

Procaine Is a DNA-demethylating Agent with Growth-inhibitory Effects in Human Cancer Cells¹

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ABSTRACT

Methylation-associated silencing of tumor suppressor genes is recognized as being a molecular hallmark of human cancer. Unlike genetic alterations, changes in DNA methylation are potentially reversible. This possibility has attracted considerable attention from a therapeutics standpoint. Nucleoside-analogue inhibitors of DNA methyltransferases, such as 5-aza-2'-deoxycytidine, are able to demethylate DNA and restore silenced gene expression. Unfortunately, the clinical utility of these compounds has not yet been fully realized, mainly because of their side effects. A few non-nucleoside inhibitors of DNA methyltransferases have been reported, including the anti-arrhythmia drug procainamide. Following this need to find new demethylating agents, we have tested the potential use of procaine, an anesthetic drug related to procainamide. Using the MCF-7 breast cancer cell line, we have found that procaine is a DNA-demethylating agent that produces a 40% reduction in 5-methylcytosine DNA content as determined by high-performance capillary electrophoresis or total DNA enzyme digestion. Procaine can also demethylate densely hypermethylated CpG islands, such as those located in the promoter region of the *RARB2* gene, restoring gene expression of epigenetically silenced genes. This property may be explained by our finding that procaine binds to CpG-enriched DNA. Finally, procaine also has growth-inhibitory effects in these cancer cells, causing mitotic arrest. Thus, procaine is a promising candidate agent for future cancer therapies based on epigenetics.

INTRODUCTION

In the last decade, transcriptional silencing of tumor suppressor genes (such as *p16^{INK4a}*, *hMLH1*, *BRCA1*) associated with the hypermethylation of the CpG islands located in their promoter regions has been accepted as a common feature of human cancer (1, 2). In recent years, a CpG island hypermethylation profile of human primary tumors has emerged, showing specific gene promoter hypermethylation of these genes that is dependent on tumor type (3, 4). However, all human neoplasms have multiple bona fide and candidate tumor suppressor genes affecting different cellular pathways that are simultaneously inactivated in the same tumor, and that contribute to the neoplastic phenotype (1–4).

The tumor suppressor genes silenced by promoter hypermethylation provide very attractive targets for the development of drugs to “wake-up” these dormant genes in the fight against cancer. In cancer cell lines, the inhibition of DNA methylation and reactivation of these genes can be accomplished by the nucleoside inhibitors 5-azacytidine and DAC, also known as decitabine (Fig. 1; Ref. 5). The re-expression of these silent genes through the use of these drugs completely restores their functionality, as has been demonstrated for *hMLH1* and *p14^{ARF}* (6, 7). The release of the repression of tumor suppressor and cell cycle genes then leads to the inhibition of tumor growth. The same drastic reduction of cell growth has also been described in a

colorectal cancer line genetically disrupted at the two major DNA methyltransferases (DNMT1 and DNMT3b), leading to demethylation and reactivation of the cell cycle inhibitor *p16^{INK4a}* (8).

One of the limitations of the nucleoside analogues in the clinical trials has been the side effects, such as thrombocytopenia and neutropenia, which are probably caused by cytotoxic effects associated with the drug's incorporation into the DNA independently of their DNA hypomethylation value. This has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA. The drug procainamide, approved by the FDA³ for the treatment of cardiac arrhythmias, has been proposed as being a non-nucleoside inhibitor of DNA methylation (9, 10). Procainamide causes global DNA hypomethylation (9, 10) and restores expression of the detoxifier gene *GSTP1* in prostate cancer cells in which it has been silenced by hypermethylation (11). This action is thought to be mediated by the binding of procainamide to GC-rich DNA sequences (12, 13). We decided to test the putative DNA hypomethylation and growth-inhibitory actions of PCA, a drug approved by the FDA for use as a local anesthetic. Both PCA and procainamide are derivatives of 4-amino-benzoic acid, but the former is the ester with 2-(diethylamino)ethanol and the latter is the amide with 2-(diethylamino)ethylamine. These distinct compounds have different hydrogen-bonding behavior, and it is thought that their interactions with proteins, DNA, and other biomolecules are not the same.

