Preferential response of cancer cells to zebularine

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Summary

The frequent silencing of tumor suppressor genes by altered cytosine methylation and chromatin structural changes makes this process an attractive target for epigenetic therapy. Here we show that zebularine, a stable DNA cytosine methylation inhibitor, is preferentially incorporated into DNA and exhibits greater cell growth inhibition and gene expression in cancer cell lines compared to normal fibroblasts. In addition, zebularine preferentially depleted DNA methyltransferase 1 (DNMT1) and induced expression of cancer-related antigen genes in cancer cells relative to normal fibroblasts. Our results demonstrate that zebularine can be selective toward cancer cells and may hold clinical promise as an anticancer therapy.

Introduction

Abnormal hypermethylation of the promoters of numerous cancer-related or tumor suppressor genes important in cellular proliferation is commonly found in primary neoplasms and tumor cell lines (Baylin et al., 2001; Baylin and Herman, 2000; Jones and Baylin, 2002; Jones and Laird, 1999). Because epigenetic processes are potentially reversible, pharmacologic inhibitors of DNA methylation provide a conceptually attractive and rational approach to reestablish the antiproliferative and other crucial cellular functions that are abnormally silenced by hypermethylation.

The first described specific inhibitors of DNA methylation, 5-Azacytidine (5-Aza-CR) and its deoxy analog, 5-Aza-2′-deoxycytidine (5-Aza-CdR), were both originally synthesized as cancer chemotherapeutic agents (Jones and Taylor, 1980; Sorm et al., 1964). Both are pyrimidine ring analogs of cytidine and 2′-deoxycytidine, respectively, but have nitrogen atoms in place of the C-5 pyrimidine carbon atoms. 5-Aza-CR is primarily activated by uridine-cytidine kinase and can be incorporated into both RNA and DNA, whereas 5-Aza-CdR is activated by deoxycytidine kinase and is only incorporated into newly synthesized DNA (Bouchard and Momparler, 1983; Taylor et al., 1984). Once incorporated into DNA, both compounds can form covalent complexes with DNA methyltransferases (DNMTs), leading to a depletion of active enzymes (Bouchard and Momparler, 1983; Santi et al., 1983, 1984; Taylor et al., 1984). Clinically, these agents have already shown utility for the treatment of leukemia and myelodysplastic syndromes (Lubbert, 2000). Nevertheless, these mechanism-based inhibitors of DNMTs have some drawbacks. Both drugs are quite toxic in vitro and in vivo, and they are unstable in aqueous solution, making them difficult to administer both experimentally and clinically (Beisler, 1978).

We recently characterized zebularine as a novel mechanism-based inhibitor of DNA methylation, exhibiting great stability in acidic and neutral solutions and minimal toxicity both in vitro and in vivo (Cheng et al., 2003). As a cytidine analog containing a 2-(1H)-pyrimidinone ring, zebularine was initially developed as a cytidine deaminase inhibitor, because it lacks the amino group on C-4 of the pyrimidine ring (Kim et al., 1986; Laliberte et al., 1992). Similar to 5-Aza-CR and 5-Aza-CdR, zebularine has been shown to form a tight, covalent complex with bacterial methyltransferases in vitro (Hurd et al., 1999; Zhou et al., 2002). However, a major concern with the usage of nucleoside analogs

SIGNIFICANCE

Demethylating agents have great potential clinical promise in reversing the hypermethylation evident in human cancers. However, the use of these agents is often limited by their cytotoxicities and nonspecific effects on both normal and cancer cells, as well as by their inabilities to permanently reverse methylation. Our study shows that a stable DNA cytosine DNA methylation inhibitor, zebularine, preferentially targets cancer cells compared to normal fibroblasts, in terms of incorporation into DNA, reduction of DNA methyltransferase levels, and induction of gene expression. Thus, zebularine is selective toward cancer cells and may represent a candidate drug for epigenetic therapy.

as inhibitors of DNA methylation is the potential for nonspecific effects toward normal cells.

Zebularine can be administered to T24 cells in a continuous fashion to effectively sustain demethylation of the *p16* 5′ region and other methylated loci (Cheng et al., 2004). The purpose of this study was to compare the effects of continuous zebularine treatment on normal fibroblasts and cancer cells. We studied the incorporation of zebularine into DNA and its effects on the growth, methylation, and DNMT protein levels in a panel of four normal fibroblasts and seven cancer cell lines. Lastly, we performed a gene expression microarray chip on four normal fibroblasts and three cancer cells. We found that a larger number of genes were activated in the cancer cells than in the normal fibroblasts, and most of these genes that were activated belonged to a cluster of tumor antigen genes, including *MAGE*-, *GAGE*-, and *XAGE*-type cancer-testis antigens. The augmentation of tumor antigen genes by zebularine suggests that this drug may have antitumor potential in combination with immunotherapy.

Results

Zebularine preferentially inhibits the growth of cancer cells

A major concern for the clinical application of nucleoside analogs is their nonspecific effects toward both normal and cancer cells. Previously, we have demonstrated the growth suppression of T24 bladder cancer cells after continuous treatment with zebularine (Cheng et al., 2004). To further investigate the growth inhibitory effects of continuous zebularine treatment, we extended our study to a panel of seven human cancer cell lines including T24, HCT15, CFPAC-1, SW48, HT-29, PC3, and CALU-1, as well as including four normal human fibroblast cell lines (LD98, T-1, LD419, and CCD-1070Sk). Interestingly, continuous treatment with zebularine substantially retarded the growth of all human cancer cell lines, but the growth of normal human fibroblasts was less affected (Figure 1A). We next examined whether this growth inhibitory effect was associated with upregulation of the mRNA for *p21(WAF1)* and/or *p27(KIP1)*, which are inhibitors of cyclin-CDK complexes involved in G_1 and S phase progression (Gong et al., 2003; Gotz et al., 1996; Tam et al., 1994). Growth inhibition in cancer cells by zebularine was found to be associated with a 2- to 7-fold induction of the *p21* mRNA levels, whereas *p27* mRNA levels remained relatively unchanged (Figure 1A). Both *p21* and *p27* mRNA were unaffected by zebularine in all four normal fibroblasts. All of the cancer cells were therefore more responsive than normal fibroblasts to the growth-suppressive effects of zebularine, and these effects were seemingly *p21*-dependent.

