

Procainamide reverses hypermethylation of *GSTP1* (glutathione S-transferase) CpG islands in human prostate cancer cells*

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*The effect of a nonnucleoside inhibitor of DNA methylases on methylation of *GSTP1* CpG islands was studied in human prostate cancer cells. The RNeasy® Mini Kit was used to purify high-quality RNA for quantitative real-time RT-PCR analysis. QIAGEN® PCR and RT-PCR technologies were used to detect *GSTP1* mRNA. Genomic DNA for methylation-specific PCR (MSP) was isolated from lymph node carcinoma of the prostate (LNCaP) human prostate cancer (PCA) cells using the QIAamp® DNA Mini Kit, and genomic DNA for bisulfite genomic sequencing was isolated from LNCaP PCA xenograft tumors using the DNeasy® Tissue Kit.*

Changes in DNA methylation often affect gene function. CpG dinucleotides clustered into islands around the regulatory region of genes affect the transcriptional regulation of these genes. Hypermethylation of CpG islands by DNA methylases has been shown to be associated with gene inactivation and plays an important role in the development of cancer. Reversal of DNA methylation at these sites is a potential therapeutic strategy, as this reversal may restore expression of transcriptionally silenced genes.

LNCaP PCA cells do not express *GSTP1*, due to *GSTP1* CpG island hypermethylation. Lack of *GSTP1* expression leaves prostate cells vulnerable to damage from both oxidant and electrophile carcinogens. Inactivation of *GSTP1* expression by DNA hypermethylation is the most common somatic genome alteration reported in human prostate cancers. This paper describes the use of procainamide, a nonnucleoside inhibitor of DNA methylases, to reverse DNA hypermethylation and restore *GSTP1* expression in LNCaP human PCA cells.

Materials and methods

Total RNA was isolated from LNCaP PCA cells using the RNeasy Mini Kit. Quantitative

real-time RT-PCR to detect *GSTP1* mRNA was performed using the Omniscript™ RT Kit and the QuantiTect™ SYBR® Green PCR Kit.

Genomic DNA for MSP was isolated from LNCaP PCA cells using the QIAamp DNA Mini Kit. DNA for bisulfite genomic sequencing was isolated from LNCaP PCA xenograft tumors using the DNeasy Tissue Kit. Bisulfite-treated DNA was amplified using standard methods (see reference 1), and subsequent PCR products were purified from 1% agarose gels using the QIAquick® Gel Extraction Kit.

Results and discussion

LNCaP PCA cells were used to study the effect of procainamide and 5-aza-dC on *GSTP1* methylation both in vitro and in vivo, as these cells contain only hypermethylated *GSTP1* CpG island alleles and do not have detectable levels of *GSTP1* mRNA or *GSTP1* polypeptides. Prolonged treatment of LNCaP PCA cells with 5-aza-dC, a nucleoside analog inhibitor of DNA methylases, is known to reverse *GSTP1* DNA hypermethylation and to restore expression of *GSTP1* mRNA. To determine whether procainamide reversed *GSTP1* methylation of LNCaP PCA cell DNA in vitro, growing cultures were treated with procainamide, *N*-acetyl- ►

Reference

1. Lin X, et al (2001) Reversal of *GSTP1* CpG island hypermethylation and reactivation of π -class glutathione S-transferase (*GSTP1*) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* **61**, 8611.

* Data excerpted from Lin, X., Asgari, K., Putzi, M.J., Gage, W.R., Yu, X., Cornblatt, B.S., Kumar, A., Piantadosi, S., DeWeese, T.L., De Marzo, A.M., Nelson, W.G. (2001) Reversal of *GSTP1* CpG island hypermethylation and reactivation of π -class glutathione S-transferase (*GSTP1*) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* **61**, 8611 (reference 1). Published with permission of the American Association for Cancer Research.

Reactivation of GSTP1 Expression in LNCaP PCA Cells

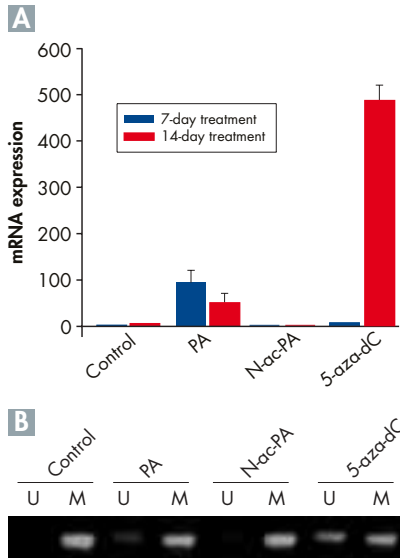


Figure 1 Reactivation of *GSTP1* expression in LNCaP PCA cells detected by quantitative real-time RT-PCR **A** and reduction in *GSTP1* CpG island allele hypermethylation detected by MSP **B**. LNCaP PCA cells were untreated (**control**), or treated with procainamide (**PA**), N-acetyl-procainamide (**N-ac-PA**), or 5-aza-dC. Unmethylated (**U**) and hypermethylated (**M**) MSP products were detected.

procainamide, or 5-aza-dC continuously for 2 weeks. Quantitative real-time RT-PCR of RNA isolated from the cell cultures showed that *GSTP1* mRNA could be detected in LNCaP PCA cells following treatment with procainamide or 5-aza-dC, but not with N-acetyl-procainamide (Figure 1A). Analysis of genomic DNA from the cell cultures treated with procainamide or 5-aza-dC by MSP showed detectable levels of unmethylated *GSTP1* CpG island alleles (Figure 1B).

