Reversal of GSTP1 CpG Island Hypermethylation and Reactivation of \( \pi \)-Class Glutathione S-Transferase (GSTP1) Expression in Human Prostate Cancer Cells by Treatment with Procainamide

Xiaohui Lin, Kekule Asgari, Mathew J. Putzi, Wesley R. Gage, Xiang Yu, Brian S. Cornblatt, Arunima Kumar, Steven Piantadosi, Theodore L. DeWeese, Angelo M. De Marzo, and William G. Nelson

Abstract

Among the many somatic genome alterations present in cancer cells, changes in DNA methylation may represent reversible “epigenetic” lesions, rather than irreversible “genetic” alterations. Cancer cell DNA is typically characterized by increases in the methylation of CpG dinucleotides clustered into CpG islands, near the transcriptional regulatory regions of critical genes, and by an overall reduction in CpG dinucleotide methylation. The transcriptional “silencing” of gene expression associated with such CpG island DNA hypermethylation presents an attractive therapeutic target: restoration of “silenced” gene expression may be possible via therapeutic reversal of CpG island hypermethylation. 5-Aza-cytidine (5-aza-C) and 5-aza-deoxycytidine (5-aza-dC), nucleoside analogue inhibitors of DNA methyltransferases, have been widely used in attempts to reverse abnormal DNA hypermethylation in cancer cells and restore “silenced” gene expression. However, clinical utility of the nucleoside analogue DNA methyltransferase inhibitors has been limited somewhat by myelosuppression and other side effects. Many of these side effects are characteristic of nucleoside analogues that are not DNA methyltransferase inhibitors, offering the possibility that nonnucleoside analogue DNA methyltransferase inhibitors might not possess such side effects. Human prostate cancer (PCA) cells characteristically contain hypermethylated CpG island sequences encompassing the transcriptional regulatory region of GSTP1, the gene encoding the \( \pi \)-class glutathione S-transferase (GSTP1), and fail to express GSTP1 as a consequence of transcriptional “silencing.” Inactivation of GSTP1 by CpG island hypermethylation, the most common somatic genome alteration yet reported for human PCs, occurs early during human prostatic carcinogenesis and results in a loss of GSTP1 “caretaker” function, leaving prostate cells with inadequate defenses against oxidant and electrophile carcinogens. We report here that the drug procainamide, a nonnucleoside inhibitor of DNA methyltransferases, reversed GSTP1 CpG island hypermethylation and restored GSTP1 expression in LNCaP human PCA cells propagated \textit{in vitro} or \textit{in vivo} as xenograft tumors in athymic nude mice.

Introduction

Somatic changes in DNA methylation present in cancer cells have long tantalized researchers interested in the development of rational cancer treatments. Similar to other somatic genome alterations present in cancer cells, including gene deletions and gene mutations, DNA methylation changes often affect gene function; methylation of CpG dinucleotides clustered into CpG islands encompassing the transcriptional regulatory region of genes has been associated with transcriptional “silencing” of many critical genes in cancer cells (1, 2). Unlike other somatic genome alterations in cancer cells, however, DNA methylation changes typically do not disrupt DNA sequence. For this reason, somatic changes in DNA methylation in cancer cells are thought to be potentially reversible “epigenetic” genome lesions, rather than irreversible “genetic” genome alterations. The enzymes responsible for maintaining CpG dinucleotide methylation patterns throughout DNA replication and mitosis are DNA methyltransferases, enzymes capable of transferring methyl groups from S-adenosyl-methionine to cytosine bases located in self-complementary CpG dinucleotides in DNA. DNA methyltransferases also appear to be critical contributors to cancer development, because DNA methyltransferase expression has been found to be required for \textit{c-fos} transformation \textit{in vitro} (3), and \textit{Apc}Min/+ mice carrying disrupted Dnmt1 alleles have been reported to develop fewer intestinal polyps \textit{in vivo} (4).

