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Epigenetic mechanisms of drug resistance: Drug-induced DNA hypermethylation and drug resistance

(DNA methylation/epimutants/3′-azido-3′-deoxythymidine/5-fluoro-2′-deoxyuridine/gene silencing)

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ABSTRACT **In a model system employing Chinese hamster V-79 cells, the DNA synthesis inhibitor 3′-azido-3′-deoxythymidine (BW A509U, AZT) was shown to induce genome-wide DNA hypermethylation, low-frequency silencing of thymidine kinase (TK; EC 2.7.1.21) gene expression, and resistance to AZT. Twenty-four hours of exposure of V-79 cells to 150 μM AZT led to >2-fold enhancement of genomic 5-methylcytosine levels and produced TK⁻ epimutants at a rate ≈43-fold above background. Such AZT-induced TK⁻ epimutants were shown to be severely reduced in their capacity to activate AZT to its proximate antiviral form, AZT 5′-monophosphate, as compared with the TK⁺ parental cell line from which they were derived. TK⁻ clones isolated under these conditions were shown to be 9- to 24-fold more resistant to the cytotoxic effects of AZT than the parental TK⁺ cell line and showed collateral resistance to 5-fluoro-2′-deoxyuridine. Three of four TK⁻ epimutants could be reactivated at very high frequency (8–73%) to the TK⁺ AZT-sensitive phenotype by 24 hr of exposure to the demethylating agent 5-azadeoxycytidine (5-azadC), implying that drug-induced DNA hypermethylation, rather than classical mutation, was involved in the original gene-silencing event in these three clones. These 5-azadC-induced TK⁺ revertants concomitantly regained the ability to metabolize AZT to its 5′-monophosphate. RNA slot blot analyses indicated that the four AZT-induced TK⁻ clones expressed 8.9%, 15.6%, 17.8%, and 11.1% of the parental level of TK mRNA. The three clones that were reactivatable by 5-azadC showed reexpression of TK mRNA to levels 84.4%, 51.1%, and 80.0% that of the TK⁺ parental cell line. These experiments show that one potential mechanism of drug resistance involves drug-induced DNA hypermethylation and resulting transcriptional inactivation of cellular genes whose products are required for drug activation.**

The usefulness of drugs used in the treatment of cancer and chronic viral infections is frequently limited by the development of clinical drug resistance (1–4). 3′-Azido-3′-deoxythymidine (AZT) is a drug with potential anticancer and proven antiviral activity. In a manner similar to the widely used anticancer agent 1-β-D-arabinofuranosylcytosine (araC), AZT is activated by cellular enzymes to its nucleotide form and then incorporated into DNA during S phase, where it terminates further chain elongation (5). As an experimental anticancer agent, AZT is used to take advantage of a putative overexpression of enzymes involved in DNA synthesis and repair which may occur as one aspect of the development of resistance to cisplatin (6–8). For example, DNA polymerase (Pol) β, which is primarily involved in the repair of damaged DNA, is apparently less discriminating in its choice of nucleotides than is Pol α, which conducts replicative DNA synthesis with a much higher level of fidelity (9). Repair tracts within cisplatin-resistant tumor cells expressing elevated levels of Pol β may thus tend to be terminated by incorporation of AZT to a greater degree than would similarly exposed normal host tissues (6–8). Similarly, cisplatin-resistant tumors expressing elevated levels of thymidine kinase (TK), the enzyme responsible for activating AZT to its proximate cytotoxic form, would also be expected to show enhanced sensitivity to AZT (6).

The potential for AZT as a cancer chemotherapy agent is apparently not limited to tumors expressing resistance to cisplatin. Thus, based upon a similar biochemical rationale, phase I trials are underway studying AZT combined with 5-fluorouracil and leucovorin (10), and combinations of AZT and 5-fluorouracil, cisplatin, and methotrexate (11).

