Original article

Silylation of deoxynucleotide analog yields an orally available drug with anti-leukemia effects

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Abstract

DNA methyltransferase inhibitors have improved the prognosis of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). However, because these agents are easily degraded by cytidine deaminase (CDA), they must be administered intravenously or subcutaneously. Recently, two orally bioavailable DNA methyltransferase inhibitors, CC-486 and ASTX727, were approved. In previous work, we developed 5-O-trialkylsilylated decitabines that resist degradation by CDA. However, the effects of silylation of a deoxynucleotide analog and enzymatic cleavage of silylation have not been fully elucidated. Enteric administration of OR21 in a cynomolgus monkey model led to high plasma concentrations and hypomethylation, and in a mouse model, oral administration of enteric-coated OR21 led to high plasma concentrations. The drug became biologically active after release of decitabine (DAC) from OR21 following removal of the 5'-O-trisilylate substituent. Toxicities were tolerable and lower than those of DAC. Transcriptome and methylome analysis of MDS and AML cell lines revealed that OR21 increased expression of genes associated with tumor suppression, cell differentiation, and immune system processes by altering regional promoter methylation, indicating that these pathways play pivotal roles in the action of hypomethylating agents. OR21 induced cell differentiation via upregulation of the late cell differentiation drivers CEBPE and GATA-1. Thus, silylation of a deoxynucleotide analog can confer oral bioavailability without new toxicities. Both in vivo and in vitro, OR21 exerted anti-leukemia effects, and had a better safety profile.
than DAC. Together, our findings indicate that OR21 is a promising candidate drug for phase 1 study as an alternative to azacitidine or decitabine.

**Keywords:** silylation, hypomethylating, orally available, OR-2100, myelodysplastic syndrome, acute myeloid leukemia
Introduction

Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are characterized by impaired differentiation of hematopoietic stem cells (HSCs) or hematopoietic progenitor cells (1,2). Somatic mutations and aberrant DNA hypermethylation that silence cancer-regulating genes (e.g., tumor-suppressor genes) in these cells (3) are involved in pathogenesis and progression of MDS and AML (4–8). Epigenetic alterations lead to reduced hypermethylation, which can in turn cause re-expression of silenced tumor-regulating genes. These alterations represent novel therapeutic targets for both of these cancers (9).

Although the clinically available DNA methyltransferase (DNMT) inhibitors azacitidine (AZA) and decitabine (DAC) have improved the prognosis of MDS and AML (10,11), they are easily degraded by cytidine deaminase (CDA), limiting their bioavailability after oral administration; consequently, they must be administered intravenously or subcutaneously. The efficacy and safety of oral AZA or DAC, as well as the combination of these drugs with CDA inhibitors, has been evaluated in clinical trials (12–14), resulting in the approval of CC-486 (oral AZA) and ASTX727 (DAC plus cedazuridine, a CDA inhibitor) (13,14).

Resistance to CDA is crucial for achieving elevated plasma concentrations of these drugs, which would in turn increase efficacy (15). Silylation of glycoproteins is known to influence their stabilities and therapeutic effects (16,17); however, the effects of silylation of deoxynucleotide analogs and enzymatic cleavage of silylation have not been fully elucidated. In previous work, we developed 5-O-trialkylsilylated DACs that
were resistant to CDA and exerted anti-cancer effects against solid tumors and adult T-cell leukemia/lymphoma (18,19). In this study, we assessed the oral bioavailability and efficacy of 5-O-trialkylsilylated DAC for treatment of MDS and AML both in vitro and in vivo.

Materials and methods

Reagents

5-O-trialkylsilylated DAC (OR2100, 2003, 2007, 2008, 2009, 2010, 2102, 2103, 2104, and 2201) was obtained from Ohara Pharmaceutical (Shiga, Japan). AZA and DAC were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were dissolved in dimethylsulfoxide (DMSO) and stored at -20°C.

Pharmacokinetic (PK) studies in cynomolgus monkeys and mice

All procedures were performed at NISSEI BILIS. The study was compliant with Ohara Pharmaceutical policies on bioethics in animal work, and all procedures were compliant with the relevant national and international guidelines for the care and use of experimental animals. Procedures for PK studies are described in Supplementary methods.

Cell lines and cultures

The MDS-L cell line was derived from the non-leukemic phase of a patient with MDS with refractory anemia-ringed sideroblasts (20). SKM1 cells (derived from the blast
cells of a patient with MDS) were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). HL60, THP-1, KG1a, and Kasumi-1 cell lines (derived from AML patients) were purchased from the American Type Culture Collection (Rockville, MD, USA). AZA-resistant HL60 (HL60R) cells were established. Detailed cell culture methods are described in Supplementary methods.

Primary patient samples

Primary samples were obtained from patients with MDS or AML. Experiments were approved by the Institutional Review Board of Saga University, and all procedures involving human participants were undertaken in accordance with the principles of the Declaration of Helsinki.

Western blot analysis

Western blot analyses were performed to assess the protein levels of DNMT1, UCK1, UCK2, and DCK. Procedures are described in Supplementary methods.