Nucleoside analogue inhibitors of DNA methyltransferases, such as 5-aza-C\(^3\) and 5-aza-dC\(^3\), have been widely used in attempts to reverse abnormal DNA methylation changes in cancer cells and restore “silenced” gene expression (5, 6). Unfortunately, despite some apparent successes using preclinical models and some promising results in early clinical trials, the clinical utility of these compounds has not yet been fully realized (6). One of the limitations of the nucleoside analogue methyltransferase inhibitors in clinical trials has been treatment-associated side effects, such as myelotoxicity with resultant neutropenia and thrombocytopenia, which are characteristic of other nucleoside analogues in general, including nucleoside analogues that are not DNA methyltransferase inhibitors (6). Another concern about the use of nucleoside analogues as DNA methyltransferase inhibitors has been that incorporation of the nucleoside analogues into genomic DNA might lead to mutations and/or cancer development (7–12). This has prompted efforts at discovery and development of nonnucleoside analogue DNA methyltransferase inhibitors, such as DNA(MT)1 anti-sense preparations and other agents, which might attenuate DNA methyltransferase activity with less treatment-associated toxicity (13, 14). We report here that the drug procainamide, a nonnucleoside inhibitor of DNA methyltransferases (15) approved by the United States Food and Drug Administration for the treatment of cardiac arrhythmias, reversed GSTP1 CpG island hypermethylation, the most common somatic genome change in human prostate cancer (PCA; Refs. 16–18), and restored GSTP1 expression in LNCaP human PCA cells propagated \textit{in vitro} or \textit{in vivo} as xenograft tumors in athymic nude mice.

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2 To whom requests for reprints should be addressed, at Bunting-Blaustein Cancer Research Building, Room 151, 1650 Orleans Street, Baltimore, MD 21231-1000. Phone: (410) 614-1661; Fax: (410) 502-9817; E-mail: bnelson@jhmi.edu.

3 The abbreviations used are: 5-aza-C, 5-aza-cytidine; 5-aza-dC, 5-aza-deoxycytidine; GSTP1, \( \pi \)-class glutathione S-transferase; RT-PCR, reverse transcription-PCR; MSP, methylation-specific PCR; CMV, cytomegalovirus.
Materials and Methods

Propagation of LNCaP Human PCA Cells in Vitro and in Vivo and Treatment of LNCaP Cells in Vitro and in Vivo with Procarcinamide and with 5-aza-dC. LNCaP PCA cells (19), which contain hypermethylated GSTP1 CpG island alleles and fail to express GSTP1 (18), were propagated in vitro by incubation in RPMI 1640 (Mediatech) supplemented with 10% FCS (Life Technologies, Inc.) and in vivo by inoculation of 10^6 cells in 0.1 ml of saline solution admixed with 75% Matrigel into the s.c. region of the flanks of athymic mice (20). Cultured LNCaP PCA cells were treated with procarcinamide (Sigma Chemical Co.) at a concentration of 100 μm, treated with N-acetylprocarcinamide (Sigma Chemical Co.) at concentration of 100 μm, or treated with 5-aza-dC (Sigma Chemical Co.) at a concentration of 10 μm, continuously for 2 weeks. Fresh medium with fresh drugs were provided after 1 week. Mice carrying visible LNCaP xenograft tumors, 2–4 weeks after LNCaP PCA cell inoculation, were treated with weekly i.p. injections of procarcinamide, at doses of 0.5 or 1 mg (in 0.1 ml of PBS), of 5-aza-dC, at doses of 17.5 or 35 μg (in 0.1 ml of PBS), or of PBS alone (0.1 ml) for 7 weeks. To assess the effects of procarcinamide and 5-aza-dC on tumor size and on GSTP1 CpG island hypermethylation and GSTP1 expression, the mice were sacrificed 2 weeks after the last treatment injection. For each animal, body weight was determined, tumor size was ascertainment by caliper measurement, and the tumor tissues were excised and fixed in 10% formalin.