Effects of zebularine on *p16* gene expression and demethylation

To further evaluate the enhanced growth inhibition of cancer cells by zebularine, we next analyzed the reexpression of the *p16* gene, which is known to be abnormally silenced by DNA methylation in all of these cancer cell lines. The *p16* gene was expressed and was mostly unmethylated in the normal fibroblasts, and the mRNA expression was largely unaffected by zebularine treatment (Figure 1B). On the other hand, the *p16* gene was induced in T24, HCT15, CFPAC-1, SW48, and HT-29, but not in PC3 or CALU-1 cancer cells (Figure 1B). The methylation levels of the 5′ region of the *p16* gene were decreased by zebularine treatment in the five cell lines that were inducible (Figure 1B). Interestingly, the *p16* gene was eventually activated in CALU-1 cells after 22 days of continuous treatment (data not shown), suggesting that these cells are more resistant to the demethylating effects of zebularine. These results also

A

Cell Lines	Doubling Time (Hrs) Zebularine (-)	(+)	Percent Increase	*p21* Expression Zebularine (-)	(+)	*p27* Expression Zebularine (-)	(+)	*GAPDH* Expression Zebularine (-)	(+)
Normal Fibroblasts									
LD98	38	46	21						
T-1	58	68	17						
LD419	38	43	13						
CCD-1070Sk	58	65	12						
Cancer Cells									
T24	21	30	44						
HCT15	26	37	41						
CFPAC-1	38	51	32						
SW48	24	39	63						
HT-29	25	42	68						
PC3	39	59	53						
CALU-1	32	42	33						

B

Cell Lines	*p16* Expression Zebularine (-)	(+)	*p16* 5′ Region Methylation (%) Zebularine (-)	(+)	*M4-4* Methylation (%) Zebularine (-)	(+)	*D4Z4* Methylation (%) Zebularine (-)	(+)
Normal Fibroblasts								
LD98			8	8	88	86	55	54
T-1			16	10	93	91	52	52
LD419			12	11	89	88	57	55
CCD-1070Sk			10	2	99	99	69	63
Cancer Cells								
T24			92	76	89	67	82	70
HCT15			89	55	92	53	91	56
CFPAC-1			82	56	86	63	86	66
SW48			84	42	69	56	84	78
HT-29			89	72	92	74	87	78
PC3			95	96	90	87	84	83
CALU-1			85	82	89	87	75	69

Figure 1. Effects of zebularine on growth suppression and methylation in normal and cancer cells

A panel of normal fibroblasts (LD98, T-1, LD419, and CCD-1070Sk) and cancer cell lines (T24 bladder cancer, HCT15, SW48, and HT-29 colon cancer, CFPAC-1 pancreatic cancer, PC3 prostate cancer, and CALU-1 lung cancer cells) were either untreated or treated in the presence of 10^{-4} M zebularine continuously for 8 days.

A: The effects of zebularine on cell growth were analyzed in these cell lines by comparing doubling times before and after drug treatment. The percent increase represents the increase in doubling time of cells after treatment as compared to the untreated control cells. Expression levels of *p21* and *p27* mRNAs were determined by RT-PCR analysis. *GAPDH* mRNA expression levels were measured to control for relative cDNA input.

B: The effects of zebularine on *p16* gene expression and methylation of various loci. The expression level of *p16* mRNA was determined by RT-PCR analysis. The *GAPDH* mRNA expression levels were the same ones shown above in **A**. Methylation status of the *p16* 5′ region, *M4-4*, and *D4Z4* was quantitated by Ms-SNuPE analysis (as described in the Experimental Procedures). Methylation percentage represents the average of three individual CpG sites in each region as assayed from two independent experiments.

suggest that the growth inhibitory effects of zebularine may be partially due to the upregulation of *p21* gene in all seven cancer cell lines and/or the induction of *p16* in T24, HCT15, CFPAC-1, SW48, and HT-29 cells.

We then assayed the methylation levels of two other loci that are known to be methylated in both normal and cancer cell lines. *M4-4* is a single copy sequence located in a CpG island that was previously characterized in our laboratory (Cheng et al., 2004), while *D4Z4* is a subtelomeric repeat sequence (Kondo et al., 2000). Both *M4-4* and *D4Z4* are highly methylated in all cell lines, and our results show that measurable demethylation was observed only in T24, HCT15, CFPAC-1, SW48, and, to a lesser extent, HT-29 cells, and minimal changes were observed in the methylation status in all normal fibroblasts, PC3, and CALU-1 cancer cells (Figure 1B). Consistent with the finding for the methylation of the *p16* 5′ region, the methylation level of these loci was mostly unaffected by zebularine in all normal fibroblasts, as well as the two cancer cell lines that were refractory to the induction of the *p16* gene by zebularine.