Pyrosequencing assay of long interspersed nucleotide element-1 (LINE-1) and the γ-globin promoter methylations

Pyrosequencing assays of LINE-1 methylation were performed to assess global methylation (19). Procedures are described in Supplementary methods. The methylation status of γ-globin promoter status was assessed in samples (peripheral
blood) sequentially collected on day 0, 1, 5, 8, 12, 15, 19, 22, 26, and 29 after initiation of 5 day duodenal administration of 10.9 μmol/kg OR21, as previously described (21).

Cell proliferation

Cell proliferation was evaluated by CCK-8 assay (Djindo, Kumamoto, Japan) and half-maximal inhibitory concentration values (IC$_{50}$) were determined by non-linear regression using the CalcuSyn software (Biosoft, Cambridge, UK). Procedures for cell proliferation analyses are described in Supplementary methods.

Flow cytometry

Antibodies used for flow cytometry are listed in Supplementary methods. Stained samples were assessed using a FACSVerse cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA).

Genome-wide DNA methylation analysis

Genome-wide DNA methylation analysis was performed using Infinium Human Methylation EPIC Bead Chips (Infinium EPIC; Illumina; San Diego, CA, USA) as previously described (18). β-values ranged from 0 (unmethylated) to 1 (fully methylated). DNA methylation data were deposited in GEO under accession number GSE148314.
Gene expression microarray analyses

Procedures for gene expression microarray analyses are described in Supplementary methods. Microarray data were deposited at GEO under accession number GSE141677.

Mice

All animal experiments were approved by the Institutional Review Board of Saga University and performed according to the university’s institutional guidelines. BALB/c Rag-2/JAK3 double-deficient (BRJ) mice (22) were housed in a specific pathogen-free barrier facility; BRJ mice were used for xenograft transplantation experiments. BRJ mice received twice-weekly administration of vehicle (1% DMSO), DAC, or OR21 after xenotransplantation. Procedures for animal experiments are described in Supplementary methods.

Statistical analysis

All statistical analyses were performed using the EZR software package (Saitama Medical Center, Jichi Medical University) (23). $P < 0.05$ was considered statistically significant.
Results

5-O-trialkylsilylated DACs have appropriate log P values for oral bioavailability and hypomethylating effects in the MDS cell line.

In this study, we developed 5-O-trialkylsilylated DACs (Figure S1A). Oral absorbability is assessed according to the log P value, defined as the ratio of a chemical’s concentrations in the octanol vs. aqueous phase of a two-phase octanol/water system (24). The log P values of 5-O-trialkylsilylated DACs were +1.64 to +5.20; the appropriate log P value for oral bioavailability is +0 to +3. Determination of LINE-1 methylation levels by pyrosequencing, which measures methylation (the ratio of T to C) at each CpG position within a sequence after bisulfite treatment, yields a representative picture of global genomic DNA methylation (25,26). Treatment with 100 nM OR2100 (OR21), 2003, 2008, 2009, 2102, or 2201 significantly decreased LINE-1 methylation levels in MDS-L (Figure S1B). Based on these drug screening experiments, we defined OR21 as a promising 5-O-trialkylsilylated DAC, as in previous reports (18,19).

Structure of OR21

OR21, a DAC prodrug, is an oligonucleotide consisting of DAC nucleoside 5’-O-trisilylate, which is protected from degradation by CDA (Figure 1A). The log P value of OR21 is +2.14, indicating likely oral availability. The stability of the base
moiety of OR21 is almost the same (half-life, 20 h) as that of DAC in an aqueous environment (pH 6.0–7.5), and the in vitro stabilities of AZA and DAC are also similar (27).

Pharmacokinetics and pharmacodynamics of OR21 in cynomolgus monkeys

Initially, we investigated the pharmacokinetics of OR21 in six cynomolgus monkeys. Sequential plasma samples were taken from monkeys given the prodrug, and plasma drug concentrations were measured over time. OR21 is easily degraded by gastric acid because of its chemical structure, but direct duodenal administration of OR21 to cynomolgus monkeys resulted in absorption of the drug and subsequent release of DAC. After administration of 6.6 µmol/kg OR21, the area under the plasma drug concentration–time curve (AUC) was 0.30 (± 0.18) µM·h, and after administration of 6.6 µmol/kg DAC, the AUC was 0.01 (± 0.01) µM·h (Figure 2A, B). A higher dose of DAC did not result in enteric bioavailability (Figure S2). Thus, OR21 exhibited higher enteric absorbability than DAC. We next evaluated the pharmacodynamics of OR21. After intravenous administration (i.v.) of 10.9 µmol/kg DAC, the AUC was 0.49 (±0.17) µM·hr, and after duodenal administration (d.a.) of 10.9 µmol/kg OR21, the AUC was 0.25 (± 0.14) µM·hr (n=4 monkeys) (Figure 2C). After 5 day intravenous administration of 10.9 µmol/kg DAC and duodenal administration of 10.9 µmol/kg OR21, γ-globin promoter methylation (average three CpG sites at -53, +6, +17) levels (Cont, 88.3 ± 1.35%) was significantly reduced on day 8 (DAC, 66.5 ± 2.52%, P=0.02;
OR21, 79.5±4.50%, P=0.02) (Figure 2D). In this setting, the demethylating effect of DAC (i.v.) was stronger than that of OR21 (d.a.), possibly due to differences in AUC: 9.0 mg/kg (26.3 μmol/kg) OR21 (d.a.) achieved an AUC of 0.45 ± 0.23 μM·hr, comparable to the AUC of 1.67 mg/kg DAC (i.v.) (Figure S2). These results indicated that OR21 has both enteral absorbability and hypomethylating effects. We next evaluated the in vitro and vivo efficacy of OR21 for AML and MDS. (At that time, the appropriately-sized enteric-coated OR21 for administration in vivo was not available, as the enteric-coated OR21 developed for monkeys was too large to administer to mice).