Our results demonstrate that PCA acts as an inhibitor of DNA methylation in breast cancer cells, causing global genomic DNA hypomethylation and demethylation and reactivation of tumor suppressor genes with hypermethylated CpG islands. We observed that this effect is associated with, and possibly mediated by, PCA binding strongly to CpG-rich DNA. Finally, we found that PCA suppresses growth in these breast cancer cells simultaneously with the occurrence of demethylating events. These findings support the possible use of PCA and its derivatives in epigenetics-based cancer therapies.

MATERIALS AND METHODS

Cell Culture. The human breast cancer cell line, MCF-7, obtained from the American Type Culture Collection, was grown in DMEM with 4.5 g/liter of glucose (Invitrogen), supplemented with 10% fetal bovine serum, penicillin/streptomycin, and amphotericin B (Invitrogen) as recommended by the supplier.

Drug Treatments. Twenty-four h after seeding, cells were washed with PBS (Sigma), the medium was replaced, and drug solutions were added to the desired final concentration. Except as otherwise specified, 72 h later, cells were washed and harvested. DAC (Sigma) was dissolved in water to a final concentration of 1.0 mM and was filtered for sterilization, aliquoted, and stored at –80°C. PCA hydrochloride and procainamide hydrochloride (Sigma) were dissolved in water to a final concentration of 0.27 M, filtered for sterilization, and stored at –20°C. Whenever needed, an aliquot of PCA solution was diluted to a final concentration of 10 mM.

³ The abbreviations used are: FDA, Food and Drug Administration; DAC, 5-aza-2'-deoxycytidine (decitabine); DAPI, 4',6-diamidino-2-phenylindole; dsDNA, double-strand DNA; GA, synthetic CpG-rich oligonucleotide; PCA, procaine; dmC, 2'-deoxy-5-methylcytidine; RAR β 2, retinoic acid receptor β 2; ssDNA, single-strand DNA; TUNEL, terminal deoxynucleotide transferase dUTP nick end labeling; CE, capillary electrophoresis; HPCE, high-performance CE; RT-PCR, reverse transcription-PCR.

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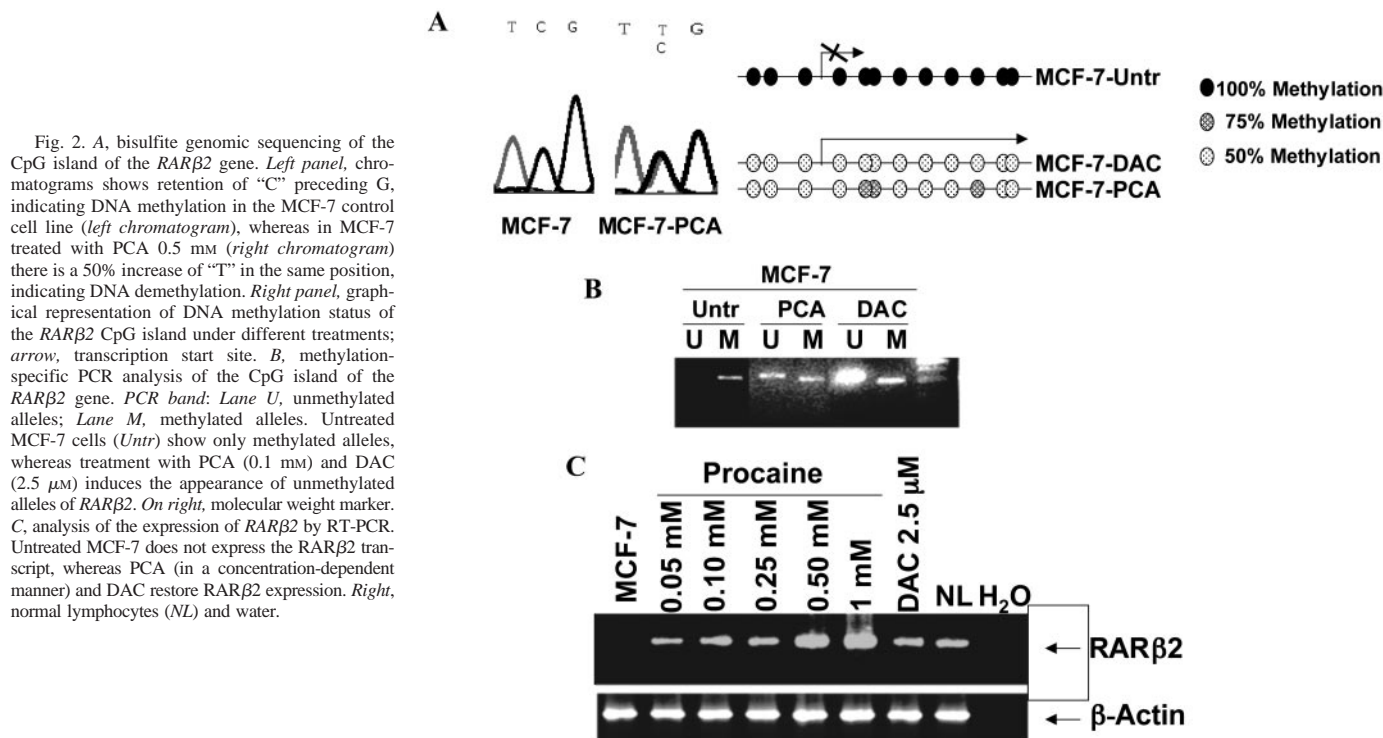