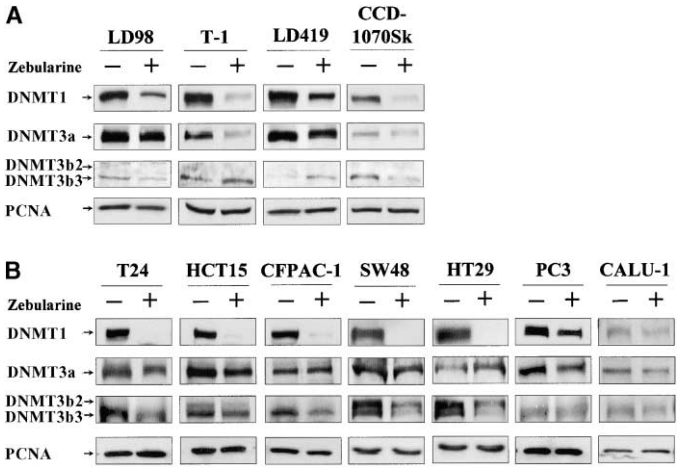

Figure 2. Effects of zebularine on DNMT protein levels in normal and cancer cells

Western blot analysis of DNMT1, -3a, and -3b2/3 protein levels after continuous zebularine treatment (10^{-4} M) for 8 days in a panel of (**A**) normal fibroblasts (LD98, T-1, LD419, and CCD-1070Sk) and (**B**) cancer cell lines (T24 bladder cancer, HCT15, SW48, and HT-29 colon cancer, CFPAC-1 pancreatic cancer, PC3 prostate cancer, and CALU-1 lung cancer cells). Either DNMT3b2 or -3b3 represents the predominant isoform in that specific cell line. Cell lysates were obtained from treated and untreated control cells (as described in the Experimental Procedures) and were analyzed by Western blot analysis with specific antibodies for DNMT1, DNMT3a, and DNMT3b proteins.

Depletion of DNMT levels by zebularine

The mechanism by which zebularine induces demethylation almost certainly requires the incorporation of the drug into the DNA and the formation of covalent complexes with DNMT(s) as is the case with both 5-Aza-CR and 5-Aza-CdR (Bouchard and Momparler, 1983; Hurd et al., 1999; Santi et al., 1984; Taylor et al., 1984; Zhou et al., 2002). Different assays have been developed to track the levels of extractable DNMTs and individual DNMTs bound to genomic DNA using antibodies specific for each DNMT (Liu et al., 2003; Velicescu et al., 2002). We had previously shown the effects of continuous treatment with zebularine on the protein levels of DNMTs in T24 bladder cancer cells using Western blot analysis (Cheng et al., 2004). It was of obvious interest to analyze the protein levels of DNMT1, -3a, and -3b in the normal and cancer cells before and after continuous zebularine treatment. Zebularine treatment decreased the levels of extractable DNMT1 in all normal fibroblasts, as well as PC3 and CALU-1 cancer cells; however, the presence of residual bands on the blots suggested that methylation capacity was not completely lost (Figures 2A and 2B). On the contrary, zebularine caused almost complete depletion of DNMT1 in T24, HCT-15, CFPAC-1, SW48, and HT-29 cancer cells (Figure 2B). The levels of DNMT3a and -3b2/3 proteins in all cell lines were less consistently affected. The mRNA levels of each DNMT, as measured by semiquantitative RT-PCR, remained unchanged after zebularine treatment (data not shown), suggesting that the depletion of DNMT proteins was due to trapping of the enzymes to the zebularine-incorporated DNA rather than an inhibition of transcription or cell proliferation. Cells that were refractory to the demethylating effects of zebularine showed partial depletion of DNMT1, -3a, and -3b. For instance, PC3 and CALU-1, as well as the normal fibroblasts, showed only partial depletion of DNMT1. These data suggest that a complete depletion of DNMT1 is likely a critical requirement for demethylation and gene induction to occur.

Cancer cells preferentially incorporate zebularine into DNA

The differential effects of the drug on cancer cells might be due to preferential incorporation of zebularine into DNA. In our first approach to this issue, we measured the levels of the uridine/cytidine kinase activity, since this "salvage" enzyme is responsible for phosphorylating zebularine to the monophosphate level, which is the first step for eventual incorporation into nucleic acids. The uridine/cytidine kinase activity levels in the cancer cell lines were generally (3- to 40-fold) higher than that in the normal fibroblasts (Figure 3). The only exception was the CALU-1 cell line, which had low uridine/cytidine kinase activity. We next directly measured the level of radioactive zebularine incorporated into the DNA in each cell line. Preliminary experiments (data not shown) confirmed that incorporation was dependent on cell division and showed that incorporation of 2-[^{14}C]-zebularine was almost completely inhibited by the presence of a 1% level of nonradioactive cytidine, confirming that the initial activation of the drug is probably dependent on uridine/cytidine kinase. Our results showed a similar trend to the uridine/cytidine kinase activity levels, in that cancer cells showed higher levels (2- to 50-fold) of incorporation than the normal fibroblasts. These results were generally consistent with the fact that zebularine had greater inhibitory effects and demethylating effects on cancer cell lines than normal fibroblasts, except for PC3 and CALU-1 cells, which both have moderate levels of DNA incorporation, yet minimal effect on DNA methylation. These latter results suggest that incorporation of the fraudulent base into DNA is not sufficient to inhibit DNA methylation in itself and that there may be other factors that dictate the final outcome.

