA and DAC, respectively (Figures 4B and S4C). Thus, we think the mechanisms of acquired resistance to AZA and DAC were same in both cell lines and the differences of cellular phenotypes of each resistant subclone were due to the expression status of UCK2 and DCK.

However, there is an issue left unsolved; why the UCK2 and DCK expression status was different in AZA-R and DAC-R cells derived from TL-Om1 and MT-2. TL-Om1 cells derived from patients with acute ATL and MT-2 cells were derived from umbilical cord blood cells transformed by co-culture with PBMCs from a patient with ATL. MT-2 cells highly express viral protein Tax, which contributes to leukemogenesis. Both cell line does not depend on IL-2 in their growth. We tried to identify biological factors, which determine expression status of UCK2 and DCK, but failed to discover such a determinant. Both of UCK2 and DCK are involved in pyrimidine synthesis, especially salvage pathway. So, we now speculate that nucleotides metabolism might affect the dependency and expression level of UCK2 and DCK.

Cytarabine and gemcitabine are deoxycytidine analogs used clinically as chemotherapy agents. Both drugs undergo monophosphorylation catalyzed by DCK, which is the rate-limiting step during processing of these compounds to the active forms.[18,27,28] It is important to note that loss or inactivation of DCK has been observed in cytarabine- and gemcitabine-resistant cell lines established in vitro.[29-31] However, UCK2 also catalyzes phosphorylation of 3′-ethynylcytidine (TAS-106), another cytidine analog and is a key enzyme that processes it into the active form.[32] The triphosphate of TAS-106 is an active metabolite that inhibits RNA synthesis by blocking RNA polymerases. A splice site mutation in UCK2 (IVS5+5G>A), which produces a truncated protein, was identified in a TAS-106-resistant cell line.[33] Thus, we think that DCK and UCK2 are related to susceptibility to cytidine analogs.

Although expression of *DCK* and *UCK2* was reduced in DAC-R MT-2 cells, other resistant cells, in which DCK and UCK2 protein expression was reduced or absent, showed expression of *DCK* and *UCK2* that was comparable with that of their parental cells (Figure 5A). Among them, DAC-R and AZA-R TL-Om1 showed genomic mutations in DCK and UCK2. Although a heterozygous deletion mutation in DCK was observed in WGS (Figure S6B), only very low levels of a wild-type PCR product were observed in TL-Om1_DAC-R (Figure 5B,C). Since we did not isolate a single cell clone, the wild-type form of TL-Om1_DAC-R was derived from a minor cell population with a wild-type allele. We think that most TL-Om1_DAC-R cells harbored a homozygous deletion in the *DCK* gene. We also think that

since the deletion mutation contained exon 7 (which contains a termination codon) and the 3′-untranslated region, protein expression disappeared.

A missense mutation, which results in amino acid substitution Arg192Gln, was observed in *DCK* of DAC-R TL-Om1 cells (Figure 5B). Although the variant allele frequency was low (26%) (Figure S6B), the mutation was predicted to be damaging based on the Sorting Intolerant from Tolerant scores. Since no DCK protein expression was observed in DAC-R TL-Om1, the biological significance of the mutation remains unclear. By contrast, a splice donor site mutation in *UCK2* was identified in AZA-R TL-Om1 cells, which led to production of an aberrant spliced form with a 21 bp insertion (seven amino acids; Figure 5D-F). It remains unclear why the translated product was not observed on western blots (Figure 3C).

Several other candidate molecules involved in acquired resistance to pyrimidine analogs have been reported. Cytidine deaminase (CDA) catalyzes the hydrolytic deamination of cytidine to uridine. Thus, it inactivates cytidine analogs (including AZA, DAC, cytarabine and gemcitabine). CDA activity in serum predicts severe/lethal toxicity caused by cytarabine in patients with AML receiving induction therapy.[34] Conversely, overexpression of CDA increased tolerance to cytidine analogs in various cells, such as hematopoietic progenitor cells[35] and cancer cell lines derived from AML,[36] MDS[36] and gastric cancer.[37] However, expression of *CDA* in normal T-cells is very low (The Blood Atlas, http://www.proteinatlas.org),[38] and our microarray data showed that *CDA* expression was undetectable in both parental and resistant cells (data not shown). In addition, although OR21 escapes CDA-mediated degradation through sylilation,[14] the efficacy of OR21 was almost the same as that of DAC (Figure 1B). Taken together, the data suggest that CDA is not related to acquired resistance in our resistant cell lines.

Alternatively, we observed increased expression of DCTD in DAC-R MT-2 (Figure 3B,C). Since DCTD acts as a deoxycytidylate deaminase, we hypothesized that inactivation of DAC was induced more efficiently in DAC-R than in parental cells. However, knockdown of *DCTD* in DAC-R cells did not affect susceptibility to DAC (Figure S5B,C). Gemcitabine is also inactivated by DCTD, and protein expression determined by immunohistochemical staining is neither prognostic nor predictive in patients with pancreatic adenocarcinoma who had had postoperative chemotherapy with either gemcitabine or 5-fluorouracil (5-FU) with folinic acid.[39] Based on these results, we think the amount of DCTD might not affect the efficacy of cytidine analogs.