OR21 induces global DNA hypomethylation by depleting DNMT1

Hypomethylating agents (AZA and DAC) deplete DNMT1. Consistent with this, DNMT1 levels were markedly lower in two MDS (MDS-L, SKM1) and two AML (HL60, THP-1) cell lines treated with OR21 (100 or 500 nM) than in untreated cells (Figure 1B and Figure S3A–C). LINE-1 methylation levels were higher in both MDS-L and SKM1 cells (88.9% and 82.7%, respectively). Treatment with 100 nM OR21 significantly decreased LINE-1 methylation levels in MDS-L [74.2% (P < 0.01)] and SKM1 [66.9% (P = 0.01)] cells (Figure 1C); the effect was equivalent to that of 100 nM DAC (75.2% reduction in MDS-L cells and 66.9% in SKM1 cells). By contrast, 100 nM AZA did not decrease the LINE-1 methylation level in MDS-L; consistent with a previous report, 100 nM of AZA had weak demethylating effects relative to DAC or OR21 (19). LINE-1 regions were also hypermethylated in AML cell lines (KG1a,
Kasumi-1, HL60, and THP-1); in this case, 500 nM OR21 tended to induce global hypomethylation (Figure 1D). These results indicate that OR21 induces global genomic DNA hypomethylation in MDS and AML cell lines by depleting DNMT1, and that the effect is equivalent to that of DAC.

**OR21 inhibits growth and induces apoptosis in MDS and AML cells**

OR21 inhibited growth of both MDS and AML cells in a dose-dependent manner (Figure S4A); the effect was equivalent to that of DAC. The dose-response curves and IC\(_{50}\) values for all three agents after 72 h of treatment are shown in Table S1. OR21 induced apoptosis of MDS and AML cells, again in a dose-dependent manner (Figure S4B). In addition, OR21 induced apoptosis of primary MDS and AML cells (Figure S4C). These results indicate that OR21 exerts anti-tumor effects against MDS and AML cells.

**Via regional demethylation, OR21 upregulates expression of genes associated with immune system processes, tumor suppression, and cell differentiation**

Vehicle-treated HL60 and MDS-L cells shared a high proportion of hypermethylated and hypomethylated sites, and OR21-treated cells had reduced hypermethylation \(\beta\) values, indicating that OR21 induced DNA demethylation (Figure 3A, B). In the three cell lines (HL60, SKM1, and MDS-L), a heatmap comparing log\(_2\) fold changes in gene expression following OR21 treatment revealed several enriched genes, which played roles in immune system processes (e.g., PRAME and CT45A), tumor suppression
(e.g., MT1E, F, TGFβ, and ALOX5AP), and cell differentiation (e.g., EVI2A, B, and TYROBO) (Figure 3C, Figure S5A–C). These genes were demethylated to a greater extent than others (Figure 3D–F, Figure S6A, B), indicating that their expression levels were regulated by promoter DNA methylation. The demethylating effect of 500 nM OR21 was similar to that of 500 nM AZA (Figure S7A–C). (OR21 is a prodrug of DAC; thus the demethylating effect and gene expression profiles of OR21-treated cells were very similar to those of DAC-treated cells, as previously described (18). AZA was used as a control). MDS and AML are characterized by impaired cell differentiation of HSCs and hematopoietic progenitor cells (1); therefore, induction of cell differentiation is a potential therapeutic strategy for both forms of cancer.

OR21 induces cell differentiation via upregulation of the late cell differentiation drivers CEBPE and GATA-1

CD11b is defined as a myeloid cell differentiation marker, whereas immature cells expressed HLA-DR. Hence, we used these surface markers to assess the potential of OR21 to induce cell differentiation in MDS or AML cells. OR21 upregulated expression of CD11b and downregulated expression of HLA-DR in MDS-L cells (Figure S8A). SKM1 and HL60 cells also exhibited upregulation of CD11b after OR21 treatment (Figure 4A and S8B). These cells also underwent morphological changes, including a reduced nuclear–cytoplasmic ratio, larger cell size, and higher numbers of multi-lobed nuclei (Figure 4B and S8C), indicating that OR21 induced cell differentiation, particularly granulocytic differentiation. Hence, we used real-time
quantitative reverse transcription–PCR to identify the transcription factors responsible for cell differentiation. OR21 upregulated expression of the *CEBPE* and *GATA-1* mRNAs in MDS-L and SKM1 cells (Figure 4C and S8D). These results indicate that OR21 induces cell differentiation, possibly by upregulating expression of the late cell differentiation drivers *CEBPE* and *GATA-1*.