A strategy similar to that described above for circumvention of resistance to cisplatin is involved in the use of AZT to limit human immunodeficiency virus type 1 (HIV-1) infection, since the HIV-1 reverse transcriptase (RT) is several orders of magnitude less discriminating in its choice of nucleotides than host Pol α (5, 9). Recent work on the mechanism underlying the development of clinical resistance to AZT during treatment of AIDS patients has focused upon mutation of the RT gene of HIV-1 (3, 4). Such mutations are thought to produce an alteration in the RT protein which reduces the extent to which AZT may be used as a substrate in the reverse transcription of the HIV-1 RNA genome into DNA. This model is attractive, since the mutation rate within RNA genomes is many orders of magnitude greater than that within DNA genomes (12–16). Based upon analogy with known mechanisms of resistance to closely related agents such as araC (17), however, cell-mediated mechanisms of acquired resistance to AZT may also exist and may play an important role in the clinical pathobiology of long-term exposure to AZT in both cancer and AIDS patients.

Drug-induced DNA hypermethylation occurs as part of the cellular response to toxic drug exposures that inhibit DNA synthesis (18–21). It was suggested that such drug-induced DNA hypermethylation may be capable of silencing genes by transcriptional inactivation and conferring upon cells resistance to drugs requiring metabolic activation by the protein encoded by the silenced gene (19–21). Drug-induced DNA hypermethylation is not solely a tissue culture phenomenon, since malignant cells isolated from cancer patients undergoing *in vivo* exposure to cytotoxic chemotherapy agents (e.g., hydroxyurea, araC) were also found to have undergone genome-wide DNA hypermethylation (21). In the present work we show that resistance to AZT can occur by AZT-induced DNA hypermethylation which transcriptionally in-

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Abbreviations: araC, 1-β-D-arabinofuranosylcytosine; 5-azadC, 5-aza-2′-deoxycytidine; AZT, 3′-azido-3′-deoxythymidine; AZTMP, AZT 5′-monophosphate; AZTDP, AZT 5′-diphosphate; AZTTP, AZT 5′-triphosphate; BrdUrd, 5-bromo-2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; HAT, hypoxanthine/aminopterin/thymidine; HIV-1, human immunodeficiency virus type 1; Pol, DNA polymerase; RT, reverse transcriptase; TK, thymidine kinase.

activates the cellular TK gene, preventing metabolic activation of AZT to its proximate antiviral form, AZT 5'-monophosphate (AZTMP). The AZT-resistant phenotype was observed to be reversible in three of four TK^- clones as a result of exposure to the demethylating agent 5-aza-2'-deoxycytidine (5-azadC), indicating an epigenetic rather than mutational basis for this mechanism of acquired resistance to AZT in these clones. Following Jeggo and Holliday (22), we therefore refer to these hypermethylation-induced clones as "epimutants." We propose that such drug-induced DNA hypermethylation and the subsequent generation of epimutants by potentially reversible gene-silencing events may be an important mechanism of acquired resistance to any drug requiring metabolic activation to elicit a therapeutic effect.

MATERIALS AND METHODS

Cell Culture. Chinese hamster V-79 cells were obtained from Morgan Harris (University of California at Berkeley). Both the TK^- and TK^+ parental cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum.

Chemicals. Non-labeled AZT was kindly provided by Kathy Pattishaw (Burroughs–Wellcome Research Laboratories, Research Triangle Park, NC). [2-^{14}C]AZT (51.4 mCi/mmol, 0.6 mCi/ml in 2% aqueous ethanol; 1 mCi = 37 MBq) and 5-azadC were purchased from Sigma. 5-Methyldeoxycytidine 5'-triphosphate was purchased from Pharmacia. Tissue culture media and other reagents were purchased from Sigma, and fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS).

Analysis of DNA Hypermethylation. Cells in logarithmic phase (2.5×10^5 per 60-mm tissue culture dish) were exposed to the indicated concentrations of AZT for 48 hr in the presence of [6-^{3}H]uridine (1 μCi/ml; 50 Ci/mmol; ICN). DNA 5-methylcytosine levels were quantitated as described (20).

