Reactivation of Tumor Suppressor Genes by the Cardiovascular Drugs Hydralazine and Procainamide and Their Potential Use in Cancer Therapy

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Abstract

Purpose: The purpose of this study is to evaluate the demethylating and tumor suppressor-reactivating activity of hydralazine and procainamide.

Experimental Design: MDA-231, MCF-7, and T24 cell lines were treated for 5 days with 10 μM hydralazine or 10 μM procainamide. 5-aza-deoxycytidine at 0.75 μM was used as positive control. BALB/c nu/nu mice xenografted with MDA-231 cells were treated with these drugs for 7 days by i.p. route. Methylation was assessed by PCR after digestion with methylation-sensitive enzymes for the ER and with methylation-specific PCR for retinoic acid receptor (RAR)β and p16 genes. Gene expression was evaluated by reverse transcription-PCR and Western blot. The duration of the gene re-expressing effect of hydralazine was analyzed on T24 cells. Functionality of the re-expressed proteins was evaluated by the induction of the estrogen-responsive gene ER, RARβ, and p16 genes in cultured cells. Both drugs also demethylated and re-expressed ER gene in mice. Hydralazine re-expressed the p16 gene longer as compared with 5-aza-deoxycytidine. The re-expressed genes were functional. In addition, the treatment with oral hydralazine demethylated and re-expressed the RARβ and p16 genes in the cervical and head and cancer patients.

Conclusions: These cardiovascular drugs have a promising tumor suppressor-reactivating action and could potentially be used in clinic as an anticancer treatment, most likely to increase the efficacy of current biological or chemotherapy treatments.

Introduction

Cancer development is driven from genetic and epigenetic changes at the cellular level that activate and inactivate oncoproteins and tumor suppressor genes, respectively. TSGs are inactivated by gene deletions and mutations, however, transcriptional silencing by epigenetic changes is another well-established mechanism of inactivation (1). Thus, methylation at CpG islands suppresses the expression of TSGs such as p16 (2), retinoblastoma (3), VHL (4), E-cadherin (5), hMLH1 (6), BRCA1 (7), and many others, including the ER (2) gene (8). In eukaryotic cells, methylation of cytosines occurs predominantly in genomic regions poor in CpG (9). On the contrary, discrete regions located at the 5’ regulatory regions of genes, termed CpG islands, are always unmethylated in normal cells with the exception of transcriptionally silent genes on the inactive X-chromosome (10) and some imprinted genes (11). The knowledge of specific genetic abnormalities in tumors has led to the developing of molecular-targeted therapies as exemplified by gene therapy directed to restoration of TSG activity (12), blocking oncoproteins, or their products with either antisense oligonucleotides (13), kinase inhibitors (14), and monoclonal antibodies (15) among other approaches.

Reversion of gene promoter methylation of TSGs is an attractive target for anticancer treatment. A large body of experimental evidence demonstrates that re-expression with demethylating drugs of silenced TSGs leads to a strong inhibitory effects on cancer cell growth in vitro and in vivo (16). These concepts are supported by the transforming ability of the forced expression of DNA-methyltransferase gene activity (17), as well as by the reversion of the malignant phenotype by antisense oligonucleotides against this gene (18).

These findings pave the way for the clinical testing of demethylating agents in cancer, however, a constrain for their use is the potent carcinogenic and mutagenic properties, as well as the associated toxicity of 5-azacytidine and its analog 5-aza-CdR, two well-known hypomethylating agents (19), being unlikely that these agents will find widespread clinical use. Be-
cause of that, there is an urgent need to find out less toxic demethylating agents.

The antihypertensive and antiarrhythmic agents hydralazine and procainamide, respectively, are known inhibitors of DNA methylation that have been used to hypomethylate T cells in experimental systems. This hypomethylation produces overexpression of lymphocyte-function-associated antigen 1 that makes T cells autoreactive. This property has been demonstrated in vitro and in vivo models (20). More recently, Kaplan et al. (21) have demonstrated that the lymphocyte function-associated antigen 1 overexpression induced by DNA-methylation inhibitors is caused by demethylation at the CD11a gene promoter. Here, we demonstrate that hydralazine and procainamide are able to demethylate and re-express TSGs in experimental and clinical settings.

Materials and Methods

Cell Lines, Tissue Specimens, and Reagents. DMEM culture media and fetal calf serum were purchased from Life Technologies, Inc. (Grand Island, NY). Hydralazine, procainamide, 5-Aza-CdR, and all-trans-retinoic acid were obtained from Sigma (St. Louis, MO). MDA-231, MCF-7, and T24 cell lines were obtained from American Type Culture Collection. Cells were grown in DMEM supplemented with 10% FCS at 37°C and 5% CO2.