Fig. 2. A, bisulfite genomic sequencing of the CpG island of the *RARβ2* gene. Left panel, chromatograms shows retention of “C” preceding G, indicating DNA methylation in the MCF-7 control cell line (left chromatogram), whereas in MCF-7 treated with PCA 0.5 mM (right chromatogram) there is a 50% increase of “T” in the same position, indicating DNA demethylation. Right panel, graphical representation of DNA methylation status of the *RARβ2* CpG island under different treatments; arrow, transcription start site. B, methylation-specific PCR analysis of the CpG island of the *RARβ2* gene. PCR band: Lane U, unmethylated alleles; Lane M, methylated alleles. Untreated MCF-7 cells (Untreated) show only methylated alleles, whereas treatment with PCA (0.1 mM) and DAC (2.5 μM) induces the appearance of unmethylated alleles of *RARβ2*. On right, molecular weight marker. C, analysis of the expression of *RARβ2* by RT-PCR. Untreated MCF-7 does not express the *RARβ2* transcript, whereas PCA (in a concentration-dependent manner) and DAC restore *RARβ2* expression. Right, normal lymphocytes (NL) and water.

PBS, and stored at 4°C until counting; to count, we used three random fields in two different experiments. To establish the mitotic index, understood as the relative number of cells in metaphase or anaphase with respect to the total number of cells, cells were stained with DAPI (Sigma), and the nuclei were visualized by microscopy. Again, three random fields in two different experiments were used. For apoptosis analysis, we performed the TUNEL assay according to the manufacturer’s instructions (Roche Diagnostics).

RESULTS

PCA Causes Global Genomic DNA Hypomethylation in the MCF-7 Breast Cancer Cell Line. The human breast cancer cell line, MCF-7, was treated with a range of concentrations of PCA (0.005, 0.01, 0.05, 0.1, and 0.5 mM) for 72 h and the 5-methylcytosine DNA content before and after each treatment was measured by HPCE, as described previously (14, 15). Examples of the analysis are shown in Fig. 1, B–D. Whereas the untreated MCF-7 cells had a $2.87 \pm 0.04\%$ 5-methylcytosine DNA content, we observed a directly proportional reduction of methylcytosine DNA groups according to increasing doses of PCA. The greatest reduction, of 41% (absolute value 1.68 ± 0.074), was attained at the highest concentration (Fig. 1E). We used MCF-7 cells treated with DAC as an internal control to validate the DNA-demethylating events; DAC was a much stronger DNA demethylating agent even at lower concentrations than was PCA (Fig. 1C). The global DNA demethylation induced by PCA and DAC was also corroborated by running gels of DNA digested, respectively, with the methyl-isoschizomer enzymes *MspI* and *HpaII* (which share the same target, although *Hpa* does not cut it if the CpG is methylated) and the *McrBC* enzymes, which only cut methylated CpGs (data not shown).