**OR21 prolongs survival of MDS and AML model mice**

Next, we used a mouse xenograft model to evaluate the anti-MDS efficacy of OR21. For this purpose, we intravenously injected SKM1 cells into BALB/c Rag-2/JAK3 double-deficient (BRJ) mice. Starting on day 7 post-transplantation, the mice were treated twice weekly with vehicle OR21 (Figure 5A). On day 28, the number of human CD45-positive cells in peripheral blood (PB) was lower in OR21-treated mice than in vehicle-treated mice [vehicle, 6.40%; OR21, 0.31% (P = 0.0597); Figure 5B]. In addition, OR21 prolonged survival [median overall survival; vehicle, 29 days; OR21, NR at day 53 (P = 0.0246); Figure 5C]. All OR21-treated mice survived with engraftment of SKM1 cells at day 53 (Figure S9).

Next, we used a mouse xenograft model to evaluate the anti-AML efficacy of OR21. In these experiments, BRJ mice were injected intravenously with HL60 cells and treated twice weekly with vehicle, DAC (1.0 mg/kg), or OR21(2.7 mg/kg) starting on day 5 post-transplantation (Figure 5D). On day 37, the number of human CD45-positive cells in PB was significantly lower in OR21-treated mice than in vehicle-treated mice [vehicle, 2.96%; DAC, 1.67% (P = 0.48); OR21, 0.75% (P=0.049)].
and the OR21-treated mice exhibited prolonged survival [median overall survival; vehicle, 44 days; DAC, 46.5 days (P = 0.164); OR21, 49 days (P = 0.005); Figure 5E, F]. In addition, OR21 induced significant hypomethylation of LINE-1 regions in bone marrow cells [vehicle, 83.7%; DAC, 56.3% (P < 0.01%); OR21, 63.0% (P < 0.01%)]. OR21-treated mice did not develop anemia, whereas DAC-treated mice were anemic at the time of sacrifice [hemoglobin levels: vehicle, 17.5 (± 1.20) g/dL; DAC, 15.6 (± 1.22) g/dL; OR21, 17.1(± 0.31) g/dL; Figure 5G, H]. OR21 (5.4 mg/kg) prolonged survival, whereas a high dose of DAC (2.0 mg/kg) did not [median overall survival; vehicle, 48 days; DAC, 32 days (P = 0.040); OR21, 57 days (P < 0.001); Figure 5I]. Notably, DAC-treated mice died earlier than those in the other groups, possibly due to severe toxicity relative to the vehicle. These results suggest that high dose of OR21 was safer and more efficacious than a high dose of DAC. Significant cell differentiation was observed in mice exposed to late treatment (from day +28) with OR21 (vehicle, 43.3%; DAC, 75.7%, p=0.229; OR21, 65.5%; P = 0.038; Figure 5J). These results confirmed the anti-tumor effects of OR21 in the MDS and AML mouse models.

OR21 is effective against AZA-resistant AML cells in vitro and in vivo

Patients with MDS or AML who fail to respond to AZA treatment have limited therapeutic options, and their prognosis is extremely poor. Hence, we investigated the efficacy of OR21 against AZA-resistant AML. AZA-resistant HL60 (HL60R) cells survived in the presence of 10 µM AZA. However, OR21 depleted DNMT1 protein,
inhibited growth, and induced apoptosis and differentiation in these cells to a similar extent as DAC and more effectively than AZA \textit{in vitro} (Figure S10A–C and S11). OR21 also significantly prolonged survival in the HL60R xenograft model (Figure S10D, p=0.0188); by contrast, DAC-treated mice exhibited severe toxicity (Figure S12A, B).

Mechanism of AZA resistance

To investigate the mechanism underlying AZA resistance, we compared the protein levels of nucleoside-metabolizing enzymes between HL60 and HL60R. HL60R had lower levels of uridine-cytidine kinase 2 (UCK2) (Figure S13A). However, UCK2 mRNA levels and promoter methylation in proximal regions did not differ significantly between the two cell lines (Figure S13B, C). These observations suggested that depletion of UCK2 was associated with AZA resistance, but that the underlying mechanisms might not involve transcriptional regulation via altered methylation. The majority of downregulated genes in HL60R were involved in immune system processes or mitochondrial pathways (Figure S13D and Table S3). HL60 methylome analysis revealed that HL60R cells were much more demethylated than HL60 (Figure S13E); meanwhile, the downregulated genes were less demethylated than the upregulated genes (Figure S13F, G). Pathway analysis of genes upregulated >2.0-fold revealed that genes associated with cell adhesion were upregulated in HL60R cells (Figure S14A, B). Among them, the chemokine receptor CXCR4 (Figure S14C, D) was upregulated at the mRNA and protein levels in HL60R. HL60R xenotransplant
mice developed extramedullary disease (Figure S14E), suggesting that increases in cell adhesion activity are associated with elevated malignant potential. The changes in HL60R may be the consequence of transcriptional regulation via altered methylation.

