Immunotoxin resistance via reversible methylation of the DPH4 promoter is a unique survival strategy

Hui Wei[a], Laiman Xiang[a], Alan S. Wayne[a,b], Oleg Chertov[c], David J. FitzGerald[a], Tapan K. Bera[a], and Ira Pastan[a,1]

[a]Laboratory of Molecular Biology and [b]Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and [c]Protein Chemistry Laboratory, Advanced Technology Program, SAIC-Frederick, National Cancer Institute, Frederick, MD 21702

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HA22 is a recombinant immunotoxin composed of an anti-CD22 Fv fused to a portion of *Pseudomonas* exotoxin A. HA22 produced a high rate of complete remissions in drug-resistant hairy cell leukemia and has a lower response rate in pediatric acute lymphoblastic leukemia (ALL). To understand why patients with ALL have poorer responses, we isolated an ALL cell line that is resistant to killing by HA22. The resistance is unstable; without HA22 the cells revert to HA22 sensitivity in 4 mo. We showed that in the resistant cell line, HA22 is unable to ADP ribosylate and inactivate elongation factor-2 (EF2), owing to a low level of DPH4 mRNA and protein, which prevents diphthamide biosynthesis and renders EF2 refractory to HA22. Analysis of the promoter region of the *DPH4* gene shows that the CpG island was hypomethylated in the HA22-sensitive cells, heavily methylated in the resistant cells, and reverted to low methylation in the revertant cells. Our data show that immunotoxin resistance is associated with reversible CpG island methylation and silencing of *DPH4* gene transcription. Incubation of sensitive cells with the methylation inhibitor 5-azacytidine prevented the emergence of resistant cells, suggesting that this agent in combination with HA22 may be useful in the treatment of some cases of ALL.

DNA methylation | drug resistance | ADP-ribosylation | diphthamide synthesis | epigenetic regulation

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy, accounting for approximately 25% of childhood cancer in the United States (1). Despite advances in curative treatment, ALL is a leading cause of cancer-related mortality in pediatrics, and survivors are at risk for multiple late effects (2–4). Novel therapies that can overcome chemotherapy resistance and decrease nonspecific toxicities are needed.

The B-lineage differentiation antigen CD22 is a relevant target for B-precursor ALL (5). We have developed two recombinant anti-CD22 immunotoxins composed of the Fv of the anti-CD22 antibody RFB4 fused to a 38-kDa fragment of *Pseudomonas* exotoxin A (PE38) (6). These agents bind to CD22, after which they are internalized via receptor-mediated endocytosis, processed by furin releasing the toxin portion, which is transferred to the endoplasmic reticulum and translocated to the cytosol. Cytotoxicity is caused by toxin-mediated ADP ribosylation of elongation factor-2 (EF2), leading to inhibition of protein synthesis and induction of programmed cell death (6). A five enzyme (DPH1–5) posttranslational modification of histidine 715 produces mature EF2 (7). The modified histidine, termed "diphthamide," is ADP ribosylated by *Diphtheria* toxin and PE38. The function of diphthamide has not been clearly delineated but may be related to maintenance of translational fidelity (8). Knockouts of *dph1*, *dph3*, or *dph4* are embryonic lethal for mice (9–11), but when similar knockouts are engineered into established cell lines there is no major phenotype. The function of the DPH4 protein is not fully understood, but it contains a DNAJ domain and may function as a cochaperone. Further, little is known about the regulation of the genes encoding DPH1–5.

Our first-generation anti-CD22 immunotoxin, BL22 or CAT-3888, was shown to have an acceptable toxicity profile in children with ALL (5), but clinical activity was modest, in contrast to adults with hairy cell leukemia (HCL), in whom BL22 produced a 50% complete response rate (12). A second-generation immunotoxin with a higher affinity for CD22 (moxetumomab pasudotox, HA22, CAT-8015) is more active in vitro, although not all childhood ALL blasts are sensitive to HA22 (13, 14). A pediatric phase I trial of moxetumomab pasudotox is ongoing (ClinicalTrials.gov ID NCT00659425). Complete responses were observed in some, but not all, children with chemotherapy-refractory ALL (15). Thus, HA22 is a promising agent for the treatment of ALL. Here we undertook preclinical studies to uncover possible mechanisms of immunotoxin resistance and predictors of clinical responses to HA22.