Treatments at 24, 48, and 72 h were developed to precisely define the optimum time points for demethylation (Fig. 1F). Parallel treatments with procainamide and DAC were also performed as positive controls. The maximum demethylating effect of PCA is observed at 72 h, although a similar level is observed at 48 h. For procainamide, the highest demethylation level was already evident after 24h of

treatment, whereas for DAC, the demethylation is progressive, the strongest loss seen at 72 h.

PCA Induces Demethylation of the CpG Island of the Tumor Suppressor Gene *RARβ2* and Restores Its Expression. Once the overall DNA demethylation effect had been found, we decided to test the effects on a particular hypermethylated locus. We chose the CpG island of the *RARβ2*, which our group and many others have reported as being hypermethylated in this cell line, in association with its transcriptional silencing (9, 17, 20, 23, 24), and in which methylation-mediated silencing is a common feature of many human primary tumors (25–27). The treatment of MCF-7 cells with PCA in concentrations higher than 0.01 mM (data not shown) led to the demethylation of the *RARβ2* promoter-associated CpG island and restored the expression of the *RARβ2* transcript. This hypomethylation of the CpG island was demonstrated by bisulfite genomic sequencing around the transcription start site, comparing untreated MCF-7 cells with PCA-treated cells (Fig. 2A). These results were corroborated by methylation-specific PCR with primers covering the same area (Fig. 2B). Again, we used MCF-7 cells treated with the classical DNA demethylating agent DAC as positive controls for the hypomethylation events at the *RARβ2* CpG island (Fig. 2, A and B).

The induction of demethylating events in the CpG island of the *RARβ2* gene was associated with the re-expression of the mRNA of *RARβ2*, which RT-PCR revealed to be absent from the untreated MCF-7 cells (Fig. 2C). The degree of gene reactivation (similar to the total loss of 5-methylcytosine DNA content) was directly proportional to the dose of PCA administered to the cells (Fig. 2C). As a positive control of restoration of gene expression, we used MCF-7 cells, treated with DAC, that also re-expressed the transcript (Fig. 2C).

PCA Directly Binds to CpG-rich DNA. We wondered about the mechanism of action of PCA by which DNA is demethylated. In contrast to DAC, PCA is not a nucleoside inhibitor (see structure in Fig. 1A); thus, we thought that, rather than through incorporation into DNA, it might exert its effects by binding to GC-rich DNA, as do procainamide and *N*-acetyl-procainamide (12, 13). To test this hy-