Quantitation of TK Gene Silencing and Activation. Following hypermethylating doses of AZT (100 or 150 μM for 48 hr) to TK^+ parental V-79 cells, the level of TK gene silencing events was quantitated by subsequent continuous exposure to 5-bromo-2'-deoxyuridine (BrdUrd, 100 μg/ml). After 2 weeks of selection in BrdUrd, TK^- clones were quantitated with an Artek (Palo Alto, CA) automated colony counter. In reactivation experiments, putative hypermethylation-induced TK^- epimutants were cloned, seeded into 75-cm^2 tissue culture flasks at 10^6 cells per flask, and subsequently exposed to 5 μM 5-azadC for 24 hr. After removal of 5-azadC, cells were permitted to recover for 24 hr and then seeded into 60-mm tissue culture dishes at 10^3 cells per dish. Twenty-four hours later, selection medium containing 1× hypoxanthine/aminopterin/thymidine (HAT; GIBCO) was substituted for control medium, and cultures were subsequently changed weekly into fresh HAT-containing medium. After 2 weeks of selection, TK^+ revertants were quantitated with an Artek automated colony counter as described above.

Analysis of Acquired Resistance to AZT or 5-Fluoro-2'-deoxyuridine (FdUrd). Cells in logarithmic phase (10^3 per 60-mm dish) were exposed to the indicated concentrations of AZT for 48 hr or FdUrd for 24 hr, at which time drug was removed, fresh culture medium was added, and clonal growth was permitted to continue for an additional 2 weeks. At this time, cultures were rinsed in phosphate-buffered saline fixed in 100% methanol for 15 min, and stained in 0.4% (wt/vol) Giemsa reagent for 15 min. Sensitivity to AZT or FdUrd was then assayed using an Artek automated colony counter set at a discrimination level of 0.2 mm and a sensitivity setting of 76.

HPLC Analysis of AZT Nucleotide Pools. V-79 cells in log phase (2.5×10^5 per 60-mm dish) were exposed to [2-^{14}C]AZT (1 μCi/ml) for 1 hr. After neutralization, trichloroacetic acid-extracted nucleotide pools were directly injected onto a Whatman SAX strong anion-exchange column and eluted under the following conditions: 0–2 min, 90% solvent A/10% solvent B; 2–20 min, a linear gradient to 90% solvent B. Solvent A consisted of deionized ultrapure water, and solvent B consisted of 1 M KH_2PO_4, pH 5.5. A Bio-Rad gradient-module HPLC equipped with a model 1706 UV/visible variable wavelength detector (detection performed at 254 nm) was used to establish a flow rate of 1.5 ml/min at a pressure of 80 kg/cm^2. Fig. 1 shows the elution profile of nucleotide pools extracted from [2-^{14}C]AZT-pretreated V-79 cells. Radioactivity eluted as 5'-mono-, 5'-di-, and 5'-triphosphates was quantitated by scintillation counting. The authenticity of the putative AZTMP, AZTDP, and AZTTP was demonstrated in a separate analysis in which fractions corresponding to these metabolites were collected, hydrolyzed for 40 min at 180°C in 88% formic acid to liberate free thymine, and analyzed by Aminex A-9 chromatography (20). Radioactivity originally isolated as putative 5'-mono-, 5'-di-, and 5'-triphosphate forms of AZT was shown to be coeluted with free thymine, retaining the label originating at the 2-carbon of the pyrimidine ring of AZT (Fig. 1 *Inset*). Thus, the radioactivity that was eluted within the nucleoside 5'-mono-, 5'-di-, and 5'-triphosphate regions of the original column procedure was shown to represent AZTMP, AZTDP, and AZTTP. Endogenous ATP was used as an internal standard.

RESULTS

Fig. 2 illustrates two points: (*i*) AZT induced significant enhancement of the level of genomic DNA methylation in V-79 cells exposed to toxic concentrations of this drug and (*ii*) this induction of DNA hypermethylation correlated with a significant increase in the number of putative TK^- epimutants. Thus, a 2-fold increase in 5-methylcytosine content correlated with a 43-fold increase in the number of putative TK^- epimutants produced. The background level of conversion to the TK^- phenotype was on the order of 4×10^{-6}. The frequency of this phenomenon was thus increased to $\approx 2 \times 10^{-4}$ following exposure to 150 μM AZT. When the number of TK^- epimutants produced by exposure to AZT was plotted