5-Aza-CdR, Hydralazine, and Procainamide Treatment. MDA-231, MCF-7, and T24 cells were plated at densities of 5 x 105 cells/100-mm dish, respectively. On day 2, cells were treated with DMEM containing either no drugs or 0.75 μM 5-Aza-CdR, 10 μM hydralazine, or 10 μM procainamide. Drugs were freshly prepared before use. The media containing the drugs was maintained during the 5 days of treatment. On day 6, the cells were harvested using a cell scraper for analysis of gene and protein expression, as well as for DNA methylation assays. These experimental conditions applied for the MDA-231 and T24 cell lines. For MCF-7 cells, the media containing the drugs was changed at day 6, and then, the cells were treated additionally for 24 h with all-trans-retinoic acid at a concentration of 1 mM. For the time course, demethylation experiment MCF-7 and T24 cells were treated with hydralazine for 5 days and harvested for methylation analysis at 24, 48, 72, 96, and 120 h.

RT-PCR. RNA from drug-treated and -untreated cells was obtained using the TriReagent Life Technologies, Inc., RNA extraction kit following the manufacturer instructions. One μg of total RNA was used for reverse transcription, which was performed with a RT-PCR kit (Perkin-Elmer, Branchburg, NJ) following the manufacturer instructions. For ER gene, the PCR reactions were performed as described previously (22). The primers used were 5'-GGAGACATGAGAGCTGC-CAAC-3' and 5'-CCAGCAGCATGTGGAAGATC-3' in a total reaction volume of 20 μl. The PCR program used was 94°C for 12 min, followed by 24 cycles at 94°C for 1 min, 56°C for 30 s, and 72°C for 40 s. The p16 gene was amplified as previously described (23) using the primers sense 5'-AGCCTTCCGGCT-GACCTGCTGG-3' and antisense 5'-CTGCCCCATCATCATCATGACCTGG-3'. For the RARβ gene, the conditions were as previously described (24) using the primers: sense 5'-GACTG-

Western Blots. For protein analysis, harvested cells were centrifuged, washed with 1× PBS, recentrifuged, and resuspended in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 150 mM NaCl, and 1% NP40] containing protease inhibitors (10 μg/ml aproteinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, and 10 μg/ml pepstatin A). Fifty μg of total protein were electrophoresed on a 12% SDS/PAGE gel, transferred to polyvinylidene difluoride membranes, and immunoblotted with the corresponding antibodies: ER; p16; RARβ; and actin for loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Membranes were incubated with the primary antibody for 1 h at room temperature, followed by incubation with IgG-horseradish peroxidase-conjugated secondary antibodies (Amersham International, Buckinghamshire, United Kingdom) for 30 min at room temperature. Bands were visualized by enhanced chemiluminescence (Amersham International).

Methylation Analysis. The analysis of methylation for the ER gene promoter was performed by PCR after digestion of the sample DNA with methylation-sensitive HpaII and methylation-insensitive MspI enzymes as described previously (25). Briefly, DNA was purified with a Wizard purification kit (Promega, Madison, WI), and then, 1 μg/sample was digested overnight in a volume of 20 μl with either 10 units of MspI or HpaII according to the manufacturer instructions. Then, 2 μl of the digestion mix, equivalent to 100 ng of DNA, were then amplified in a volume of 20 μl using the primer sequences 5'-TCTCCCCCTACTCCCCCTGC-3' and 5'-GAAATCAAAAAACAGCCTACC-3' that amplify a product of 292 bp of the p0 ER gene promoter. The protocol of amplification consisted of 95°C for 5 min, 25 cycles of 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension of 7 min at 72°C.

Absence of bands from samples digested with MspI indicates that the digestion worked as this enzyme cuts irrespective of the methylation status, no amplification in reactions digested with HpaII is indicative of demethylation, whereas amplification indicates methylation.

Methylation status of p16 and RARβ genes was analyzed by methylation-specific PCR as described previously (26, 27). Briefly, 1 μg of DNA in a volume of 100 μl of each sample was denatured with freshly prepared NaOH at a final concentration of 0.2 M and modified according to the manufacturer instructions of the DNA Modification kit (Intergen, Purchase, NY). The PCR mixture contained 2 μl of 10× PCR buffer, 0.5 units of Taq Gold polymerase, deoxyribonucleotide triphosphates (each 1.25 mM), 300 ng of primers/reaction, and bisulfite-modified DNA or unmodified DNA in a final volume of 20 μl. Products were visualized in a 2% agarose gel under UV light.