DNA methylation is a well-known mechanism of epigenetic regulation in mammalian cells (16). DNA methylation is catalyzed by three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b). Hypermethylation of CpG residues in the promoter region is usually associated with transcriptional inactivation, whereas demethylation results in increased transcription (17). Epigenetic modification by CpG methylation of regulatory genes might contribute to the emergence of drug resistance in cancer cells. This deregulation of gene expression by CpG methylation can often be reversed using the DNA methylation inhibitor 5-azacytidine (azacytidine), which inhibits DNMTs and is used to treat myelodysplastic syndrome. Sharma et al. (18) reported a reversible resistance to cisplatin associated with global changes in histone modification. The drug-resistant cells were eliminated by treatment with a histone deacetylase inhibitor, although the specific mechanism by which the cells became resistant to cisplatin was not established. These studies highlight the fact that epigenetic modification can be associated with reversible drug resistance in cancer cells.

Results

We isolated an HA22-resistant cell line by intermittent exposure to HA22 (*Materials and Methods*). Cytotoxicity assays showed that the parental cell line was sensitive to HA22, with an IC_{50} of 1.1 ng/mL (Fig. 1*A*), whereas the resistant line (HAL-01-R) was not killed by 100 ng/mL HA22 (Fig. 1*A*). Further, HAL-01-R cells exhibited cross-resistance to diphtheria toxin and immunotoxin HB21-PE40 that targets the human transferrin receptor (Fig. 1*B*) but was sensitive to two other protein synthesis inhibitors, cycloheximide and ricin (Fig. 1*B*). The resistant cell line was maintained for several months in HA22 and had approximately the same growth rate as the parental cells (Fig. 1*C*). When the

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[1]To whom correspondence should be addressed. E-mail: pastani@mail.nih.gov.

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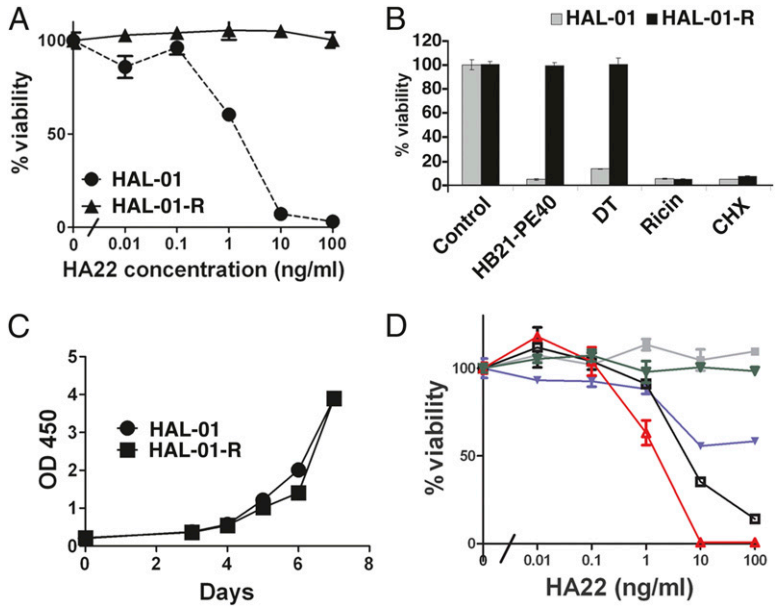