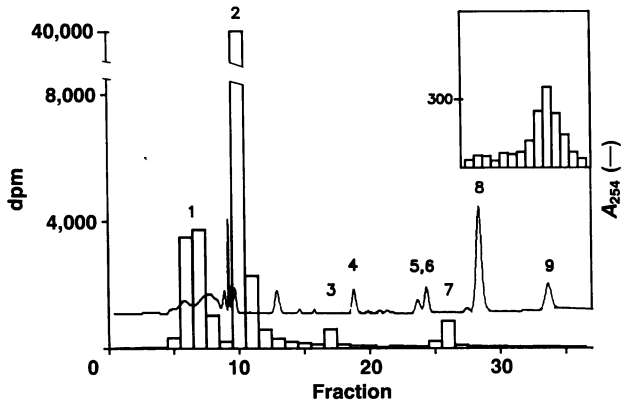

FIG. 1. HPLC analysis of AZT nucleotide pools. TK^+ V-79 cells, TK^- epimutants produced from them following hypermethylating doses of AZT, or TK^+ revertants produced following exposure of the TK^- epimutants to 5-azadC were incubated for 1 hr with [2-^{14}C]-AZT, and nucleotide pools were extracted and analyzed. The elution profile was as follows: 1, unmetabolized AZT plus glucuronidated and other, undefined metabolites of AZT; 2, AZTMP; 3, AZT 5'-diphosphate (AZTDP); 4, adenosine 5'-diphosphate; 5, guanosine 5'-diphosphate; 6, uridine 5'-triphosphate; 7, AZT 5'-triphosphate (AZTTP); 8, adenosine 5'-triphosphate; 9, guanosine 5'-triphosphate. (*Inset*) Radioactivity originating within the pyrimidine ring of putative AZTMP, AZTDP, and AZTTP, following formic acid hydrolysis to the free base, comigrates with thymine.

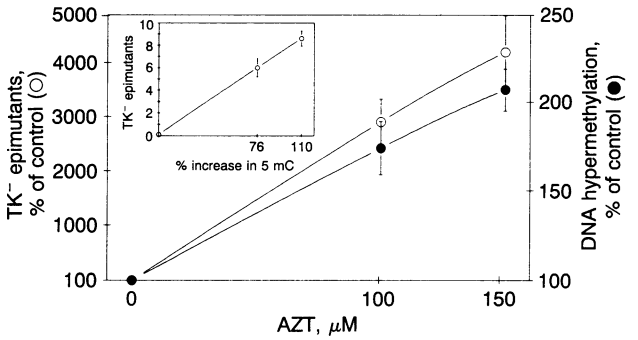

FIG. 2. Correlation between level of AZT-induced DNA hypermethylation and induction of TK⁻ epimutants. For DNA hypermethylation studies, V-79 cells were exposed to the indicated concentrations of AZT for 48 hr, in the presence of [6-^{3}H]uridine (1 μCi/ml). Results are expressed as the mean percent of control for four independent determinations of 5-methylcytosine (5mC)/total cytosine, ± SEM; control cells had levels of 5mC (% of total C) of 1.74 ± 0.01. The background level of TK gene silencing, assumed to be due to classical mutation, was ≈4×10^{-6}. A total of 2×10^7 viable cells were analyzed for each group. For induction of TK⁻ epimutants, V-79 cells were exposed to the indicated concentrations of AZT for 48 hr, then replated at 10^5 viable cells per 60-mm dish. Selection conditions for identification of TK⁻ epimutants consisted of continuous exposure to BrdUrd at 100 μg/ml. Results are presented as the mean percent of control for 10 independent determinations, ± SEM.

against the percent increase in DNA methylation (as compared with controls) occurring in cultures exposed to 100 and 150 μM AZT, it was seen that the induction of TK⁻ epimutants was directly related to the degree of AZT-induced DNA hypermethylation over the dose range studied (Fig. 1 *Inset*).

AZT-induced TK⁻ clones 1–4 showed IC_{25} values of 122, 83, 190, and 128 μM AZT, respectively, compared with an IC_{25} value of 30 μM for the TK⁺ parental cell line from which they were derived (Fig. 3). Upon challenge with 150 μM AZT, clones 1–4 showed 9-, 11-, 24-, and 10-fold increases, respectively, in resistance to AZT as compared with parental TK⁺ cells (Fig. 3). Comparable increases in resistance to FdUrd were observed in these clones (Fig. 4). Thus, clones 1 and 2 showed IC_{25} values for FdUrd of >5 μM; clone 3, ≈2.5 μM; and clone 4, ≈3 μM (compared with an IC_{25} of 0.7 μM for TK⁺ parental cells).