Zebularine substantially alters gene expression in human cancer cells

We also wanted to investigate the effects of continuous zebularine treatment on global gene expression profiles in normal and cancer cells. A high-density oligonucleotide gene expression

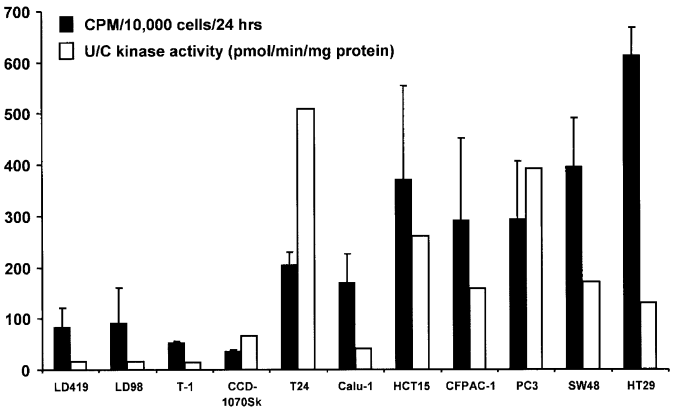

Figure 3. Level of incorporation of 2-[^{14}C]-zebularine into DNA and level of uridine/cytidine kinase activity in normal and cancer cells

Cells in logarithmic phase were plated onto 60 mm dishes and treated 24 hr later with 2-[^{14}C]-zebularine to measure the level of incorporation. Twenty-four hours after the treatment, the cultures were harvested, and the radioactivity incorporated into DNA was determined. The incorporation results are expressed as counts per minute per 10,000 cells (adjusted for population doubling) (black bars). The levels of uridine/cytidine kinase activity are measured (as described in the Experimental Procedures) and are expressed as pmol/min/mg of proteins (white bars). Results represent the average values of two or three separate experiments. Error bars represent the standard deviation of three separate determinations.

microarray analysis on four normal fibroblasts (LD98, LD419, T-1, and CCD-1070k) and three cancer cell lines (T24, CFPAC-1, and HCT15) showed that a common set of 12 genes were upregulated by ≥2-fold after 8 days of continuous drug treatment in all three cancer cell lines, but not in the normal fibroblasts (Table 1A). Interestingly, eight of these (67%) belonged to a cluster of tumor antigens, which include *MAGE*-, *GAGE*-, and *XAGE*-cancer-testis antigens (Table 1A). The level of induced expression varied from 2-fold for *MAGEA1* to a maximum of 315-fold for *SPANXA1* in T24 cells. The microarrays did not detect the upregulation of *p16* or *p21* that was found earlier (Figure 1), possibly due to technical issues related to the fact that multiple transcripts are present in the *p16* gene, making it difficult for the software to score the low signal intensities. We cannot, however, explain the discrepancy between the results obtained for *p21* expression. However, we previously verified that genes detected as being increased on microarrays were also shown to be upregulated by RT-PCR (Liang et al., 2002).

A total of 6 out of 13,300 genes were found to be downregulated in all three cancer cell lines by continuous zebularine treatment (Table 1B). Similarly, only one gene out of 13,000 genes examined was found to be upregulated or downregulated in common in all four normal fibroblasts (Tables 1C and 1D). This result supports the idea that zebularine is relatively nontoxic in that only a small number of genes were globally affected by zebularine. Our earlier work on the global effects of gene expression by 5-Aza-CdR in both T24 and LD419 cell lines (Liang et al., 2002), as well as others, has also shown that a cluster of tumor antigens was activated in cancer cells but not in normal cells (Karpf et al., 2004). Altogether, our data suggest the potential utilization of zebularine in the upregulation of these tumor antigens, which constitute promising targets for immunotherapy (Gillespie and Coleman, 1999; Weber et al., 1994).

Discussion

An important concern for the clinical application of DNA methylation inhibitors is whether they will nonselectively affect normal as well as cancer cells. Here we have shown that zebularine can preferentially target cancer cells compared to normal fibroblasts, in terms of incorporation into DNA, growth inhibition, demethylation, and depletion of DNMTs. We are currently testing the generality of these findings by testing the response of normal epithelially derived cells to zebularine. While it is possible that they will not react in the same way as fibroblasts, our earlier studies using mice given daily doses of zebularine showed little evidence of toxicity (Cheng et al., 2003). Zebularine may therefore be selective toward cancer cells and have potential as an anticancer therapy.

Although five of the seven cancer cells that were treated with zebularine showed *p16* induction, all of them showed an upregulation of the *p21* but not the *p27* gene. These CDK inhibitors are known to be differentially regulated (Gong et al., 2003; Ilyin et al., 2003), and DNA methylation is unlikely to play a role in the modulation of their expression, because both genes were expressed even in the untreated cells. The negative effects of zebularine on the growth of all five cells might therefore be due to an induction of *p21* and/or the *p16* gene. The p21 protein can directly arrest DNA replication in response to DNA damage by binding to proliferating cell nuclear antigen (PCNA) (Waga et al., 1994). The increased *p21* expression points to a novel mechanism for zebularine in addition to its effects on DNA methylation. Nevertheless, other mechanisms, such as the induction of apoptosis, may play an important role in the growth regulation of the cancer cells treated by zebularine (Nieto et al., 2004).

The preferential effects of zebularine for cancer cells are probably due to differential metabolism compared to normal cells. The drug requires activation by phosphorylation before it can be incorporated into nucleic acids, and the conversion of the nucleoside to the monophosphate is the first step in this process, which is catalyzed by the uridine/cytidine kinase enzyme. Once phosphorylated, the 2-(1H)-pyrimidinone ring can be incorporated into RNA and into DNA after the reduction by ribonucleotide reductase of the ribose moiety to deoxyribose. Our data support the idea that uridine/cytidine kinase may serve as a potential gateway enzyme that predetermines the amount of zebularine that eventually gets into the DNA and probably RNA. Since lower levels of this enzyme were present in the normal cells, we would indeed expect less zebularine incorporation into both RNA and DNA of normal fibroblasts. This was strongly supported by the incorporation data. Nevertheless, the level of uridine/cytidine kinase activity does not correlate perfectly with the level of radioactive zebularine incorporation into the DNA, probably due to fact that other enzymes in this pathway might alter the incorporation into the DNA.