Fig. 1. Properties of resistant cells as shown with water-soluble tetrazolium (WST) assay results. (*A*) HAL-01 and HAL-01-R cells were treated with HA22 for 3 d. (*B*) HAL-01 and HAL-01-R cells were treated for 3 d with 100 ng/mL of HB21-PE40, diphtheria toxin (DT), 1 μg/mL cycloheximide (CHX), or 10 ng/mL ricin. (*C*) HAL-01-R has a similar growth rate compared with parental cell line HAL-01. (*D*) HAL-01-R cells were cultured in normal medium without HA22 for indicated number of days, followed by treatment with the indicated concentrations of HA22 for 3 d. —▲—, HAL-01; —▼—, HAL-01-R in HA22; —▬—, HAL-01-R without HA22 for 32 d; —▼—, HAL-01-R without HA22 for 58 d; —■—, HAL-01-R without HA22 for 120 d. Each data point represents the average value from three samples.

resistant line was grown without HA22, the cells slowly reverted to sensitivity (Fig. 1*D*). After 120 d the IC_{50} was 9.5 ng/mL.

To elucidate the mechanism of resistance, we analyzed several steps in the immunotoxin pathway. We measured the number of CD22 binding sites on the cell surface and found that there was no decrease in CD22 expression (Fig. 2*A*). We measured HA22 internalization by flow cytometry using HA22 labeled with Alexa 647 and found that the rates of HA22 internalization were not decreased (and actually were slightly increased) in the resistant cell line, as was the total amount accumulated (Fig. 2*B*). These

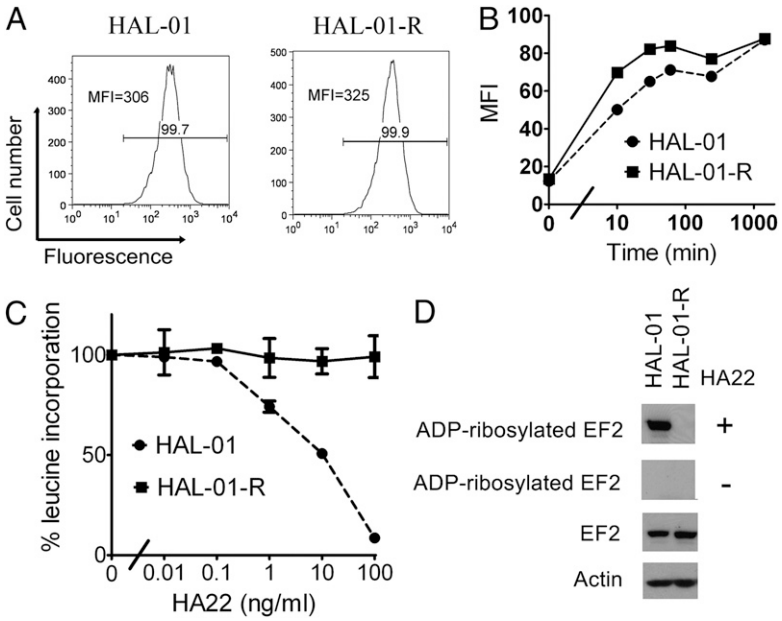

Fig. 2. EF2 refractory to ADP ribosylation renders resistant cells nonresponsive to HA22. (*A*) CD22 expression. Cells were incubated on ice with RFB4 (mouse anti-CD22 antibody) or control mouse IgG1, followed by goat anti-mouse antibody-phycoerythrin (PE) and analyzed by FACS. MFI, geometric mean of fluorescence intensity. (*B*) Time course of immunotoxin internalization. HAL-01 and HAL-01-R cells were incubated with HA22-Alexa 647 at 37 °C for 10 and 30 min and 1, 4, and 24 h. Internalized immunotoxin is shown as the geometric mean fluorescence. (*C*) HA22 does not inhibit protein synthesis in resistant cell line HAL-01-R. Cells were treated with HA22 for 20 h. Protein synthesis was measured by incorporation of ^{3}H-leucine. Mean triplicate values are shown. (*D*) HA22 can ADP ribosylate EF2 in parental cells but not in resistant cells. Cell lysate (30/μg) was incubated with or without 100 ng of HA22 for 60 min at 25 °C. Samples were analyzed by Western blot with streptavidin HRP conjugate to detect biotin-ADP ribose-EF2. EF2 and actin are shown as loading controls.

data show that resistance is not due to a defect in binding or internalization of HA22.