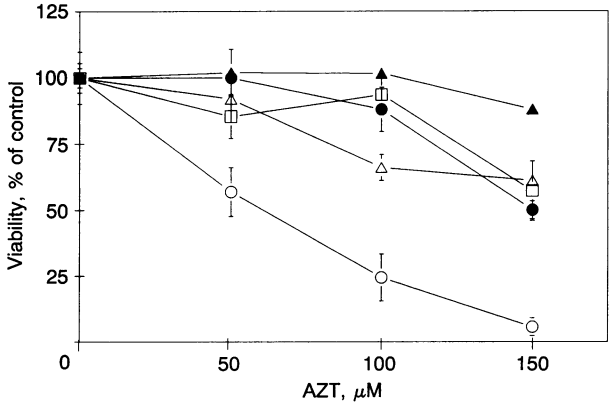

FIG. 3. Resistance to AZT in hypermethylation-induced TK⁻ subclones and parental V-79 cells. Individual cultures (10^3 cells per 60-mm dish) were exposed to the indicated concentrations of AZT for 48 hr, and viability was determined by quantitating clonogenic capacity with an automated colony counter. The plating efficiency of V-79 TK⁺ parental cells in medium without AZT in this series of experiments was ≈89%. Results are presented as the mean of four determinations, ± SEM. Where error bars are not apparent, SEM values were smaller than the corresponding symbol. ●, Clone 1; △, clone 2; ▲, clone 3; □, clone 4; ○, TK⁺ parental cells.

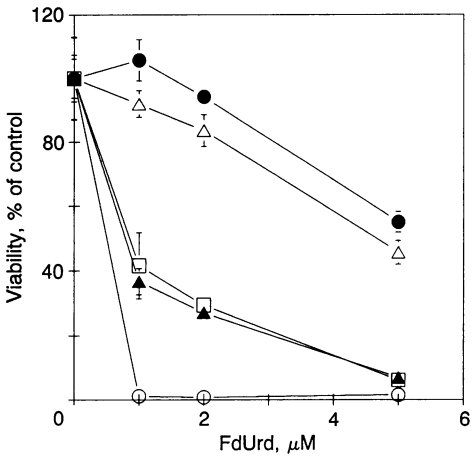

FIG. 4. Resistance to FdUrd in hypermethylation-induced TK⁻ subclones and parental V-79 cells. Individual cultures (10^3 cells per 60-mm dish) were exposed to the indicated concentrations of FdUrd for 24 hr, and viability was determined as for Fig. 3. The plating efficiency of V-79 TK⁺ parental cells in this series of experiments in medium without FdUrd was 88%. Results are expressed as the mean of four determinations, ± SEM. Where error bars are not apparent, SEM values were smaller than the corresponding symbol. Symbols are as in Fig. 3.

To be effective as an antitumor agent or as a chain terminator for reverse transcription of HIV-1 RNA genomes, AZT must be activated to AZTMP by the product of the cellular TK gene, and subsequently to AZTDP and AZTTP by the product of the thymidylate kinase gene. It was thus important for us to measure the capacity of our putative AZT-induced TK⁻ epimutants to activate AZT to its proximate antitumor/antiviral form, AZTMP, and to AZTDP and AZTTP as well.