Immunodeficient athymic nude mice with LNCaP PCA xenograft tumors were studied to determine whether the effect of procainamide on *GSTP1* DNA hypermethylation could be reproduced in vivo. Following treatment of the mice with either procainamide or 5-aza-dC, the tumors were excised and stained with anti-*GSTP1* antibody. Procainamide was found to be as effective as 5-aza-dC at restoring *GSTP1* expression (Figure 2). A significant ($P < 0.0001$) increase in the proportion of cells expressing *GSTP1* mRNA after treatment with procainamide or 5-aza-dC was seen.

Bisulfite genome sequencing analysis was used to determine the level of *GSTP1* DNA methylation, in both LNCaP PCA cells cultivated in vitro and as xenograft tumors in mice. Following treatment of the mice with procainamide or 5-aza-dC, there was a decrease in the level of *GSTP1* DNA hypermethylation (Figure 3). This suggests that procainamide restores *GSTP1* expression by inhibiting DNA methylases, which results in a decrease in hypermethylation of *GSTP1* CpG islands. Additional studies will be needed to determine whether procainamide and 5-aza-dC differ in their ability to reactivate specific genes that have been silenced by CpG island hypermethylation.

Percentage of Cells Expressing GSTP1 Protein

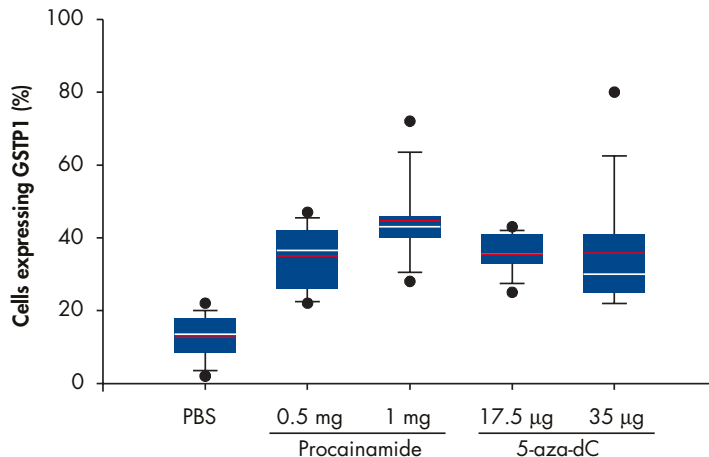
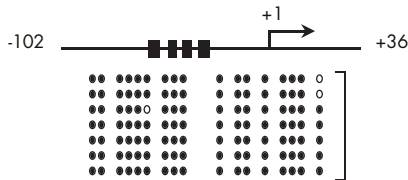


Figure 2 Reactivation of *GSTP1* expression in LNCaP PCA cells in vivo. Immunodeficient mice with LNCaP PCA tumors were treated with PBS, procainamide, or 5-aza-dC for 7 weeks. **White line**: median; **red line**: mean; **•**: outliers.

Reduction in *GSTP1* CpG Island Methylation

A LNCaP PCA cells in vitro



B LNCaP PCA cells in vivo

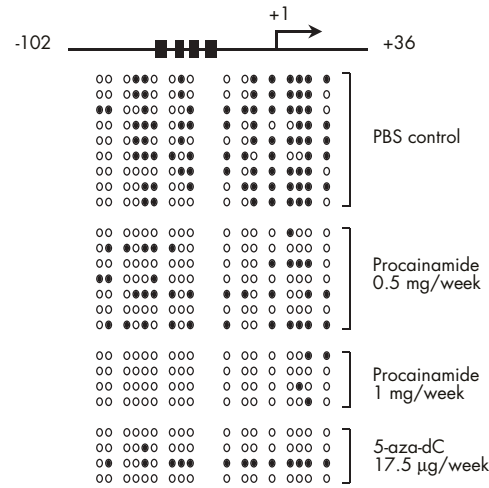


Figure 3 Reduction in *GSTP1* CpG island hypermethylation in DNA isolated from LNCaP PCA cells both in vitro **A** and in vivo as LNCaP PCA xenograft tumors **B**. DNA was analyzed using bisulfite genome sequencing analysis. ●: methylated CpG; ○: unmethylated CpG.

Conclusions

◆ Quantitative RT-PCR of high-quality RNA using the Omniscript RT Kit and QuantiTect SYBR Green PCR Kit suggested that procainamide might reactivate genes that have been silenced by CpG island hypermethylation in cancer cells.

◆ Analysis of genomic DNA, isolated from LNCaP PCA cells using the QIAamp DNA Mini Kit or isolated from LNCaP PCA xenograft tumors using the DNeasy Tissue Kit showed that procainamide reduced *GSTP1* CpG island methylation in both LNCaP PCA cells and LNCaP PCA xenograft tumors in mice. ■

Further reading

Goessl, C. et al. (2000) Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res.* **60**, 5941.

Genomic DNA was extracted from several bodily fluids and prostatic tissue using the QIAamp DNA Blood Mini Kit and the QIAamp Tissue Kit (now the QIAamp DNA Mini Kit). Using methylation-specific PCR (MSP) with fluorescently labeled primers specific for methylated and unmethylated target sequences of the glutathione S-transferase P1 gene, it was possible to detect 200 prostate cancer cells among 2.2×10^7 non-malignant leukocytes.

Silva, J.M. et al. (1999) Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations *Cancer Res.* **59**, 3251.

The QIAamp DNA Blood Mini Kit was used to extract DNA from 1 ml of plasma using a specified protocol. The isolated DNA was tested by PCR for polymorphic markers, p53 mutations, and aberrant methylation. A high proportion of breast cancer patients had plasma DNA similar to tumor DNA at diagnosis, and this was correlated with pathological parameters.

Literature