The next step in the immunotoxin pathway that can be accurately measured is the inactivation of EF2, which leads to inhibition of protein synthesis. Fig. 2*C* shows that ^{3}H-leucine incorporation was not reduced by HA22 in the resistant cells, indicating that either the toxin did not reach the cytosol or that EF2 could not be inactivated by the toxin. There is no assay that measures the translocation of the toxin fragment that contains the ADP ribosylation activity into the cytosol. Instead, we determined if cellular EF2 could function as a substrate for toxin-mediated ADP-ribosylation. We added HA22 and 6-biotin-17-NAD to cell-free extracts prepared from sensitive or resistant cells and probed for biotin-ADP-ribose associated with EF2. Using this assay, it is possible to compare the extent of EF2 modification from each cell type. The Western blot in Fig. 2*D* shows that HA22 can catalyze the incorporation of ADP ribose into EF2 in sensitive HAL-01 cells but not in resistant cells. The amount of EF2 was unchanged. Because His715 must be modified to diphthamide to be ADP ribosylated by toxin, this finding indicates that either a defect in diphthamide biosynthesis or a modification of amino acids in the diphthamide loop causes the resistance. We prepared EF2 cDNA and sequenced the ORF from resistant cells and did not find any mutations.

We measured the levels of mRNA that encode each of the five diphthamide enzymes (DPH1–5) by quantitative PCR (qPCR) and found that only the levels of DPH4 mRNA were substantially reduced in resistant cells (Fig. 3*A* and Table S1). Immunoblots showed that the level of DPH4 protein was also substantially reduced (Fig. 3*B*). To show that low DPH4 levels alone can cause toxin resistance, we used shRNA to knock down DPH4 mRNA and protein in HAL-01 cells (Fig. 3 *C* and *D*) and found a reduction in the amount of EF2 that could be ADP ribosylated (Fig. 3*E*) and an increase in resistance to killing by HA22 (Fig. 3*F*). These findings show that a reduction in DPH4 protein is sufficient to cause HA22 resistance.

To determine whether His-715 of EF2 was modified to diphthamide in the resistant cells, we isolated EF2 by diethylaminoethyl (DEAE) chromatography and gel electrophoresis and digested the EF2 band by trypsin. The peptides were analyzed by MALDI-MS. Fig. 4 shows the results for selected peptides from cells. MS/MS analysis of *m/z* 1745.861 from HAL-01-R cells identifies a peptide (FDVHDVTLHADAIHR) that contains an unmodified His-715 residue that was not found in the analysis of *m/z* 1745.844 from HAL-01 cells. Instead, analysis of *m/z* 1828.908 and 1836.179 from HAL-01 cells demonstrated that both ions correspond to a diphthamide-modified peptide but with loss of a trimethylamino group that could have occurred because of an elimination reaction (19) during protein isolation, in the case of *m/z* 1828.908 (Fig. S1), and during MALDI analysis, in the case of the metastable ion 1836.179. Thus, direct sequence analysis of EF2 in the resistant cells shows it is not modified by diphthamide.

DNA Hypermethylation in the DPH4 Promoter Region Resulted in DPH4 Down-Regulation. Drug resistance can occur by multiple mechanisms (20). Because the resistant cell line reverted to sensitive when grown without HA22, it seemed likely that the resistance was due to a regulatory or epigenetic change and not a structural mutation. Because the ALL cell line is diploid, it seemed unlikely that the DPH4 locus could be reversibly inactivated by a single mutation affecting the transcription of the gene. A common epigenetic change in cancer cells is CpG methylation in the promoter region that diminishes transcription of the affected gene.