Nucleotide pool analysis (Fig. 5) showed that, with respect to activation to AZTMP, hypermethylation-induced clones showed levels corresponding to 4.2% (clone 1), 4.9% (clone 2), 0.8% (clone 3), and 0.6% (clone 4) of the parental TK⁺ cell line from which they were derived. Total AZTDP and AZTTP production was also reduced in the hypermethyla-

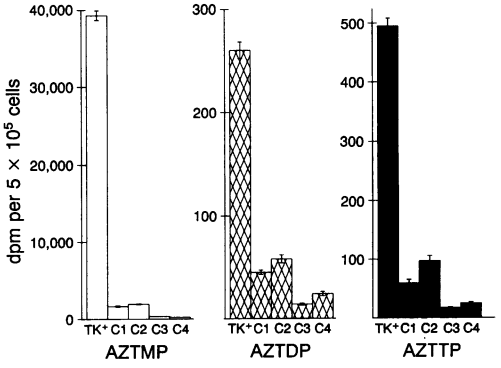

FIG. 5. Ability of hypermethylation-induced clones to metabolize AZT to its 5′-mono-, 5′-di-, and 5′-triphosphate forms. V-79 cell cultures (2.5×10^5 cells per 60-mm dish) were exposed to [2-^{14}C]AZT (1 μCi/ml) for 1 hr, and nucleotide pools were extracted and analyzed. Where error bars are not apparent, SEM values were smaller than the corresponding symbol. Radioactivity (dpm) in AZTMP, AZTDP, and AZTTP was as follows: TK⁺ parental cells, 39,264 ± 1102, 261 ± 15, and 495 ± 22, respectively; TK⁻ clone 1, 1655 ± 143, 45 ± 4, and 59 ± 10, respectively; TK⁻ clone 2, 1936 ± 120, 58 ± 6, and 96 ± 16, respectively; TK⁻ clone 3, 319 ± 35, 14 ± 2, and 17 ± 2, respectively; TK⁻ clone 4, 226 ± 14, 24 ± 3, and 25 ± 4, respectively. ATP was used as an internal standard.

tion-induced TK⁻ epimutants, to levels of 17.2% and 12.0% (clone 1); 22.3% and 19.6% (clone 2), 5.3% and 3.4% (clone 3), and 9.3% and 5.1% (clone 4). It is evident from these data that the primary level of inhibition of AZT metabolism to its antiviral form in our model system is at the level of TK gene expression, rather than thymidylate kinase gene expression, because 5′-monophosphates represent a much smaller percentage of TK⁺ parental activity than do di- and triphosphates.

AZT induction of putative TK⁻ epimutants might have occurred via inactivation of the TK gene locus by classical mutation, and not as a hypermethylation-mediated epigenetic event. To test this possibility, each of the four isolated clones was exposed to the demethylating agent 5-azadC. Exposure to 5-azadC reactivated the TK⁺ phenotype in three of the four clones with a frequency of 8–73% (Table 1). Clone 4 was not reactivatable by 5-azadC, even after several attempts, suggesting the possibility of a mutational basis for this TK gene silencing event. This reversion frequency is many orders of magnitude greater than that which could be expected as a result of back mutation to the TK⁺ genotype. This suggests strongly that the original gene silencing event involved methylation-mediated silencing of TK gene expression and not classical mutation.

Transcriptional inactivation of the TK gene locus was responsible for the induction of AZT-resistant TK⁻ epimutants (Table 2). AZT-resistant clones 1–4 showed 8.9%, 15.6%, 17.8%, and 11.1% of the TK-specific mRNA found in an equivalent concentration (20 μg) of parental TK⁺ RNA. Following exposure to 5-azadC, levels of TK mRNA increased in the three revertible clones (nos. 1–3) to 84%, 51%, and 80% of the original TK⁺ parental cell-line level. These reactivation events represented increases in TK gene expression ranging from 3- to nearly 10-fold in the 5-azadC-induced revertants compared with the AZT-induced TK⁻ epimutants from which they were derived.

All three 5-azadC-reactivated TK⁺ revertants showed a substantial increase in their ability to metabolize AZT, ranging from 3-fold to 34-fold at the 5′-monophosphate level, 2-fold to 15-fold at the diphosphate level, and zero to 2-fold at the triphosphate level, as compared with the TK⁻ epimutants from which they were derived (Table 3). Here again, the major change occurred at the monophosphate level rather than at the di- or triphosphate level, suggesting that TK and not thymidylate kinase represented the affected silenced (and reactivated) gene in this model system.