Detection of GSTP1 mRNA Using Quantitative RT-PCR. Total RNA was isolated from LNCaP PCA cells using an Rneasy RNA isolation kit (Qiagen). GSTP1 mRNA was detected by quantitative RT-PCR using an iCycler iQ Thermal Cycler (Bio-Rad). Before PCR, cDNA was synthesized from 1 μg of RNA using an Omniscript RT kit (Qiagen). PCR reaction mixtures included cDNA from 125 ng of RNA, sense (5′-GGGAGAGT-GCCTTCACATAAGT-3′) and antisense (5′-GGAGAGCCTCCACCTCGAC-3′) primers, and the Master Mix from a QuantiTect SYBR Green PCR kit (Qiagen). PCR cycles involved incubation at 94°C for 30 s, at 55°C for 30 s, and then at 72°C for 30 s. Cloned GSTP1 cDNA was used as a standard for quantification. As a control, TBP mRNA, encoding TATA binding protein, was also detected by quantitative RT-PCR, using specific sense (5′-CAC-GAACCCAGGGCAGCTATT-3′) and antisense (5′-TTTCTTCTCCTGCCAGA-GCTTGAG-3′) primers and the same PCR reaction mixture (21). PCR cycles for TBP cDNA detection involved incubation at 94°C for 30 s, at 55°C for 30 s, and then at 72°C for 30 s. Cloned TBP cDNA was used as a standard for quantification. Each of the PCR assays was run in triplicate; GSTP1 and TBP mRNA copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the iCycler iQ Thermal Cycler.

Immunohistochemical Detection of GSTP1 Polypeptides in LNCaP PCA Xenograft Tissue Sections. Formalin-fixed, paraffin-embedded LNCaP PCA xenograft tissue sections were counterstained with hematoxylin.

Results

GSTP1 mRNA was detected by quantitative RT-PCR (see Materials and Methods). As a control, TBP mRNA expression was assessed by quantitative RT-PCR (see Materials and Methods). Each of the PCR assays was run in triplicate; GSTP1 and TBP mRNA copy numbers were estimated from the threshold amplification cycle numbers using software supplied with the iCycler iQ Thermal Cycler.

Analysis of Methylation Status of GSTP1 Promoter. Bisulfite treatment of genomic DNA was performed by sodium bisulfite treatment. Bisulfite-treated DNA was subjected to MSP using primers selective for methylated (5′-ACCACTAAACACCAACAC/GAC-TAAAACAT-3′) and unmethylated (5′-TGACTTCGCGTGCGCGCG-3′) alleles, using reaction conditions that have been described previously. To permit DNA sequencing of individual GSTP1 CpG island alleles, the PCR products were purified by electrophoresis on 1% agarose gels (Life Technologies, Inc.), isolated from the agarose (using a QIAquick gel extraction kit; Qiagen), recovered by ethanol precipitation, and then cloned by ligation into pCR 2.1Topo cloning vectors (using a TOPO kit; Invitrogen), followed by introduction into TOP10 One Shot competent bacteria. Plasmid DNAs isolated from independent drug-resistant bacterial clones (a minimum of four clones for each PCR reaction product) were subjected to DNA sequence analysis using a cycle sequencing approach with M13 sequencing primers dye-labeled terminators (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit; Perkin-Elmer) and an ABI automated sequencer.

Effects of Procarcinamide and N-Acetylprocarcinamide. To ascertain whether procarcinamide and N-acetylprocarcinamide exposure might trigger trans-activation of the GSTP1 promoter in LNCaP PCA cells or other human cancer cells, LNCaP PCA cells, PC-3 PCA cells (25), MCF-7 breast cancer (BCA) cells (26), and HCT116 colorectal cancer (CRC) cells (27) were transfected with pCMV β-Gal (Promega), with

Fig. 1. Reactivation of GSTP1 expression in LNCaP PCA cells in vitro by treatment with procarcinamide and with 5-aza-dC. Growing cultures of LNCaP PCA cells were treated with procarcinamide (PA; 100 μm), N-acetylprocarcinamide (N-Ac-PA; 100 μm), 5-aza-deoxycytidine (5-aza-dC; 10 μm), or left untreated (Control) for 2 weeks. For each treatment condition (A), RNA isolated from LNCaP PCA cells after 1 week (7 day treatment), or after 2 weeks (14 day treatment; B) of exposure was subjected to analysis for GSTP1 mRNA expression by quantitative RT-PCR (see “Materials and Methods”). Data shown are GSTP1 mRNA copy number/TBP mRNA copy number; bars, ± SE. In addition, for N-Ac-PA treatment condition in B, genomic DNA was isolated from LNCaP PCA cells after 2 weeks of treatment was subjected to analysis for GSTP1 CpG island methylation using a MSP assay (see “Materials and Methods”). Displayed are PCR products generated with primers specific for unmethylated GSTP1 CpG island alleles (U) and for hypermethylated GSTP1 CpG island alleles (M).
Results and Discussion