The incorporation of the 2-(1H)-pyrimidinone ring into DNA is almost certainly required for the depletion of DNMT1 and the inhibition of DNA methylation that we observed in five of the seven cancer cells. A disparity between the lack of demethylation and zebularine incorporation was observed in both CALU-1 and PC3 cancer cells by day 8 of continuous zebularine treatment. We do not have an explanation for this observation at present; however, treatment of CALU-1 cells with continuous zebularine eventually reactivated the *p16* gene by day 22, which suggests that there may be a different threshold for each cell

Table 1. Genes altered after zebularine treatment of the normal fibroblast and cancer cell lines

				FC						
				Fibroblasts				Cancer cells		
Probe ID	Unigene	Gene symbol	Map location	LD98	LD419	T1	CCD-1070Sk	T24	Cf-Pac	HCT15
A: Genes upregulated ≥2-fold in all cancer cell lines, but not in fibroblast cell lines, 8 days after continuous zebularine treatment										
Cancer-related antigens										
207325_x_at	Hs.72879	MAGEA1	xq28	1.1	−2.8	1.0	−2.3	2.1	5.3	10.6
206218_at	Hs.113824	MAGEB2	xp21.3	1.1	1.6	1.1	1.6	2.5	2.6	4.6
207663_x_at	Hs.176661	GAGE3	xp11.4-p11.2	−1.4	−1.5	1.4	−1.3	6.1	4.0	7.0
208155_x_at	Hs.272484	GAGE6	xp11.4-p11.2	1.2	−1.4	−1.9	−1.2	2.8	9.8	11.3
208235_x_at	Hs.278606	GAGE7	xp11.2-p11.4	1.7	−3.2	1.4	−3.0	2.6	9.2	17.1
206640_x_at	Hs.251677	GAGE7B	xp11.4-p11.2	−4.9	1.2	1.4	−1.1	3.2	9.8	24.3
220057_at	Hs.112208	XAGE1	xp11.23	−1.4	1.3	−1.2	1.1	7.5	7.5	48.5
220922_s_at	Hs.334464	SPANXA1	xq27.1	−1.1	1.1	1.1	1.1	315.2	445.7	13.9
Other										
204351_at	Hs.2962	S100P	4p16	−1.7	−1.6	1.4	−4.0	11.3	6.1	6.5
201739_at	Hs.296323	SGK	6q23	−1.3	1.1	−1.6	1.3	2.1	2.8	2.6
200924_s_at	Hs.79748	SLC3A2	11q13	−1.5	−1.1	−1.1	1.1	2.8	3.7	2.0
214358_at	Hs.449863	ACACA	17q21	1.1	1.3	1.3	−1.4	10.6	2.3	2.0
B: Genes downregulated ≥2-fold in all cancer cell lines, but not in fibroblast cell lines, 8 days after continuous zebularine treatment										
203081_at	Hs.99816	CTNNBIP1	1p36.22	1.1	1.2	−1.1	−1.1	−2.3	−2.6	−2.5
205014_at	Hs.1690	HBP17	4p16-p15	−2.6	−1.1	1.3	2.3	−8.6	−5.7	−4.0
202769_at	Hs.79069	CCNG2	4q21.21	−1.3	−1.1	1.1	2.1	−3.2	−2.0	−2.3
203453_at	Hs.2794	SCNN1A	12p13	−1.2	−1.9	1.0	−1.4	−2.0	−2.5	−3.7
213279_at	Hs.348350	DHRS1	14q11.2	−1.1	1.0	−1.2	1.0	−2.0	−2.0	−2.8
219562_at	Hs.3797	RAB26	16p13.3	−1.3	1.4	1.1	−1.7	−5.3	−3.7	−4.9
C: Genes upregulated ≥2-fold in all fibroblast cell lines, but not in cancer cell lines, 8 days after continuous zebularine treatment										
204637_at	Hs.119689	CGA	6q12-q21	4.3	2.1	2.8	5.7	−1.3	1.7	1.5
D: Genes downregulated ≥2-fold in all fibroblast cell lines, but not in cancer cell lines, 8 days after continuous zebularine treatment										
221019_s_at	Hs.29423	COLEC12	18pter-p11.3	−2.0	−3.0	−13.9	−2.0	1.5	1.2	−1.3

FC, fold change.

line in terms of the amount of zebularine incorporation required in order for demethylation and gene activation to occur. The threshold levels might be quite variable between each cell line, since other factors, such as the rate of DNA repair, rate of zebularine mutation, rate of DNA synthesis, ploidy numbers, *trans*-acting factors, and potentially others may influence these cells. Nevertheless, the cancer cells in general showed a greater level of incorporation of zebularine, which is consistent with the growth inhibitory and demethylating effects observed in most of these cells.