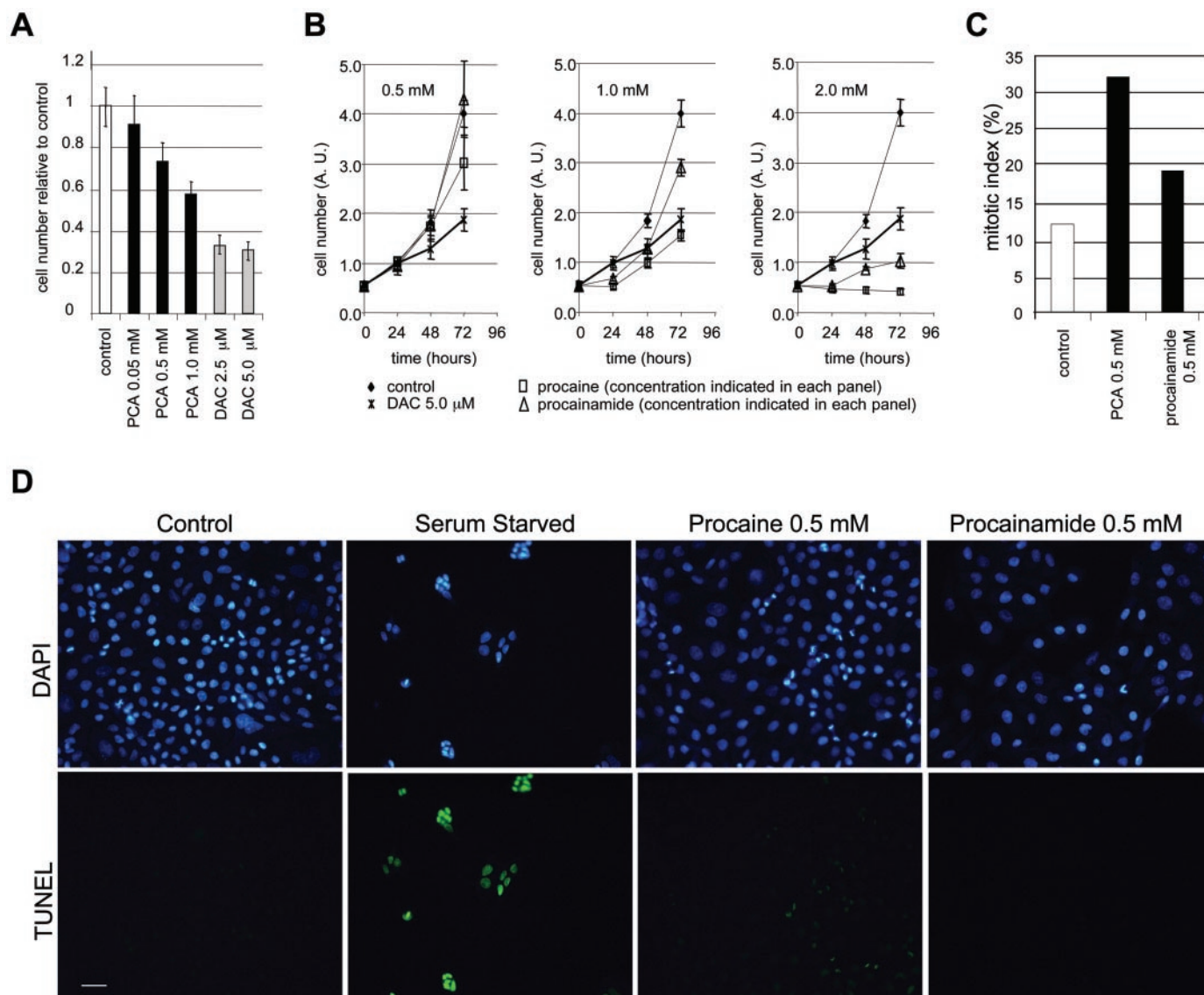


Fig. 4. PCA induces cell growth arrest in M phase. *A*, cell counting expressed as the number of cells relative to the untreated MCF-7 cells. *B*, cell counting (arbitrary units) of MCF-7 cells treated with procainamide (Δ) and PCA (\square) at the indicated concentrations in each panel. DAC 5.0 μ M [GRAPHIC] and untreated (\blacklozenge) were included as controls in all cases. *C*, mitotic index, the number of cells in metaphase, anaphase, or telophase (mitotic figures) with respect to the total number of cells. *D*, apoptotic measurement by double staining with DAPI and TUNEL assay of MCF-7 control cells that were PCA treated (0.5 mM) and procainamide treated (0.5 mM). Cells undergoing serum starvation for 72 h were used as positive control for apoptotic induction. DAPI staining also shows an increase of mitotic figures in PCA-treated cells. Bar, 25 μ m.

but, instead, binds to DNA. Thus, PCA may be an example of an agent that demethylates DNA and reactivates methylated genes with less potential side effects. It is important to mention that the doses of PCA that achieve significant demethylation and growth-inhibitory effects in our study are of the same order as those administered in conjunction with antineoplastic drugs (30–33) or radiotherapy (34, 35). Most important, PCA has even proved to protect against chemotherapy-related nephrotic and hepatic toxicities (31).

Our study supports a role for PCA as a promising DNA-hypomethylating drug with growth-inhibitory effects in cancer cells. Its long-established and safe use as a local anesthetic, with well-known pharmacological characteristics, may stimulate its prompt transition to preclinical and early clinical trials for epigenetics-based cancer treatments.

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