DISCUSSION

Previous work demonstrated that drug-induced DNA hypermethylation constitutes one response of cells to cytotoxic levels of DNA synthesis-inhibiting drugs, both *in vitro* and *in vivo* (19–21). Since physiologic DNA methylation represents one mechanism by which cells silence genes (23–26), such drug-induced DNA hypermethylation may play a role in the development of resistance to drugs requiring metabolic activation (21). In the present work we observed that at cytotoxic concentrations of AZT, a >2-fold enhancement in total genomic DNA methylation occurred in V-79 cells, concomitant with a 43-fold enhancement in the induction of AZT-resistant, TK⁻ subclones compared with nontreated controls (Fig. 2). (Under the conditions reported here, AZT resistance appeared to be primarily due to hypermethylation inactivation of the TK gene, rather than to inactivation of other genes whose protein products are also required for AZT activation—e.g., thymidylate kinase.) The IC_{25} value for AZT (Fig. 3) or FdUrd (Fig. 4) in these subclones was substantially increased and within a range that would confer drug resistance *in vivo*. Since three of four TK⁻ AZT-resistant subclones could be reactivated at high frequency for TK⁺ expression and resensitization towards AZT by exposure to the demethylating agent 5-azadC, the initial gene silencing clearly was an epigenetic rather than a mutational event in these three clones (Table 1).

Occurring concomitantly with their loss of sensitivity to AZT or FdUrd, all drug-induced TK⁻ epimutants lost their ability to activate AZT to its proximate antitumor/antiviral form, AZTMP (Fig. 5). 5-azadC-induced reversion to the TK⁺ phenotype correlated directly with ability to reexpress TK-specific mRNA (Table 2) and the ability to activate AZT

Table 1. Reactivation of TK⁺ phenotype following pulse exposure to 5-azadC

Clone	Plating efficiency in HAT medium, % of TK⁺ parental cells
1	8 ± 3
2	21 ± 4
3	73 ± 3
4	NR

TK⁻ subclones 1–4 were exposed to a pulse dose of 5-azadC (5 μM, 24 hr), and then selected for their ability to grow in HAT-containing medium. Results obtained represent analysis of 10^4 cells (ten 60-mm dishes containing 10^3 cells each) and are expressed as the mean of five independent determinations of plating efficiency of 5-azadC-exposed cultures in HAT-containing medium, ± SEM. The plating efficiency of V-79 parental TK⁺ cells was ≈89%, and colonies enumerated in clones 1–3 ranged in numbers from 8×10^2 to 7×10^3. NR, not reactivatable.

Table 2. mRNA expression in AZT-induced TK⁻ epimutants and in 5-azadC-induced TK⁺ revertants derived from them

Clone	mRNA, % of TK⁺ parental cells: AZT-induced TK⁻ epimutant	5-azadC-induced TK⁺ revertant
1	8.9	84.4
2	15.6	51.1
3	17.8	80.0
4	11.1	—

Twenty micrograms of total RNA from each clone was blotted to nylon membrane and probed with an oligolabeled fragment from the 3′ end of the Chinese hamster V-79 TK gene. Densitometric scans of the resulting films were quantitated by the method of peak heights. The level of TK mRNA expressed in the TK⁺ parental cell line was taken as 100% of the expected expression level, and the values given above represent individual percentages of this level of expression. Clone 4 was not reactivatable by 5-azadC. Measurements of $\log(r_0 t)$ indicated that the percent abundance of TK transcripts in the unsynchronized TK⁺ parental cell line analyzed in logarithmic phase was ≈0.5%.

Table 3. Ability of 5-azadC-induced TK⁺ revertants to metabolize AZT to its 5′-mono-, 5′-di-, and 5′-triphosphate forms

5-azadC-derived revertant clone	Fold increase over TK⁻ epimutant: AZTMP	AZTDP	AZTTP
$TK^+_{aza\text{-}1}$	3.0 ± 0.007	3.0 ± 0.21	1.2 ± 0.10
$TK^+_{aza\text{-}2}$	2.5 ± 0.16	2.3 ± 0.00	0.9 ± 0.08
$TK^+_{aza\text{-}3}$	33.9 ± 0.92	15.0 ± 0.81	2.0 ± 0.32