The growth inhibitory effects of zebularine appeared to correlate well with the level of zebularine incorporation into the DNA. The normal cells had low zebularine incorporation and exhibited minimal yet measurable effects on growth inhibition, while the cancer cells had high levels of zebularine incorporation and exhibited greater growth inhibition. This suggests that the growth inhibition seen in cancer cells but not in normal cells may have resulted directly from the incorporation of the drug into the DNA and the effects thereafter. Nonetheless, one cannot rule out the possibility that the growth-inhibitory effects may also be exerted through their incorporation into RNA, which is likely, since preliminary experiments have shown that the 2-(1H)-pyrimidinone ring can be found in the RNA of treated T24 cells at levels ten times greater than the incorporation into the DNA (T. Ben-Kasus et al., 2004, Proc. AACR, abstract). The incorporation of 5-Aza-CR into RNA is known to produce disassembly of polyribosomes, coacceptor function of tRNA, and marked inhibition of protein synthesis (Li et al., 1970; Momparler et al., 1976; Reichman and Penman, 1973). The incorporation of zebularine into RNA could thereby also conceivably explain the growth inhibitory properties of the drug in all cancer cell lines, including CALU-1 and PC3 cells. Zebularine might therefore be multifactorial in its effects, but the preferential activity in cancer cells makes it an exciting drug.

The differential depletion of DNMTs by zebularine in the normal and cancer cell lines is quite interesting. The levels of DNMT1, DNMT3a, and DNMT3b2/3 were less affected in normal fibroblasts after zebularine treatment, consistent with the observation that less zebularine is incorporated into their DNA. The cancer cells that responded to zebularine in terms of demethylation showed a complete depletion of DNMT1 and partial depletion of DNMT3a and DNMT3b2/3. We had previously shown that zebularine preferentially depletes DNMT1 over the other DNMTs in T24 cells (Cheng et al., 2004), implicating a more specific and higher affinity of this drug for DNMT1. We have also previously shown that both 5-Aza-CdR and zebularine display differential depletion of DNMTs in T24 cells using Western blot analysis (Cheng et al., 2004; Velicescu et al., 2002). The complete depletion of DNMT1 appeared to correlate with the demethylation of various loci in these cell lines, suggesting that DNMT1 depletion may be an important indicator of the demethylating ability of zebularine.

The reason for the apparent specificity of the 2-(1H)-pyrimidinone ring for the DNMT1 enzyme is unexpected, since all known cytosine-5 DNMTs appear to utilize the same mechanism of action (Cheng and Roberts, 2001). Perhaps zebularine exhibits a greater enzymatic binding affinity for DNMT1 than for the other DNMTs. However, this is still unclear and needs to be clarified. Nevertheless, it was recently shown that DNMT3b3 isoform has a reduced catalytic activity (Okano et al., 1999; Soejima et al., 2003), which might compromise its ability to complex with zebularine, thereby explaining the partial depletion of this enzyme by the drug. In addition, the partial depletion of DNMT3a by zebularine could potentially be due to the fact that DNMT3a protein is expressed throughout the cell cycle, as opposed to DNMT1 and DNMT3b, which are cell cycle regulated (Robertson et al., 2000), and the depletion of DNMT3a can therefore only occur during the S phase, in which the 2-(1H)-pyrimidinone ring is incorporated into the DNA.

Our microarray data suggest that a combinatorial approach for the treatment of human cancers could involve the use of demethylating agents to augment the presentation of specific cell surface antigens, which could then be targeted by immunotherapy. Indeed, early studies by Frost et al. (1984) showed that 5-Aza-CR could induce the formation of strongly immunogenic variants of mouse cells, providing support for this concept (Frost et al., 1984). The activation of cancer-related or tumor antigens of the MAGE family has been observed in a number of human cancers, and this was shown to correlate with genomic DNA hypomethylation (De Smet et al., 1996). Based on these observations, therapeutic strategies have been recently developed that strive to direct cellular immunity toward tumors that express MAGE antigens (Gillespie and Coleman, 1999). A caveat of this strategy is that the majority of human tumors do not express MAGE antigens; however, treatment of tumor cells with 5-Aza-CdR induces their expression (Liang et al., 2002; Weber et al., 1994). Our findings show that *MAGE* genes and other cancer-testis antigens represent a significant proportion of the genes upregulated by zebularine, and this appears to be specific to cancer but not normal fibroblasts. Our microarray data also show that only a small number of genes are downregulated in all three cancer cells or all four normal fibroblasts after continuous zebularine treatment. These downregulated genes may be directly affected by the drug itself or due to the indirect induction of negative regulatory factors, as was postulated for 5-Aza-CdR (Liang et al., 2002). Nevertheless, none of the downregulated genes belonged to the group of cancer-specific antigens. The combination regimen of zebularine followed by immunotherapy in the treatment of various human cancers will be the focus of future studies. In addition to its stability, minimal toxicity, and ability to sustain demethylation through continuous administration, zebularine is also relatively selective toward cancer cells and thus represents a promising candidate for epigenetic therapy.

Experimental procedures

Cell lines

T24 (bladder transitional carcinoma cells), HCT15, SW48, and HT-29 (colon carcinoma cells), CFPAC-1 (pancreatic carcinoma cells), CALU-1 (lung carcinoma cells), and CCD-1070Sk (human normal fibroblasts) were obtained from the American Type Culture Collection (Rockville, MD). PC3 (prostate carcinoma) cells were kindly provided by Dr. Gerry Coetzee. LD419, LD98, and T-1 (human normal fibroblasts) were established in our laboratory. T24, SW48, HT-29, LD419, LD98, and T-1 cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco/Life Technologies, Inc., Palo Alto, CA). HCT15 was cultured in RPMI 1640 medium supplemented with 10% FCS, penicillin/streptomycin, and 1× sodium pyruvate (Gibco/Life Technologies, Inc.). CFPAC-1 was cultured in IMDM medium supplemented with 10% FCS, penicillin/streptomycin, and 1× glutamine (Gibco/Life Technologies, Inc.). CALU-1 was cultured in McCoy's 5A medium supplemented with 10% FCS, penicillin/streptomycin, and 1× glutamine. PC3 was cultured in RPMI 1640 medium supplemented with 5% FCS and penicillin/streptomycin. CCD-1070Sk was cultured in MEM medium supplemented with 10% FCS, penicillin/streptomycin, 1× sodium pyruvate (Gibco/Life Technologies, Inc.), and 1× MEM nonessential amino acids (Gibco/Life Technologies, Inc.). All cultures were grown in a humidified incubator at 37°C in 5% CO_2.