The toxicity of OR21 may be lower than that of DAC, even though both drugs have the same AUC

We monitored the long-term administration of OR21 in BALB/c mice (not immunodeficient) treated with a high (2.5 mg/kg of DAC or 7.5 mg/kg of OR21) or low (1.25 mg/kg of DAC or 3.7 mg/kg of OR21) dose of the indicated drug. Although the plasma AUCs for high-dose DAC and OR21 were approximately equal, high-dose DAC resulted in greater hematopoietic toxicity, resulting in severe anemia and early death (Figure 6A, B). By contrast, mice treated with a high dose of OR21 developed anemia by Day 29, but recovered by Day 57 while still on the drug (Figure 6B). In addition, these mice did not die early: all were still alive after 80 days, whereas all mice receiving high-dose DAC died within 80 days. Neither DAC- nor OR21-treated mice exhibited signs of lethal toxicity in response to low-dose DAC or OR21; however, DAC-treated mice were more likely than OR21-treated mice to develop anemia (Figure 6C). Together, these results indicate that OR21 may be less toxic than DAC, resulting in improved tolerability even at high doses.
Enteric-coated OR21 exhibited favorable absorbability in an oral administration mouse model

Finally, we developed enteric-coated OR21 with the goal of making the drug resistant to gastric acid. We investigated the PK of enteric-coated OR21 in five ICR mice. Sequential plasma samples were taken from mice given the prodrug, and plasma drug concentrations were measured over time. Oral administration of enteric-coated OR21 to mice resulted in absorption of the drug and subsequent release of DAC. After administration of 5 mg of enteric-coated OR21, the AUC of DAC (released from OR21) was 1.06 (± 0.28) µM/h (Figure S15). Thus, enteric-coated OR21 exhibited high oral absorbability in a mouse model.

Discussion

The findings of this study reveal that OR21 is a promising hypomethylating agent that can be administered orally and exerts anti-tumor effects against MDS and AML both in vitro and in vivo. In addition, OR21 is less toxic than DAC. DAC and OR21 had similar effects in vitro in terms of hypomethylation, cell growth inhibition, and apoptosis induction; these effects were distinct from those of AZA. These differences are consistent with previous results showing that AZA is incorporated predominantly into RNA, whereas DAC is incorporated predominantly into DNA (27). Hence, the in vitro activity of OR21, which was triggered by release of DAC from OR21 after removal of the 5'-O-trisilylate substituent, might be due to incorporation of DNA rather than RNA.
Resistance to CDA is crucial for achieving elevated plasma concentrations of DNMT inhibitors. Guadecitabine, which is resistant to CDA and exerts stronger hypomethylating activity than AZA (28), has a favorable survival outcome in patients with MDS and AML who are not responsive to AZA (15). Nevertheless, guadecitabine is not orally absorbable (29). By contrast, the recently approved agents CC-486 (13) and ASTX727 (14) can be orally absorbed. Silylation of DAC confers resistance to CDA (18); accordingly, OR21 was enterically bioavailable in cynomolgus monkey, and enteric-coated OR21 was orally bioavailable in a mouse mice model. In addition, silylation of DAC and enzymatic cleavage of silylation did not result in new toxicities 

in vivo. It is worth noting that ASTEX727 consists of two compounds, DAC and cedazuridine, a CDA inhibitor, whereas OR21 is a single compound (DAC prodrug). Consequently, OR21 has enormous advantages in terms of production, i.e., it is easier and cheaper to manufacture. We have now prepared gelatin capsules filled with granules of enteric-coated OR21, and we are currently performing PK studies of this formulation in cynomolgus monkeys to determine the dose for a first-in-human clinical trial. Because formulation is a very important issue related to future development, we prefer not to present detailed PK data until the first in-human clinical trial is completed. The preliminary results suggest that OR21 could be used as an oral hypomethylating agent.

OR21-induced global DNA hypomethylation in MDS and AML cells could upregulate expression of silenced genes associated with tumor suppression, cell differentiation, and immune system processes. Among the genes that were upregulated
after OR21 treatment, TGFβ is an important tumor-suppressor gene in hematological malignancies that affects immune system process and cell differentiation (30); accordingly, TGFβ may play an important role in the anti-tumor effects of OR21. Although it is unclear how immune system process pathways are involved in the anti-tumor effects of OR21, these pathways were downregulated in AZA-resistant HL60 cells (31), suggesting that immune pathways play important roles in the anti-tumor effects of hypomethylating agents used to treat AML. Moreover, upregulation of genes associated with immune system processes and tumor suppression could alter immunogenicity or tumor recognition, thereby sensitizing various tumors (32,33). Immune checkpoint inhibitors used to treat melanoma (34), lung cancer (35), or other solid tumors (36,37) also exhibit anti-tumor effects in patients with MDS or AML (38,39). To optimize promising combination therapeutic strategies involving OR21, subsequent administration of immune checkpoint inhibitors may improve efficacy against MDS or AML.

AZA-resistant HL60R cells exhibited downregulation of the UCK2 protein without alteration of UCK2 promoter methylation or mRNA levels (40,41). UCK2 is a pivotal uridine-metabolizing enzyme (42). Furthermore, alteration of promoter methylation resulted in upregulation of genes related to cell adhesion in AZA-resistant HL60R cells, and OR21 overcame AZA resistance. Patients with MDS or AML who fail to respond to AZA treatment have limited therapeutic options, and their prognosis is extremely poor (43,44). We showed that OR21 was effective against AZA-resistant AML in vitro and in vivo, whereas DAC exerted a high degree of toxicity in vivo. These results
suggest that OR21 may yield clinically favorable outcomes in patients with MDS or AML who do not respond to AZA.