LNCaP human PCA cells have been reported to contain only hypermethylated GSTP1 CpG island alleles and to be devoid of GSTP1 mRNA and GSTP1 polypeptides (18). Furthermore, treatment of LNCaP PCA cells in vitro with 5-aza-C, a nucleoside analogue inhibitor of DNA methyltransferases, reversed GSTP1 CpG island hypermethylation and restored GSTP1 expression. The evidence that the reactivation of GSTP1 expression in LNCaP PCA cells induced by 5-aza-C treatment occurred as result of a reduction in GSTP1 CpG island methylation was that: (a) GSTP1 expression by LNCaP PCA cells expressing GSTP1 appeared only after decreased promoter methylation; rather, LNCaP PCA cells expressing GSTP1 appeared only after decreased GSTP1 promoter methylation was evident after prolonged 5-aza-C exposure (for many generations); (b) 5-aza-C-treated LNCaP PCA cells that contained unmethylated GSTP1 promoter alleles expressed GSTP1 mRNA and GSTP1 polypeptides, whether or not 5-aza-C was present in the growth medium; and (c) SssI CpG-methylease treatment of GSTP1 promoter sequences before ligation to unmethylated CAT reporter sequences resulted in a marked reduction in CAT reporter expression after transfection into LNCaP PCA cells. Thus, for the preclinical evaluation of DNA methyltransferase inhibitors, reactivation of GSTP1 expression in LNCaP PCA cells may constitute a good candidate biomarker of DNA methyltransferase inhibitor efficacy.

Both procainamide and N-acetyl-procainamide, a major procainamide metabolite, have been shown to inhibit DNA methyltransferase activity in extracts from mammalian cells (15). In addition, although the mechanism by which each of the agents effect enzyme inhibition has not been established, both clearly act differently than 5-aza-C and 5-aza-dC, appearing to bind to GC-rich DNA rather than to be incorporated into the DNA template (28, 29). To ascertain whether
proacainamide could reverse GSTP1 CpG island hypermethylation and restore GSTP1 expression in LNCaP PCA cells, growing cultures of LNCaP PCA cells were treated with procacainamide at a concentration of 100 μM, treated with N-acetyl-procainamide at a concentration of 100 μM, or treated with 5-aza-dC at a concentration of 10 μM, continuously for 2 weeks. Quantitative RT-PCR revealed that exposure to procacainamide or to 5-aza-dC, but not to N-acetyl-procainamide, resulted in the appearance of GSTP1 mRNA in LNCaP PCA cells (Fig. 1A). Of interest, although GSTP1 expression was evident after 1 week of procacainamide treatment, GSTP1 mRNA did not appear until after 2 weeks of exposure to 5-aza-dC treatment. Restoration of GSTP1 expression in LNCaP PCA cells treated with procacainamide or with 5-aza-dC was accompanied by the appearance of unmethylated GSTP1 CpG island alleles in LNCaP PCA cell DNA, as detected using a MSP technique (Fig. 1B; Ref. 23).