Cells were exposed to [2-^{14}C]AZT for 1 hr, and nucleotide pools were extracted and analyzed as described in the text. Results are expressed as the mean of three independent determinations, ± SEM. Radioactivity (cpm) in AZTMP, AZTDP, and AZTTP was as follows: $TK^+_{aza\text{-}1}$, 4982 ± 19, 135 ± 13, and 255 ± 28, respectively; $TK^+_{aza\text{-}2}$, 4910 ± 437, 133 ± 2, and 225 ± 27, respectively; $TK^+_{aza\text{-}3}$, 11,475 ± 1057; 205 ± 17, and 470 ± 110, respectively. A nucleotide concentration of 1.0 represents dpm from [2-^{14}C]AZT in the respective mono-, di-, and triphosphate fractions of the TK⁻ epimutant from which the TK⁺ revertant was derived.

to AZTMP (Table 3). This finding suggests that drug-induced DNA hypermethylation occurring as part of the toxic response to drug exposure mimics the negative transcriptional regulation characteristic of physiologic DNA methylation.

It can be noted from Figs. 2 and 3 that AZT is relatively nontoxic to V-79 cells, and very high levels of the drug (100–150 μM) were required to induce the toxicity of which DNA hypermethylation is a part. Drug-induced DNA hypermethylation very definitely is dependent upon toxic levels of drug exposure (21). The levels of AZT used in our experiments are thus above those that can be achieved clinically (27). However, the human CD4$^+$ T-lymphocyte populations that are the target for AZT antiviral chemotherapy are much more sensitive to AZT than are V-79 cells. In fact, it has been demonstrated that human tissues in general are much more sensitive to AZT than are rodent tissues (28). Further, with respect to anticancer chemotherapy, enhanced sensitivity to AZT might also be expected in human tumors expressing resistance to cisplatin by virtue of overexpression of TK or Pol genes (6–8). That epigenetic events are indeed involved in establishing a drug-resistant state *in vivo* is supported by studies showing that lymphocytic leukemia cells resistant to araC, presumably by virtue of low expression of the required activating enzyme, deoxycytidine kinase, can be reactivated for deoxycytidine kinase expression and sensitivity to araC by exposure of patients to the demethylating agent 5-azacytidine (29). This example of an apparently methylation-related silencing of deoxycytidine kinase resulting in araC resistance *in vivo* bears striking similarity to TK gene silencing resulting in AZT resistance. Both drugs are false metabolites of DNA precursors and require metabolic activation to the nucleotide form for antitumor or antiviral action. Both possess chain-termination properties once they are incorporated into DNA. Finally, resistance to both drugs can occur by virtue of methylation silencing of the gene for the activating enzyme and can be reversed by exposure to demethylating agents. It therefore appears very likely that epigenetic gene-silencing events similar to those observed in araC-treated patients will also occur in patients undergoing anticancer or antiviral chemotherapy with AZT.

Mutations within the HIV-1 RT gene may confer resistance to AZT (3, 4). The extent to which cell-mediated resistance to AZT contributes to the development of clinical resistance to AZT, either alone or in concert with viral RT gene mutations, remains to be determined. There is, however, some precedent for suggesting that optimum conditions for the development of RT gene mutations might exist within CD4$^+$ T lymphocytes that have a methylation-silenced TK gene. Thus, in our hands, TK genes silenced by drug-induced DNA hypermethylation appear not to be totally silent. As illustrated in Fig. 5 and as observed by us in other experiments using V-79 and other cell types, residual TK activity in the range of 2–10% is common. Such limited activation of AZT to its proximate antiviral form in hypermethylation-silenced TK$^-$ epimutants may permit selective pressure sufficient to encourage viral evolution toward AZT-resistant subtypes, without being overtly lethal to the virus.

Finally, in order for AZT-induced DNA hypermethylation to silence TK genes under conditions which permit cell viability, repair of AZT-terminated replication intermediates must occur. Such repair of AZT-terminated replication intermediates has been reported in mammalian cells (30).

Our results with the V-79 cell model system indicate that drug-induced DNA hypermethylation may play a role in the development of resistance to drugs requiring metabolic activation to their anticancer/antiviral forms. The contribution of such drug-induced DNA hypermethylation to *in vivo* drug resistance and the possibility and effects of its circumvention remain to be determined, however.

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