Several of the solute carrier group of membrane transport proteins, including SLC15, SLC28, SLC28 and SLC29, transport pyrimidine analogs[8] and are related to acquired resistance to Ara-C.[29] Also, MDS patients responding to decitabine show significantly higher expression of *SLC29A1* than nonresponders.[40,41] Sine AZA-R and DAC-R MT-2 cells were tolerant to AZA, DAC and OR21, we performed transcriptome analysis using a Clariom S microarray (data not shown); however, we found no significant differences.

In contrast to data derived from artificially established drug-resistant cell lines, there is limited evidence from clinical specimens to support the contribution of DCK and UCK2 to resistance to DAC and AZA, respectively. Although decreased expression of *DCK* and *UCK2* has been observed in clinical specimens from patients with AML at relapse after DAC and AZA treatment,[16] to the best of our knowledge, no reports showing a link of genetic mutations in *DCK* and *UCK2* and therapeutic effects. Since loss of UCK2 expression also occurred through a point mutation at a splice donor site (Figure 5D-F), conventional exon sequencing could not detect it. Thus, deep RNA sequencing or whole genome sequencing (WGS) are needed to validate the contribution of genetic abnormalities in UCK2 and DCK to therapeutic effects.

It is well known that monotherapy using DAC or AZA shows clinical benefit in patients with MDS and AML.[10] Recently, combination therapy with AZA or DAC plus other anticancer drugs has been tested in clinical trials.[10] Among them, combination with the Bcl-2 inhibitor Venetoclax showed clinical efficacy, tolerable safety and favorable overall response rates in patients with AML.[42] Therefore, more new regimens containing AZA or DAC will be developed and used clinically in the future. However, both cytidine analogs are easily inactivated by CDA and can only be administered by injection.

Since OR21 shows potent anti-ATL effects and enhanced oral bioavailability, with lower hematotoxicity than DAC,[7] we plan to evaluate the efficacy and safety of OR21 in patients with acute and lymphoma ATL in forthcoming clinical trials. Meanwhile, ASTX727[13] (a combination drug comprising DAC and the CDA inhibitor Cedazuridine) and SGI-110[43] (a dinucleotide of DAC and deoxyguanosine), has been developed. Both drugs are resistant to CDA-mediated degradation, and ASTX727 can be administered orally (although SGI-110 cannot). Since the pharmacological action of DAC prodrugs, including OR21 is due to released DAC, we think that the same drug resistance mechanisms will be observed in patients treated with these prodrugs. Taken together, the data presented in this article suggest that inactivation of DCK and UCK2 is one of the mechanisms to induce resistance to DAC and AZA, respectively; therefore, it is important to monitor their status during DNA demethylating chemotherapy for switching cytidine analogs to achieve better treatment outcome.

## ACKNOWLEDGMENTS

The authors would like to thank Professor Masao Matsuoka at Kumamoto University for providing the TL-Om1 cell line. Flow cytometric analyses, pyrosequencing and real-time RT-PCR were conducted at the Analytical Research Center for Experimental Sciences, Saga University. This work was supported by Japan Society for the Promotion of Science KAKENHI, Grant Numbers JP17H06956 and JP20K07593, by Nippon Shinyaku Research Grant and by OHARA Pharmaceutical Co. (who provided partial financial support).

## CONFLICT OF INTEREST

Yuki Kurahashiand Yuki Fukuda-Kurahashi are full-time employees of OHARA Pharmaceutical Co. Tatsuro Watanabe received grant from Nippon Shinyaku Co. Shinya Kimura received grant from OHARA Pharmaceutical Co about this work. Shinya Kimura also received

grants and personal fees from Bristol-Myers Squibb, Pfizer and Novartis Pharmaceuticals outside of the submitted work. The other authors have no potential conflicts of interest to declare.

## DATA AVAILABILITY STATEMENT

The microarray data generated in our study are available in GEO under accession number GSE166980 and the raw WGS data are in DDBJ under accession number DRA011609 (PRJDB11215). Other data are available from the authors upon reasonable request.

## ORCID

*Tatsuro Watanabe* https://orcid.org/0000-0002-4166-4781

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## SUPPORTING INFORMATION

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**How to cite this article:** Yoshida-Sakai N, Watanabe T, Yamamoto Y, et al. Adult T-cell leukemia-lymphoma acquires resistance to DNA demethylating agents through dysregulation of enzymes involved in pyrimidine metabolism. *Int. J. Cancer*. 2022;150(7):1184-1197. doi:10.1002/ijc.33901