Patients with MDS or AML exhibit altered HSC differentiation (1,2), and AML patients with low CEBPE gene expression level had significantly worse survival outcomes (Figure S16); therefore, induction of appropriate cell differentiation is a potential therapeutic strategy for MDS and AML (45,46). In three different cell lines, OR21 upregulated genes associated with cell differentiation, resulting in induction of myeloid cell differentiation. Induction of global DNA hypomethylation triggered upregulation of the silenced key differentiation drivers CEBPE and GATA-1, which are inactivated in MDS and AML (47,48), thereby triggering cell differentiation (49). OR21 induced anti-tumor effects in vivo by promoting both hypomethylation and cell differentiation. The effects were similar to those of DAC, although OR21 did not induce severe toxicities in mice. Long-term administration experiments confirmed that OR21 was less toxic than DAC at both high and low doses. Although it is not clear why the toxicity of OR21 is lower than that of DAC, one possible explanation is that OR21 achieves a lower peak plasma concentration: the peak plasma concentration of DAC in mice was 0.43 µM after intraperitoneal administration of 1.59 µM/kg OR21, vs. 0.64 µM after administration of 0.88 µM/kg DAC (calculated as an AUC equivalent to that of released DAC, which had an AUC of 0.38 µM/h, Table S4) (18).

Although the safety of cleaved sialylated DAC has not been fully elucidated, we confirmed that OR21 is less toxic, possibly due to differences in peak plasma concentration and AUC profiles relative to DAC administration. Meanwhile, the
efficacy of OR21 is mainly due to the release of DAC from OR21 after removal of the 5'-O-trisilylate substituent. Therefore, OR21 represents an orally bioavailable drug with anti-tumor effects against MDS and AML similar to those of DAC, and is therefore a potentially suitable treatment for patients with MDS and AML (10,50).

In conclusion, silylation of deoxynucleotide analog can provide oral bioavailability without new toxicities. OR21 exhibits high oral availability and exerts strong anti-tumor effects against MDS and AML both in vitro and in vivo; moreover, it is less toxic than DAC. We anticipate that the efficacy and safety of OR21 for treatment of MDS and AML will be verified in planned early-phase clinical trials.

**Contributors**

HU, YK, YS, KK, and YY collected and analyzed the data, performed the experiments, and wrote the manuscript. TW, Y F-K, NY, NH, HH, KT, HH, SO, TU, and SK contributed to the research design and edited the manuscript. AK performed statistical analysis. TU and SK made substantial contributions to the study conception and design. HU and SK critically reviewed the drafts. All authors approved the final version.

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References


Figure legends

Figure 1. Structure of OR21 and its effects on methylation.

Structure of 5’-O-triethylsilyl-2’-deoxy-5-azacytidine (OR2100, OR21) and decitabine (DAC) (A). Agents with a log P value of +1.0 – +5.9 are expected to be absorbed after oral administration. Analysis of protein expression in MDS-L and HL60 cells treated with azacytidine (AZA), DAC, or OR21 (B). LINE-1 methylation analysis in MDS-L and SKM1 cells, as determined by bisulfite pyrosequencing (C). MDS-L and SKM1 cells were strongly hypermethylated (88.9% and 82.7%, respectively). Treatment with 100 nM DAC or OR21 significantly decreased LINE-1 methylation levels in MDS-L [AZA, 88.8% (P = 0.867); DAC, 75.2% (P < 0.01); OR21, 74.2% (P < 0.01)] and SKM1 cells [AZA, 72.1% (P < 0.01); DAC, 58.4% (P < 0.01); OR21, 66.9% (P=0.01)] (mean ± SD; n = 3). LINE-1 methylation analysis in AML cell lines treated with 500 nM OR21. AML cell lines were also hypermethylated (KG1a, 89.1%; Kasumi-1, 90.7%; HL60, 83.7%; THP-1, 69.0%) (D). Treatment with OR21 tended to induce hypomethylation [KG1a, 82.4% (P=0.091); Kasumi-1, 78.6% (P = 0.128); HL60, 76.0% (P = 0.089); THP-1, 64.6% (P = 0.243)] (mean ± SD; n = 3).

Figure 2. Pharmacokinetics and pharmacodynamics of OR21

Sequential plasma samples were taken from monkeys given the prodrug, and plasma drug concentrations were measured over time. After administration of 6.6 µmol/kg OR21, the area under the plasma drug concentration–time curve (AUC) was 0.30 (±
After administration of 6.6 µmol/kg DAC, the AUC was 0.01 ± 0.01 µM·h (A). After administration of 10.9 µmol/kg DAC, the AUC was 0.49 ± 0.17 µM·h and after administration of 10.9 µmol/kg OR21, the AUC was 0.25 ± 0.14 µM·hr (C). After 5 day intravenous administration (iv) of 10.9 µmol/kg DAC and duodenal administration (d.a.) of 10.9 µmol/kg OR21, the γ-globin promoter methylation (average three CpG sites at -53, +6, and +17) levels (Cont, 88.3±1.35%) were significantly reduced at day 8 (DAC, 66.5 ± 2.52%, P=0.02; OR21, 79.5±4.50%, P=0.02) (D). *P < 0.05.

**Figure 3. Genes differentially expressed after OR21 treatment due to alteration of promoter DNA methylation.**

Methylome analysis revealed that OR21-treated HL60 and MDS-L cells had reduced hypermethylation β values (A, B). Heatmap comparing log2 fold changes in gene expression among three cell lines (HL60, SKM1, and MDS-L) following treatment with OR21 (C). Analysis revealed enrichment of genes involved in immune system processes (including cancer testis antigen, e.g., PRAME, TGFβ, and CT45A), tumor suppression (e.g., MT1E, F, TGFβ, and ALOX5AP), and cell differentiation (e.g., EVI2A, B, TGFβI, and TYROBO). (D, E) Each of these genes were demethylated after OR21 treatment (D). The β-values of each gene decreased (paired t-test, E) **P < 0.01. The delta β-values (OR21-treated minus vehicle-treated) of HL60 and MDS-L cells were below zero (F).