In Vivo Experiments. To evaluate whether hydralazine and procainamide demethylate re-express the ER gene in vivo, 16 nu/nu 8-week-old female mice (Harlan Teklad, Madison, WI) were injected with 6 x 106 MDA-231 cells in the flank. After 4–8 weeks, once the tumors reached a size between 0.5 and 1 cm in diameter, 4 mice/group were injected by i.p. route
with 200 μl of normal saline, 300 μg of 5-Aza-CdR, 5 mg/kg hydralazine, or 20 mg/kg procainamide daily for 7 days. At day 8, mice were sacrificed under chloroform anesthesia and tumors removed. Tumors were immediately processed for protein, RNA and DNA extraction, and then for evaluation of the ER and product expression, as well as the assessment of the methylation status as described above.

**Heritability of DNA Demethylation.** To evaluate the duration of the gene-reactivating effect of hydralazine as compared with 5-Aza-CdR, T24 cells were treated with either of these drugs for 5 days as described above. At day 6, the media was changed, and cells were successively passaged in drug-free medium. Every subsequent day cells were analyzed for the mRNA of the p16 gene by RT-PCR.

**Functional Assays of the Re-expressed Products of ER and p16 Genes.** To evaluate whether the re-expressed genes were functional, the MDA-231 and T24 cell lines were treated in culture as above described with hydralazine or 5-Aza-CdR. For the breast cancer cell line, the media containing the drugs was replaced by fresh media without phenol red (phenol red-free RPMI) containing 10% dextran charcoal-stripped FCS. After 24 h in these latter conditions, estradiol at a concentration of 10 nM was added for additional 24 h, and then, cells were analyzed for the expression of the estrogen-responsive gene PS2 as described previously (22). For the bladder cancer cell line, cells were kept for 24 h in drug-free medium after treatment (confluency ~80%) and then harvested and fixed in 75% ethanol, stained with propidium iodide, and analyzed by flow cytometry to determine the cell cycle profile.

**Clinical Evaluation of the Demethylating and Gene Re-expression Activities of Hydralazine.** To evaluate whether hydralazine was able to demethylate and reactivate expression of silenced TSGs in a clinical setting, two patients (one with cervical cancer and another with head and neck cancer) who had progressed to all available treatment were treated with hydralazine after a signed informed consent. Hydralazine was administered for 10 days by oral route (50 mg 3 times a day). Two days before commencing treatment, a punch biopsy from the tumors was taken for DNA and RNA extraction to document methylation and lack of expression of the RARβ and p16 genes, respectively. At day 11, a biopsy from the same site was taken for reanalysis of methylation and gene expression. To confirm the changes in methylation pre- and posttreatment, the PCR products of the RARβ gene were sequenced in an ABI PRISM 310 (PE Biosystems) sequencer.

**Results**

Previous studies have revealed that the ER, p16, and RARβ genes are methylated in MDA-231, T24, and MCF-7 cell lines, respectively, and also that 5-Aza-CdR is able to demethylate these genes. To determine whether other inhibitors of DNA-methyltransferase can demethylate TSGs, these cell lines were treated with hydralazine and procainamide at indicated doses for 5 days. As a positive control, 5-Aza-CdR was used. As expected, the PCR from untreated MDA-231 cells was negative in the sample digested with MspI but positive in the sample digested with HpaII, indicating cells were methylated. On the contrary, the PCR failed to amplify after digestion with HpaII from cells treated with the three demethylating drugs, which indicates demethylation (Fig. 1A). To investigate whether the demethylating ability of hydralazine and procainamide was not cell line nor gene specific, T24 and MCF-7 cells were treated with these demethylating drugs. The methylation-specific PCR using bisulfite-modified DNA for the p16 and RARβ genes demonstrated methylation in untreated but demethylation in treated cells (Fig. 1, B and C). In addition, the time course demethylation analysis done in MCF-7 and T24 cells for the RARβ and p16 genes with hydralazine showed that as expected, the unmethylated band appeared after 48 h (Fig. 1, D and E). Interestingly, the demethylation was faster for the p16 gene. Thus far, these results demonstrated that hydralazine and procainamide were able to demethylate these TSGs. To investigate whether demethylation correlated with gene reactivation, the expression of these genes was investigated at the level of RNA and protein. Untreated cell lines were negative, but after 5 days of treatment with the drugs, the cells expressed the transcript and protein (Fig. 2, A–C). To further explore the ability of hydralazine and procainamide to induce demethylation in vivo, nu/nu mice received injections of MDA-231 cells to generate tumors and, subsequently, were treated for 7 days by i.p. injections of hydralazine or procainamide. The analysis of the expression of the ER gene at protein and RNA levels as well as the methylation status of the gene demonstrated that both drugs were able to demethylate and re-express the ER gene (Fig. 3). Thus far, these results demonstrated that like 5-Aza-CdR, both
drugs demethylate and re-express TSGs in vitro and in vivo. To compare the effect on the heritability of these changes, hydralazine and 5-Aza-CdR were tested in the T24 cell line. The results showed that under identical conditions of treatment at the doses tested, hydralazine kept longer the reactivating effect on the p16 gene (Fig. 4).