To discover whether procacainamide treatment could reverse GSTP1 CpG island DNA methylation and promote GSTP1 expression in LNCaP PCA cells in vivo, immunodeficient mice carrying LNCaP PCA xenograft tumors were treated with procacainamide at doses of 0.5 or 1 mg/week, with 5-aza-dC, at doses of 17.5 or 35 μg/week, or with PBS alone for 7 weeks. Two weeks after the last treatment injection, tumor-bearing mice were euthanized and necropsied. When the LNCaP PCA xenograft tumors were excised and subjected to immunohistochemical staining for GSTP1 polypeptides, a significant (P < 0.0001) increase in the fraction of LNCaP PCA cells expressing GSTP1 was observed after treatment either with procacainamide or with 5-aza-dC (Fig. 2). In fact, procacainamide appeared as effective as 5-aza-dC at restoring GSTP1 expression at the doses and dosing schedule used (Fig. 2D). The restoration of GSTP1 expression in LNCaP PCA cells by procacainamide treatment appeared likely to be a consequence of DNA methyltransferase inhibition, because GSTP1 reactivation was accompanied by a reduction in GSTP1 CpG island hypermethylation detected by bisulfite genomic sequencing analysis (Fig. 3). Of interest, even in the absence of treatment with procacainamide or with 5-aza-dC, propagation of LNCaP PCA cells in vivo as xenograft tumors in immunodeficient mice resulted in a slight reduction of GSTP1 CpG island hypermethylation (Fig. 3) and in the reactivation of GSTP1 expression in a small number of cells (Fig. 2, A and D). The mechanism for this phenomenon, not seen when LNCaP PCA cells are propagated in vitro, was not determined. In a previous study, LNCaP PCA cells and LNCaP cells genetically modified to express high GSTP1 levels by transfection of GSTP1 cDNA exhibited similar growth properties in vitro and in vivo. Perhaps not surprisingly then, neither procacainamide nor 5-aza-dC had any dramatic effects on LNCaP PCA xenograft tumor growth properties in vivo (Fig. 2E). Nonetheless, procacainamide appeared slightly more effective than 5-aza-dC at limiting LNCaP PCA xenograft tumor growth, although procacainamide was not more effective than 5-aza-dC at reactivating GSTP1 expression (Fig. 2, D and E). Whether the mild inhibition of LNCaP PCA xenograft tumor growth by procacainamide was a consequence of DNA methyltransferase inhibition, such as via the reactivation of a “silenced” gene (or genes) other than GSTP1 involved in growth control, or was the result of some other action of the drug, was not discerned.

CpG island hypermethylation and changes in chromatin structure often appear to collaborate in the transcriptional “silencing” of critical genes in cancer cells (30–39). As a consequence, reactivation of genes exhibiting somatic CpG island hypermethylation in cancer cells has been demonstrated not only with DNA methyltransferase inhibitors but also with inhibitors of histone deacetylases and with combinations of DNA methyltransferase inhibitors and histone deacetylase inhibitors. DNA methyltransferases are not the only targets of procacainamide and N-acetyl-procainamide. To discover whether procacainamide and/or N-acetyl-procainamide might be capable of activating GSTP1 expression in LNCaP PCA cells by some mechanism other than DNA methyltransferase inhibition, a series of analyses of the effects of the agents on GSTP1 promoter regulation were undertaken. When LNCaP PCA cells were transfected with an unmethylated GSTP1 promoter/luciferase reporter plasmids and then treated with procacainamide, with N-acetyl-procainamide, with 5-aza-dC, or with the histone deacetylase inhibitor trichostatin A, increased luciferase reporter expression was seen in association with exposure to procacainamide, to N-acetyl-procainamide, and to trichostatin A (Fig. 4A). The induction of GSTP1 promoter activity by trichostatin A was far greater (~60 fold) than the induction by procacainamide (~2 fold) or by N-acetyl-procainamide (~3 fold). The effect of N-acetyl-procainamide treatment on GSTP1 promoter function was not specific; N-acetyl-procainamide triggered increases in the activities of CMV and SV40 promoters in LNCaP PCA cells (Fig. 4B) and augmented CMV promoter activity in PC-3 PCA cells, in MCF-7 BCA cells, and in HCT116 colorectal cancer (CRC) cells (Fig. 4C). To determine whether either procacainamide or N-acetyl-procainamide might affect histone acetylation or deacetylation, LNCaP PCA cells were treated with procacainamide, with N-acetyl-procainamide, or with the histone deacetylase inhibitor trichostatin A and then subjected to immunoblot analysis for acetylated histones (Fig. 5). As expected, trichostatin A treatment increased histone H4 acetylation, particularly at lysine 5 and at lysine 8 (Fig. 5). In contrast, exposure to procacainamide or to N-acetyl-procainamide