**Figure 4. OR21 induces cell differentiation in MDS and AML cells.**
Flow cytometry revealed elevated expression of CD11b (granulocytic differentiation marker) in MDS and SKM1 cell lines (MDS-L) following 96 h treatment with 100 nM azacitidine (AZA), decitabine (DAC), or OR21 (mean ± SD; n = 3) (A). OR21 induced morphologic changes consistent with granulocytic differentiation (i.e., reduced nuclear–cytoplasmic ratio, larger cell size, and higher numbers of multi-lobed nuclei) in MDS-L and SKM1 cells. May–Giemsa staining of cytospin preparations at 96 h is shown. (B)

Levels of CEBPA, CEBPB, PU.1, GATA1, and CEBPE mRNAs were measured by quantitative reverse transcription–PCR. Upregulation of CEBPE and GATA-1 was observed in MDS-L and SKM1 cells treated with DAC or OR21 (mean ± SD; n = 3) (C).

*P < 0.05, **P < 0.01.

Figure 5. SKM1 and HL60 xenograft mouse model experiments.

Schedule of SKM1 cell xenograft experiments using BALB/c Rag-2/JAK3 double-deficient (BRJ) mice (A). Day 28: flow cytometry of hCD45-positive cells in mice treated with vehicle or OR21 (B). Kaplan–Meier survival curves showing cumulative survival of mice treated with vehicle or OR21 (n = 3 per cohort) (C). Median overall survival time was 29 days (vehicle) (P=0.0246); NR = not reached. Statistical analysis was performed using the log-rank test. Schedule of HL60 cell xenograft experiments using BRJ mice (D). Day 37: flow cytometry of hCD45-positive cells in mice treated with vehicle (n = 5), decitabine (DAC) (n = 4), or OR21 (n = 4) (E). Kaplan–Meier survival curves showing cumulative survival of mice treated with vehicle, DAC, or OR21 (F). Median overall survival times were 44 days (vehicle) (n = 10), 46.5...
days (DAC, n = 8, P=0.164), and 49 days (OR21, n = 10, P=0.005). Statistical analysis was performed using the log-rank test. DAC-treated mice were more likely to develop anemia than OR21-treated mice (G). Hemoglobin (Hb) levels were 17.5 g/dL (vehicle, n = 8), 15.6 g/dL (DAC, n = 6, P = 0.092), and 17.1 g/dL (OR21, n = 7, P = 1.0). LINE-1 methylation in bone marrow cells decreased after treatment with DAC or OR21 (n = 6 per cohort) (H). Mice treated with a higher dose of DAC (2.0 mg/kg) did not live longer, whereas survival was prolonged in mice treated with OR21 (5.4 mg/kg) [median overall survival; vehicle, 48 days; DAC, 32 days (P = 0.040); OR21, 57 days (P < 0.001)] (I).

HL60 xenograft mice exposed to late treatment with OR21 (Day +28) had more CD11b-positive cells in peripheral blood (PB) on Days +35 to +40, as determined by flow cytometry (n = 4 per cohort) (vehicle, 43.3%; DAC, 75.7%, p=0.229; OR21, 65.5%; P = 0.038) (J).

**Figure 6. In vivo safety profile of OR21.**

(A) Kaplan–Meier survival curves showing cumulative survival of mice treated with vehicle, decitabine (DAC; 2.5 mg/kg), or OR21 (7.5 mg/kg) (n = 6 per cohort). (B) DAC (2.5 mg/kg) exerted high levels of toxicity, causing severe anemia by Days +29 and +57. Mice treated with OR21 (7.5 mg/kg) also developed anemia by Day +29, but recovered by Day +57 while still on the drug (n = 6 per cohort). (C) Among mice exposed to low-dose DAC (1.25 mg/kg) or OR21 (3.75 mg/kg) until Day +113, those exposed to DAC were more likely to develop anemia (n = 6 per cohort). WBC, white blood cell count; Hb, hemoglobin; platelet count, PLT.
Figure 1

A 5'-O-Triethylsilyl-2'-deoxy-5-azacytidine (OR2100, OR21)

Decitabine

B

DNMT1

β-actin

Log P = + 2.14

Log P = - 0.32

C

P = 0.006

P = 0.026

P = 0.867

D

P = 0.091

P = 0.128

P = 0.089

P = 0.01

P < 0.01

P < 0.01

5'-O-Triethylsilyl-2'-deoxy-5-azacytidine (OR2100, OR21)

Decitabine

MDS-L

HL60

SKM1

MDS-L

SKM1

KG1a Kasumi-1 HL60 THP-1

Cont AZA DAC OR21

Cont AZA DAC OR21

Cont OR21 Cont OR21 Cont OR21 Cont OR21

(100 nM) (100 nM) (500 nM) (500 nM)
Figure 2

A

OR21 2.25 mg/kg (6.6 µmol/kg)

OR21 2.25 mg/kg (6.6 µmol/kg)

AUC 0.30 ± 0.18 µM·hr

B

DAC 1.0 mg/kg (6.6 µmol/kg)

DAC 1.0 mg/kg (6.6 µmol/kg)

AUC 0.01 ± 0.01 µM·hr

C

DAC and OR21 10.9 µmol/kg

DAC and OR21 10.9 µmol/kg

D

HgF CpG Methylation (%)

HgF CpG Methylation (%)

OR21 or DAC

DAC and OR21 10.9 µmol/kg

DAC 1.67 mg/kg i.v.