Drug treatments

All normal and cancer cell lines were plated (3×10^5 cells/100 mm dish) and treated 24 hr later with 10^{-4} M zebularine continuously for 8 days. The medium was changed every 3 days thereafter along with fresh zebularine. DNA, RNA, and protein lysates were harvested at the end of the treatment period for methylation, RT-PCR, and Western blot analyses, respectively.

Nucleic acid isolation

RNA was collected and extracted from cultured cells using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol. DNA was collected as previously described (Gonzalez-Zulueta et al., 1995).

RT-PCR analysis

Total RNA (5 μg) extracted from cultured cells was reverse transcribed using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers (Amersham-Pharmacia, Piscataway, NJ) in a total volume of 25 μl. The reverse transcription (RT) was performed as previously described (Gonzalez-Zulueta et al., 1995). cDNA was amplified with primers specific for either *p16, p21, p27*, or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The RT-PCR conditions were as follows: for *p16*, 94°C for 3 min, 28–30 cycles of 94°C for 1 min, 56°C for 30 s, 72°C for 40 s, and a final extension step at 72°C for 5 min; for *p21*, 94°C for 3 min, 18–23 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final extension step at 72°C for 5 min; for *p27*, the conditions were exactly the same as *p21*, except 26–28 cycles were used; for *GAPDH*, 94°C for 1 min, 19 cycles of 94°C for 1 min, 58°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 2 min. The primer sequences are as follows: *p16* sense, 5′-AGC CTT CGG CTG ACT GGC TGG-3′; *p16* antisense, 5′-CTG CCC ATC ATC ATG ACC TGG A-3′; *p21* sense, 5′-AGG GTG ACT TCG CCT GGG AGC-3′; *p21* antisense, 5′-CAC ACA AAC TGA GAC TAA GGC AGA AGA TGT-3′; *p27* sense, 5′-GCG CCT TTA ATT GGG GCT CCG GCT AA-3′; *p27* antisense, 5′-GCT ACA TCC AAC GCT TTT AGA GGC AGA TCA-3′; *GAPDH* sense, 5′-CAG CCG AGC CAC ATC GCT CAG ACA-3′; and *GAPDH* antisense, 5′-TGA GGC TGT TGT CAT ACT TCT C-3′. RT-PCR amplification reactions of each of the expressed genes were performed with 200 ng cDNA, 10% dimethylsulfoxide (DMSO), 100 μM dNTPs, *Taq* DNA polymerase (Sigma), and 1 μM primers. The RT-PCR conditions, primers, and sequences for *DNMT1, -3a*, and *-3b* were performed as previously described (Robertson et al., 1999). All reactions were analyzed in the linear range of amplification. PCR products were resolved on 2% agarose gels.

Oligonucleotide array analysis

cRNA preparation

Total RNA (10 μg) was used as starting material for the cDNA preparation. The first and second strand cDNA synthesis was performed using the SuperScript II System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions, except using an oligo-dT primer containing a T7 RNA polymerase promoter site. Labeled cRNA was prepared using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Inc., Farmingdale, NY). Biotin-labeled CTP and UTP (Enzo) were used in the reaction, together with unlabeled triphosphates. Following the in vitro transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array hybridization and scanning

Fifteen micrograms of cRNA was fragmented at 94°C for 35 min in a buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, 30 mM magnesium acetate. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris [pH 7.6], 0.005% Triton) was heated to 95°C for 5 min and subsequently to 45°C for 5 min before loading onto the Affymetrix HG_U133A probe array cartridge. The probe array was then incubated for 16 hr at 45°C at constant rotation (60 rpm). The washing and staining procedure was performed in the Affymetrix Fluidics Station. The probe array was exposed to ten washes in 6× SSPE-T at 25°C, followed by four washes in 0.5× SSPE-T at 50°C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate (final concentration of 2 mg/ml; Molecular Probes, Eugene, OR) in 6× SSPE-T for 30 min at 25°C, followed by ten washes in 6× SSPE-T at 25°C. An antibody amplification step was followed using normal goat IgG as blocking reagent (final concentration of 0.1 mg/ml; Sigma) and biotinylated anti-streptavidin goat antibody (final concentration of 3 mg/ml; Vector Laboratories, Burlingame, CA). This was followed by a staining step with a streptavidin-phycoerythrin conjugate (final concentration of 2 mg/ml; Molecular Probes) in 6× SSPE-T for 30 min at 25°C and 10 washes in 6× SSPE-T at 25°C. The probe arrays were scanned at 560 nm using a confocal laser-scanning microscope (Hewlett Packard GeneArray Scanner G2500A; Affymetrix, Inc., Santa Clara, CA). The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software. In our analysis, genes that were induced or reduced ≥3-fold after drug treatment were categorized into different groups.