The sequence of the promoter region of DPH4 is shown in Fig. S2. It contains a single CpG island, which spans the transcriptional start site. The methylation status of the promoter region of the parental sensitive line (HAL-01), the resistant line (HAL-01-R), and

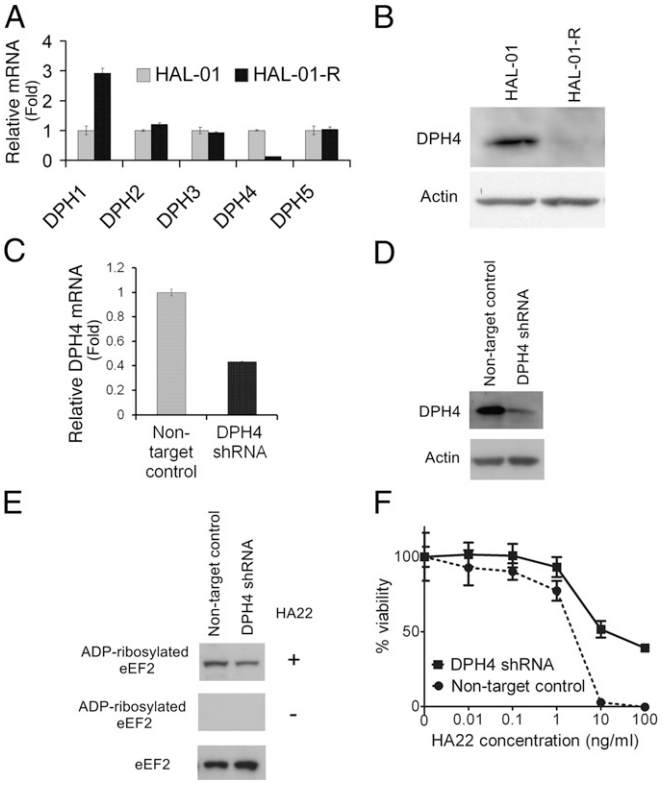

Fig. 3. Down-regulation of DPH4 resulted in altered EF2 in the resistant cell line. (*A*) DPH1–5 mRNA levels. *DPH* mRNA levels were analyzed by quantitative RT-PCR, and the data represent the average of three samples. (*B*) DPH4 protein levels. Equal amounts of cell lysate from sensitive and resistant cells were subjected to SDS/PAGE followed by Western blot with anti-DPH4 and actin antibodies. (*C–F*) Knock-down of DPH4 by shRNA. (*C*) Quantitative RT-PCR showing efficient suppression of DPH4 mRNA in HAL-01 cells by shRNA. (*D*) DPH4 down-regulation by shRNA was confirmed by Western blot. (*E*) Reduced ADP ribosylation of EF2 due to DPH4 knock-down by shRNA. ADP ribosylation was measured as described in Fig. 2*D*. (*F*) Sensitivity to HA22 of control and DPH4 knockdown HAL-01 cells was assessed by WST assays and expressed as percentage of surviving cells relative to untreated controls.

the immunotoxin sensitive revertant line (HAL-01-Rev) is shown in Fig. 5 and Table S2. CpG residues in the region −134 to +55 relative to the ATG were hypomethylated in the sensitive cells, hypermethylated in resistant cells, and hypomethylated in the revertant cells (Fig. 5 and Table S2). Thus, the methylation status was strongly correlated with the levels of DPH4 mRNA and protein. To assess whether DNA hypermethylation was a global change or specific for the DPH4 promoter, we analyzed the methylation status of 24 gene promoters that are reported to be methylated in a variety of leukemia and lymphoma cell lines and found that methylation status did not correlate with the status of the *DPH4* gene (Fig. S3). Thus, changes in DPH4 CpG methylation are gene specific.

CpG methylation is catalyzed by three DNMTs (17). Because miRNA-126 and miRNA-29 can regulate DNA methylation by targeting DNMT1 and DNMT3 (21, 22), we analyzed miRNAs from sensitive and resistant cells with an Affymetrix miRNA array system. We found that the level of miR-126 was decreased 14.3-fold in the resistant line (Table S3) and that levels of other miRNAs varied but not in any systematic way. Recently it was shown that high expression of miR-126 inversely correlates with DNMT1 levels in CD4^{+} T cells and that miR-126 can inhibit DNMT1 translation by interacting with the 3′ untranslated region of the mRNA (21). To investigate whether the decreased level of miR-126 in resistant cells might have caused an increase in DNMT1 and contributed to the increased