DAC 1.67 mg/kg i.v.

OR21 3.75 mg/kg d.a.

OR21 3.75 mg/kg d.a.

DAC 1.67 mg/kg i.v.

DAC 1.67 mg/kg i.v.
Figure 3

A

![Gene frequency distribution for HL60 cells in Control and OR-treated conditions.](HL60.png)

B

![Gene frequency distribution for MDS-L cells in Control and OR-treated conditions.](MDS-L.png)

C

Cancer antigen expression heatmap for different cell lines and conditions.

D

Bar chart showing β values for HL60 cells across different conditions.

E

Graph showing β values for HL60 and MDS-L cells in Control and OR21 conditions.

F

Box plot comparing delta β values between HL60 and MDS-L cells.

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Figure 4

**A**

![Graph](image)

**B**

![Images](image)

**C**

<table>
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<tr>
<th></th>
<th>Cont</th>
<th>AZA</th>
<th>DAC</th>
<th>OR21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CEBPA</strong></td>
<td>1.00</td>
<td>0.88 (±0.21)</td>
<td>1.08 (±0.67)</td>
<td>1.44 (±0.34)</td>
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<tr>
<td><strong>CEBPB</strong></td>
<td>1.00</td>
<td>1.08 (±1.00)</td>
<td>0.86 (±0.79)</td>
<td>1.20 (±0.89)</td>
</tr>
<tr>
<td><strong>PU.1</strong></td>
<td>1.00</td>
<td>0.94 (±0.02)</td>
<td>0.82 (±0.21)</td>
<td>1.24 (±0.44)</td>
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<tr>
<td><strong>GATA1</strong></td>
<td>1.00</td>
<td>1.58 (±0.47)</td>
<td>3.04 (±1.90)</td>
<td>5.10 (±3.32)</td>
</tr>
<tr>
<td><strong>CEBPE</strong></td>
<td>1.00</td>
<td>4.74 (±3.38)</td>
<td>6.81 (±4.21)</td>
<td>6.27 (±1.73)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>AZA</th>
<th>DAC</th>
<th>OR21</th>
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<tbody>
<tr>
<td><strong>CEBPA</strong></td>
<td>1.00</td>
<td>0.99 (±0.42)</td>
<td>0.79 (±0.33)</td>
<td>0.94 (±0.46)</td>
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<tr>
<td><strong>CEBPB</strong></td>
<td>1.00</td>
<td>0.95 (±0.40)</td>
<td>2.32 (±1.21)</td>
<td>1.31 (±0.58)</td>
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<tr>
<td><strong>PU.1</strong></td>
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<td>1.02 (±0.25)</td>
<td>0.72 (±0.09)</td>
<td>0.62 (±0.17)</td>
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<tr>
<td><strong>GATA1</strong></td>
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<td>3.99 (±1.89)</td>
<td>118.73 (±70.18)</td>
<td>42.31 (±15.80)</td>
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<tr>
<td><strong>CEBPE</strong></td>
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<td>1.74 (±0.82)</td>
<td>2.80 (±1.18)</td>
<td>2.82 (±1.42)</td>
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Figure 6

A  

Survival (%)  

<table>
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<tr>
<th>Vehicle</th>
<th>OR21 7.5 mg/kg</th>
<th>DAC 2.5 mg/kg</th>
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<tbody>
<tr>
<td>100</td>
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<td>80</td>
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<tr>
<td>0</td>
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P = 0.317

months

B

Day 29

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<th>Vehicle</th>
<th>DAC 2.5</th>
<th>OR21 7.5</th>
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<tbody>
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<td>P &lt; 0.01</td>
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<td></td>
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<td>P &lt; 0.01</td>
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<tr>
<td>P = 0.074</td>
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<td>P = 0.792</td>
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Day 57

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<th>Vehicle</th>
<th>DAC 2.5</th>
<th>OR21 7.5</th>
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<tbody>
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<td>P = 0.093</td>
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<td>P = 0.293</td>
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C

Day 113 (DAC 1.25 mg/kg, OR21 3.75 mg/kg)

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<th>WBC × 10^9/μL</th>
<th>Hb g/dL</th>
<th>PLT × 10^4/μL</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>DAC</td>
<td>OR21</td>
</tr>
<tr>
<td>P = 0.353</td>
<td>P = 0.119</td>
<td>P = 0.794</td>
</tr>
<tr>
<td>P = 0.565</td>
<td>P = 0.074</td>
<td>P = 0.924</td>
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<tr>
<td>P = 0.792</td>
<td>P = 0.876</td>
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Molecular Cancer Therapeutics

Silylation of deoxynucleotide analog yields an orally available drug with anti-leukemia effects
Hiroshi Ureshino, Yuki Kurahashi, Tatsuro Watanabe, et al.

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