It has been demonstrated that genes reactivated after 5-Aza-CdR treatment are functional. Because of the importance of this phenomenon upon the potential therapeutic use of demethylating agents, we tested whether the reactivated genes by hydralazine were functional. As it could be predicted, in the MDA-231 cell line, the re-expressed ER product induced the transcription of the PS2 gene upon stimulation for 24 h with estradiol (Fig. 5A). Likewise, in the bladder cancer cell line, the reactivation of p16 gene was able to induce G1 arrest, indicating that the gene product was functional (Fig. 5B).

It has been reported that 5-azacytidine selectively increases δ-globin synthesis in a patient with β Thalassemia (28), therefore, we treated two cancer patients with hydralazine for 10 days to confirm if our experimental results were clinically achievable. Fig. 6 shows that hydralazine demethylated and re-expressed the p16 gene in the head and neck cancer patient (Fig. 6A) and the RARβ gene in the cervical cancer patient (Fig. 6B). This demethylation was confirmed by sequencing the RARβ gene products (methylated pretreatment and demethylated posttreatment). The product amplified encompasses position 950-1095 of the promoter-exon region of this gene (GenBank accession no. X56849 as shown in Ref. 27), which has 10 methylated cytosines. All these were demethylated after treatment with hydralazine. Fig. 6, C and D, shows position 1000–1095 in which the five methylated cytosines marked with the arrow became demethylated.

**Discussion**

The demonstration that the re-expression of TSGs silenced by methylation can have therapeutic value in malignant diseases has been hampered by the unavailability of suitable drugs for clinical testing. In this study, we show that two noncytotoxic agents having current clinical use for nonmalignant conditions are able not only to demethylate but to re-express in vitro and in vivo the functional products of TSGs in cancer cell lines. In addition, we demonstrate that this effect is clinically achievable by hydralazine.

In this work, we used the ER-negative MDA-231 cell line that lacks ER gene expression because of hypermethylation of its promoter (29, 30). It has been shown that the demethylating agent 5-aza-CdR is able to re-express a functional ER protein (8). Our results demonstrate that untreated MDA-231 cells have methylated and silenced the expression of the ER gene, however, after 5 days of exposure to procainamide or hydralazine, this epigenetic change was reverted. Methylation-specific PCR after bisulfite modification is currently considered as the most simple and effective method to determine the methylation status of gene promoters. The ER gene methylation has been analyzed with this technique (31), however, we were unsuccessful in...
reproducing these results, therefore, we decided to analyze the ER gene methylation by PCR amplification after digestion with methylation-sensitive enzymes. This technique has some technical limitations that mostly depend on an efficient enzymatic digestion, however, our data clearly demonstrate that this was not the case because no bands could be amplified after digestion with HpaII from treated cells, but they were present in untreated cells. In addition, these results are supported by the identical results obtained with 5-aza-CdR, used as a positive control.

To rule out that the effect of procainamide and hydralazine could be a cell line or a gene-specific effect, we studied the p16 gene in the bladder cancer cell line T24. This cell cycle regulatory gene has been widely studied in regard to its methylation status in cancer. Previous results in this cell line have shown that 5-aza-CdR reverts its methylation and induces its re-expression in vitro and in vivo (16). Our results indicate that procainamide and hydralazine are able to re-express its gene product and also provide evidence that the reactivation of the p16 gene is because of demethylation as demonstrated by methylation-specific PCR. In addition, our results demonstrate that the gene products expressed were functional as shown by the induction of the estrogen-responsive PS2 gene after estradiol treatment and to the increase in the population of T24 cells in the G1 phase of the cell cycle. These findings additionally support the potential use of hydralazine for reactivate TSGs in cancer.