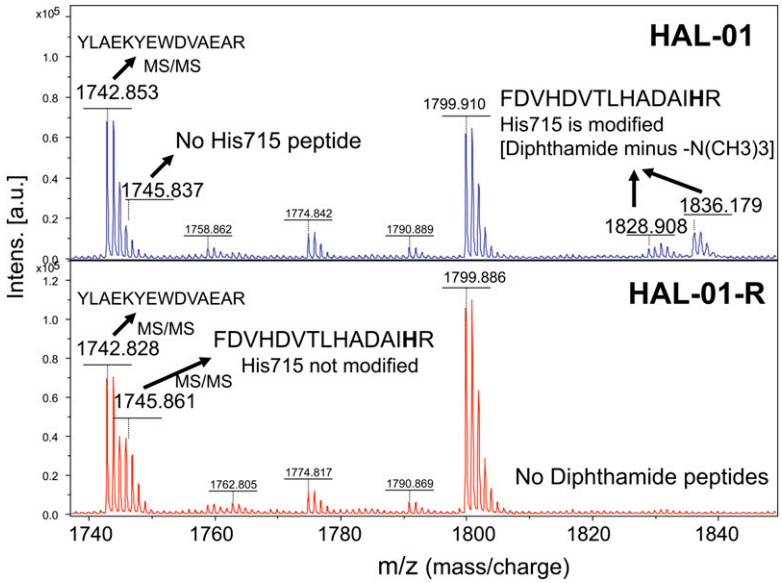

Fig. 4. MALDI of tryptic digests of EF2 gel bands from HAL-01 and HAL-01-R cells. MS/MS analysis of *m/z* 1745.861 from HAL-01-R cells allowed confident identification of EF2 peptide FDVHDVTLHADAIHR with nonmodified His-715. Corresponding analysis of *m/z* 1745.844 from HAL-01 cells yielded no identification. MS/MS analysis of *m/z* 1828.908 and 1836.179 from HAL-01 cells demonstrated that they both are ions corresponding to diphthamide-modified peptide with eliminated trimethylamino group.

methylation at the DPH4 promoter, we measured DNMT1 RNA levels and found no difference between sensitive and resistant cell lines.

DNA Methyltransferase Inhibitor Azacytidine Prevents Resistance. CpG methylation can be inhibited by azacytidine (23), which is used to treat myelodysplastic syndrome (24, 25). To determine whether reducing methylation of CpG islands in the DPH4 promoter could prevent drug resistance, we grew 12×10^6 HAL-01 cells for 18 d with HA22 at 500 ng/mL, azacytidine at 300 nM, or both together (Fig. 6). We found that HA22 killed a large number of cells, but HA22-resistant HAL-01 cells eventually emerged and grew rapidly. When azacytidine was present with HA22, no viable cells could be detected. Azacytidine by itself slowed growth by only 17%. We also found that treatment of the HA22-resistant cells with azacytidine partially restored DPH4 expression, increased sensitivity to HA22, and decreased methylation of the CpG residues (Fig. S4 and Table S2). We propose that azacytidine, by inhibiting CpG methylation, prevents the development of HA22 resistance.

Discussion

We describe here the isolation and characterization of an ALL cell line with reversible resistance to the cytotoxic effects of immunotoxin HA22 through an epigenetic mechanism. HA22 is composed of an anti-CD22 Fv linked to a toxic fragment of PE38 (6, 13, 14). We established that the resistance was due to low levels of DPH4 mRNA and protein. Without DPH4 the cell cannot modify His-715 of EF2 to diphthamide, and EF2 cannot be ADP ribosylated and inactivated by PE38, *diphtheria* toxin, or immunotoxins containing these toxins. We also showed that the CpG island in the DPH4 promoter region is hypomethylated in sensitive cells, which express DPH4, and hypermethylated in resistant cells, which do not have a diphthamide residue at His-715. Revertants return to a hypomethylated state and toxin sensitivity. Although there are many studies showing changes in CpG methylation patterns between drug-sensitive and -resistant cells (26–30), we are unaware of a study in which the methylation status and expression pattern of a specific gene is the basis of drug resistance.