Western blot analysis of DNMT protein levels

Cells were lysed by the addition of radioimmunoprecipitation (RIPA) buffer (PBS, 0.1% SDS, 0.5% nonidet P-40, and 0.5% sodium deoxycholate), and protein extracts were prepared as previously described (Velicescu et al., 2002). Approximately 60 μg total protein extract was electrophoresed and transferred to PVDF membranes overnight at 4°C as previously described (Velicescu et al., 2002). The membranes were hybridized with antibodies against human DNMT1 (1:1000 dilution; New England Biolabs, Beverly, MA), human DNMT3b (T-16; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and proliferating cell nuclear antigen (PCNA) (1:4000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) in Tris-buffered saline-Tween (TBS-T) buffer (0.1 M Tris, 1.5 M NaCl, and 1% Tween 20) with 5% nonfat dry milk overnight at 4°C. The human DNMT3a antibody was provided by Dr. Ye-Guang Hu (Shanghai, China). The membranes were washed three times with TBS-T buffer at room temperature and incubated with secondary antibodies as follows: anti-mouse-IgG-HRP (1:3000 dilution for PCNA; Santa Cruz), anti-rabbit-IgG-HRP (1:2000 dilution for DNMT1; Santa Cruz), anti-goat-IgG-HRP (1:10000 for DNMT3b; Calbiochem, San Diego, CA). All were incubated with the membrane for 1 hr at room temperature. Afterwards, the membranes were washed five times with TBS-T at room temperature. Proteins were detected with the ECL chemiluminescent detection kit (Amersham-Pharmacia, Piscataway, NJ) and by exposure to Kodak X-OMAT AR film (Rochester, NY).

Quantitation of DNA methylation levels by methylation-sensitive single-nucleotide primer extension assay

Genomic DNA (4 μg) was digested with EcoRI (Roche, Indianapolis, IN) and treated with sodium bisulfite as previously described (Cheng et al., 2003). Methylation analysis was performed using the methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) assay (Cheng et al., 2003). The bisulfite-PCR and the qualitative Ms-SNuPE assay for *p16* 5′ region were performed as previously described (Cheng et al., 2003). The bisulfite-PCR primers for other loci are as follows: for *D4Z4*, 5′-GGG TTG AGG GTT GGG TTT AT-3′ (sense) and 5′-AAC TTA CAC CCT TCC CTA CA-3′ (antisense); for *M4-4*, 5′-ATG GTT TGA GGG TTT AGA TTA GGT-3′ (sense) and 5′-ACA TCA AAA TAA ACT TCC TCT TAC CA-3′ (antisense). The PCR conditions for *D4Z4* were as follows: 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 45 s, and then extension at 72°C for 45 s. The PCR conditions for *M4-4* were as follows: 95°C for 2 min, followed by 42 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 30 s, and then extension at 72°C for 1 min. A final extension step at 72°C for 10 min followed each PCR program. The Ms-SNuPE conditions for *D4Z4* were the same as those described previously (Velicescu et al., 2002). The Ms-SNuPE conditions for *M4-4* were as follows: 95°C for 1 min, 46°C for 30 s, and 72°C for 20 s. The *D4Z4* SNuPE primers are as follows: 5′-TGA GGG TTG GGT TTA TAG T-3′, 5′-GTG GTT TAG GGA GTG GG-3′, 5′-TAT ATT TTT AGG TTT AGT TTT GTA A-3′, 5′-GAA AGG TTG GTT ATG T-3′. The *M4-4* SNuPE primers are as follows: 5′-GGG TTT AGA TTA GGT TTT TT-3′, 5′-GTA ATA AGG ATT ATT TGA ATA G-3′, 5′-TAA TAA TGT GGA TTT GTT TAA ATT-3′.

Radioactive incorporation assay

All cell lines were plated (2×10^5 cells/60 mm dish) and treated 24 hr later with 0.75 μCi/ml 2-[^{14}C]-zebularine for 24 hr. A separate dish of untreated cells was used for each cell line to determine the total number of cells per dish at the end of 24 hr treatment with a Z1 Coulter Particle Counter (Beckman Coulter Corporation, Hialeah, FL). Cells were washed three times with PBS, trypsinized, suspended in 450 μl of water, lysed with 0.3 M KOH, and then incubated at 37°C for 18 hr to hydrolyze RNA. Protein and DNA were precipitated with 90% trichloroacetic acid (TCA) on ice for 5 min, and the DNA pellet was thoroughly washed with 5% TCA and hydrolyzed in 100 μl of 5% TCA for 30 min. The samples were centrifuged, and radioactivity in 50 μl of supernatant was counted in 10 ml of scintillation fluid (Research Products International Corp., Mt. Prospect, IL) in a Packard Tri-Carb 1600 TR Liquid Scintillation Spectrometer (Downers Grove, IL).

Uridine/cytidine kinase activity

The uridine/cytidine kinase activity in normal fibroblasts and cancer cells was measured using assays modified as described by Luccioni et al. (1994).

Determination of cell doubling times

All cultured cells (3×10^5 cells/100 mm dish) were plated and treated with 10^{-4} M zebularine 24 hr later and continuously for 8 days, with fresh zebularine and media changes every 3 days. The cell number/dish was counted with a Z1 Coulter Particle Counter (Beckman Coulter Corporation, Hialeah, FL) every 2 to 3 days. Untreated cells were analyzed under similar conditions as a control. The average cell number from two plates was determined, and the mean cell numbers were plotted to define the cell population doubling times. Initial drug treatment was started 24 hr after seeding.

Acknowledgments

This work was supported by NIH grant RO1 CA 82422. D.J.W. was supported by NIH Training Grant in Basic Research in Oncology T32 CA09659.

Received: October 17, 2003
Revised: June 7, 2004
Accepted: June 23, 2004
Published: August 23, 2004

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