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**Fig. 3.** Reduction in GSTP1 CpG island hypermethylation in LNCaP DNA by treatment with procacainamide and with 5-aza-dC. Genomic DNA from LNCaP PCA cells propagated in vitro (A) and from LNCaP PCA cells propagated in vivo (B) as xenograft tumors and treated with procacainamide or 5-aza-dC was subjected to bisulfite genomic sequencing analysis for GSTP1 CpG island hypermethylation. Four to nine PCR clones from each specimen were sequenced; the GSTP1 CpG island DNA methylation pattern for each clone is displayed. •, 5′-CpG; ○, CpG.
failed to have much of an effect on overall histone H4 acetylation or on acetylation at lysine 5 or at lysine 8 (Fig. 5). Curiously, procainamide treatment, and not treatment with either trichostatin A nor with N-acetyl-procainamide, appeared to slightly increase H4 acetylation at lysine 12 (Fig. 5). Considering all of the data collected, the only evidence that procainamide or N-acetyl-procainamide might activate GSTP1 expression in LNCaP PCA cells in vivo by any mechanism other than DNA methyltransferase inhibition was that the agents were able to increase GSTP1 promoter activity slightly in LNCaP PCA cells when provided at a high concentration in vitro (Fig. 4). However, it is unlikely that either agent was able to increase GSTP1 promoter activity in LNCaP PCA cells in vivo by the same mechanism. The N-acetyl-procainamide augmentation of CMV promoter activity in vitro occurred at N-acetyl-procainamide concentrations (>100 μM; see Fig. 4D) higher than that usually achieved in vivo accompanying procainamide treatment (4–8 μg/ml or 14.7 to 29.4 μM). Thus, the reactivation of GSTP1 in LNCaP PCA xenograft tumors in vivo accompanying procainamide treatment was most likely attributable to drug inhibition of DNA methyltransferases and not to drug effects on histone acetylation or other transcriptional regulatory process.

All of the data presented in this report suggest that procainamide might serve as a nonnucleoside inhibitor of DNA methyltransferases useful for reactivating critical genes “silenced” by somatic CpG island hypermethylation in cancer cells. The data further suggest that translation of these preclinical observations to “proof-of-principal” clinical trials with procainamide may be possible using GSTP1 expression and GSTP1 CpG island methylation changes as end point biomarkers for cancers, such as PCA, BCA, and hepatocellular carcinoma, that display frequent GSTP1 CpG island hypermethylation (16, 18, 23, 40). Additional preclinical studies will be needed to ascertain whether procainamide and 5-aza-dC might differ in the propensity to reactivate
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specific genes carrying CpG island hypermethylation changes and to determine whether procainamide, similar to 5-aza-C and 5-aza-dC, might synergize with histone deacetylase inhibitors in the reactivation of “silenced” genes that cannot be reactivated solely by treatment with DNA methyltransferase inhibitors (30).

Somatic CpG island DNA methylation changes have been reported to occur very early during the pathogenesis of many human cancers, suggesting that DNA methyltransferase inhibitors might be considered for use as cancer chemoprevention drugs as well as cancer treatment drugs, if inhibitors with adequate safety profiles can be discovered (4, 41, 42). In support of this concept, 5-aza-dC treatment reduced the appearance of intestinal polyps, thought to represent premalignant lesions, in ApC (dfox)− mice (4). The recognition in this report that both 5-aza-dC and procainamide may be capable of silenced gene reactivation in cancer cells, despite exhibiting markedly different side effects, suggests that relatively nontoxic DNA methyltransferase inhibitors can likely be developed for use in cancer prevention as well as cancer treatment. The one side effect possibly shared by 5-aza-dC and procainamide may be the induction of autoimmunity (43). However, whether autoimmunity constitutes an unavoidable side effect of DNA methyltransferase inhibition is not known. Finally, although procainamide has been a fairly widely used drug for many years, no data have been published regarding the effects of the drug on cancer development. Perhaps, an epidemiology study of cancer incidence and mortality associated with procainamide treatment might disclose whether DNA methyltransferase inhibitor treatment reduces or increases cancer risks.

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References