There are many reports showing that drug resistance can be reversed by the treatment of cells or tumors with the deme-

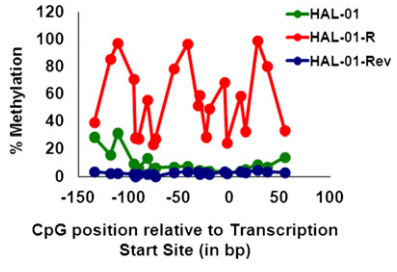

Fig. 5. CpG island methylation. Genomic DNA from sensitive, resistant, and revertant cell lines was subjected to methylation analysis at the cytosine residue of CpG dinucleotide present within the promoter region of *DPH4* gene. Data are plotted as percentage methylation for each CpG dinucleotide relative to the transcription start site. Green, HAL-01; red, HAL-01-resistant; blue, HAL-01-revertant.

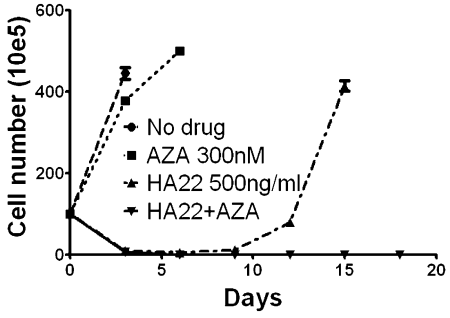

Fig. 6. Azacytidine inhibits resistance emergence in sensitive cells. HAL-01 cells were treated singly with azacytidine or HA22 alone or with a combination of azacytidine and HA22 continuously. Fresh HA22 was added every 3 d, and azacytidine was added every day. The live cell number was determined by trypan blue exclusion.

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thylating agents azacytidine or 5-aza-2'-deoxycytidine (decitabine) (31). Most studies describe an association of demethylating agent treatment with increased sensitivity to a variety of chemotherapeutic agents and do not establish the mechanism of action. Plumb et al. (27) have studied cisplatin-resistant ovarian and colon cancer xenografts and showed that the expression of hMLH, a mismatch repair gene, is associated with the reversal of drug resistance upon treatment with azacytidine. Fulda et al. (28) reported that the decreased expression of caspase-8 and hypermethylation are correlated with drug resistance in Ewings tumors and that the resistance can be reversed by azacytidine. In our report we show that the ability of HA22 to kill cells via inactivation of EF2 is lost because of an epigenetic change in the extent of methylation within the promoter region of the *DPH4* gene and that resistance is prevented by azacytidine treatment.

The mechanism of selective modification of the DPH4 promoter region is not known. Methylation of CpG islands is considered a dynamic process (32). Because we could isolate HA22-resistant cells at high (500 ng/mL) concentrations of HA22, we assume that there is not a slow increase in methylation that gives rise to the resistant phenotype, but instead that the promoter region is heavily methylated in a small number of ALL cells at all times and that these cells are HA22 resistant. These cells should not be detected in normal cell-killing experiments, in which the viability of a small number of cells is analyzed. However, when we used a large number of cells in the selection, we could isolate resistant cells. It would be of interest to be able to analyze the methylation status of single cells before exposure to HA22 to examine this hypothesis.

We are carrying out clinical trials with HA22 in chemotherapy-refractory HCL and ALL. In HCL, HA22 resistance is rarely seen, and the overall response rate approaches 90%, with more than 60% of patients at the highest dose achieving complete remissions (12). In ALL the response rate is significantly lower, and the complete responses not as durable (15). Drug resistance is an important cause of relapse in ALL, with multiple molecular mechanisms that vary with the drug and the biologic subtype (33–35). Because the methylation inhibitor azacytidine prevented the development of HA22 resistance, this combination may prevent resistance in some ALL patients. Hypermethylation was demonstrated in subtypes of childhood ALL (36, 37), and rare complete responses were reported after treatment with azacytidine and decitabine (38, 39).

Modification of EF2 to diphthamide at residue 715 occurs in many eukaryotic organisms. It was suggested that the modification maintains the fidelity of translation but is clearly not required for the viability of cultured cells. Mice with inactivation of one *DPH1* allele are viable but have an increased incidence of cancer; upon inactivation of both alleles viable progeny are not obtained (9). Likewise inactivation of both alleles of *DPH3* and *-4* are embryonic lethal (10, 11).

We used standard biochemical methods to show that the mutant cells could not make DPH4 RNA and protein and that HA22 could not catalyze the incorporation of NAD into the EF2 of mutant cells. To determine whether EF2 was modified by diphthamide we developed a method that used small-scale purification of EF2, trypsin digestion, and MS analysis and detected a peptide with unmodified His-715 in HA22-resistant cells and a diphthamide-modified peptide in normal cells. We will use this method to evaluate mechanisms of immunotoxin resistance in patients.

Materials and Methods

Reagents. HA22, BL22, and SS1P were produced as previously described (40). Azacytidine (Sigma) was dissolved in RPMI-1640 medium. MISSION lentiviral particles containing DPH4 shRNA (TRCN0000145424) and nontarget shRNA control transduction particles (SHC002V) were from Sigma. Antibodies to actin and EF2 were from Abcam and DPH4 from Santa Cruz.

Establishment of Resistant Cell Line. The ALL cell line HAL-01 (DSMZ) was maintained in RPMI-1640 medium with 10% (vol/vol) FBS. To isolate resistant cells, 2×10^7 cells were seeded in 10 mL RPMI-1640 medium with HA22 at 100 ng/mL and incubated for 72 h. Residual living cells were expanded over 2 wk in normal medium. A second round of selection was performed similarly.

Antigen Expression and Internalization of HA22. Quantitation of CD22 surface expression and HA22 internalization was performed as previously described (41, 42).

Protein Synthesis Inhibition Assay. Protein synthesis inhibition was performed as previously described (43).

Toxin-Induced ADP Ribosylation of EF2. Cells were lysed in 0.3 mL radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors, and 0.01 mL of cell lysate (30 μg) was incubated with 100 ng of HA22 in ADP ribosylation buffer [20 mM Tris·HCl (pH 7.4), 1 mM EDTA, and 50 mM DTT] with 5 mM 6-Biotin-17-NAD (Trevigen) for 60 min at 25 °C. Samples were subjected to SDS/PAGE followed by Western blotting with streptavidin HRP conjugate (Invitrogen) to detect biotin-ADP ribose-EF2.

RT and Real-Rime PCR. Measurement of mRNA levels was performed as previously described (44). Sequences are in Table S4.

Immunoblots. Cells were collected, washed with cold Dulbecco's phosphate buffered saline (DPBS) twice, and solubilized in lysis buffer [25 nM Tris·HCl (pH 7.5), 150 nM NaCl, 1.0% Nonidet P-40 (vol/vol), and 0.5% sodium deoxycholate (wt/vol)) with protease inhibitor mixture (Sigma). Detection of proteins was performed as previously described (45).

Lentiviral Infections. HAL-01 cells were infected with MISSION lentiviral particles at a multiplicity of infection of 5 (http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/learning-center/spinoculation-protocol.html). Puromycin was added at 3 μg/mL 3 d after infection. The infected cells were maintained in puromycin until used.

DNA Methylation Analysis. DNA methylation analysis of the DPH4 promoter was performed by EpigenDx (http://www.epigendx.com/). This method is described in *SI Material and Methods*.

Determination of the Extent of EF2-Diphthamide Modification. This method is described in *SI Material and Methods*.

Affymetrix miRNA Array. Affymetrix miRNA array analysis was performed by the Laboratory of Molecular Technology, SAIC-Frederick, Inc., National Cancer Institute at Frederick (http://www.saic-frederick.com).

Statistical Analysis. Data are expressed as mean ± SD. Statistical analysis was performed using the Student *t* test for comparison between two groups.

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