To provide further evidence, we also studied in the MCF-7 cell line the effects of these two drugs on the RARβ gene. This gene has been found silenced by methylation in several cell lines, as well as in primary tumors such as lung carcinoma where the methylation of this gene has prognostic implications (32). It is known that the expression of the RARβ gene is up-regulated by retinoic acid (33) and that in vitro, demethylation of the RARβ gene is not sufficient for its expression unless cells are treated with retinoic acid (34). Our results show that after 5-day exposure to the tested drugs, all-trans-retinoic acid induced the expression of the receptor in the treated cells and that this effect correlated with promoter demethylation.

An important finding of this study was that the systemic administration of hydralazine and procainamide to animals was able to demethylate and re-express the ER gene without over toxicity. Interestingly, the concentration of these two drugs at which the effect was observed in vitro and in vivo is in the therapeutic range used in clinic as antiarrhythmic and anti-hypertensive, respectively (35, 36).

It is known that 5-aza-CdR inhibits cytosine methylation by covalently binding DNA methyltransferase and that its demethylating effect is maintained for at least nine doubling times, being after this time, a gradual recuperation of methylation to the original level (37). The mechanism by which hydralazine and procainamide inhibit DNA methylation is still unknown, however, both drugs bind deoxyguanine-deoxycytosine rich DNA sequences (38), and this binding may interfere with translocation of the enzyme DNA-methyltransferase along the DNA strand. Accordingly, the demethylating effect of both drugs is comparable with 5-aza-CdR when evaluated by the methylmethionine assay and by direct quantification of deoxymethylcytosine (39).

Because procainamide and hydralazine most likely have a distinct mechanism of demethylation that 5-aza-CdR, we analyzed the effect on inheritability of the re-expression upon the p16 gene in the T24 cell line. Surprisingly, hydralazine was more effective than 5-aza-CdR for keeping re-expressed this gene longer, which is in line with a delayed demethylating effect of this drug when tested in vitro (39). The evidence would suggest that to keep genes demethylated, cells would need to be in continuous exposure to the demethylating drugs, which from a practical point of view would favor the use of hydralazine instead of 5-aza-CdR because this anti-hypertensive drug is given in daily basis for long periods of time. An important issue to be addressed is whether the sole re-expression of the sup-
pressor genes can inhibit tumor growth in the clinic. Data from 5-aza-CdR show that its antitumor activity \textit{in vitro} and \textit{in vivo} may depend not only from its demethylating activity but also from its intrinsic cytotoxic actions (40). However, both procainamide and hydralazine have also been tested as antitumor agents. Hydralazine has intrinsic cytotoxicity because of the induction of intracellular acidification and tumor-selective hypoxia (41, 42). Procainamide and its analog 3-chloroprocainamide have shown to increase the efficacy of cisplatin in some tumor models (43, 44); moreover, because of its DNA-methyltransferase inhibitory activity, procainamide changes the humoral immunological environment into a cellular one, enabling an increased antitumor efficacy when combined with IFN in renal carcinoma (45).

Recent studies have shown that in cervical carcinoma, there is hypermethylation of a vast array of genes thought to be important for the development and progression of this tumor (46, 47). Among these, the RARβ gene has been found methylated in 50 and 26% of cervical cancer cell lines and tumors, respectively. Likewise, for head and neck cancer, \textit{p16} is among other genes, silenced by methylation in 47% of primary tumors (48). Here, it is shown that hydralazine at a dose of 150 mg daily, which is even below the 200 mg commonly used as antihypertensive, is able to revert the methylation status and to re-express the RARβ and \textit{p16} genes in patients with these tumor types.

The implication of this study seems important because these drugs could have a role in cancer therapy. It is remarkable their known negligible toxicity as well as their availability for oral use and low cost. Currently, there are at least two clinical Phase I-II trials ongoing, testing the demethylating ability of 5-aza-CdR in patients with advanced malignancies, as well as...
preclinical testing of antisense molecules targeting the DNA-methyltransferase gene. The characteristics above describing hydralazine and procainamide make them suitable for clinical trials and seem a simple alternative to 5-aza-CdR and antisense oligonucleotides.

Despite that both hydralazine and procainamide were able to demethylate and reactivate the TSGs tested, we chose to perform additional experiments only in the former as our results in mice suggested that procainamide achieved only partial demethylation, however, the potency of their demethylating activity should be formally compared. In addition, because the clinical use of an antiarrhythmic agent could be more problematic than of an antihypertensive in noncardiovascular conditions, hydralazine seems to be more appropriate for additional testing.

In conclusion, we provide evidence that these two cardiovascular drugs reactive TSGs silenced by methylation and may become a potential useful therapy targeting one of the most common epigenetic abnormality found in cancer cells. Just to mention some of their potential applications, in the case of breast cancer, for instance, the re-expression of the ER protein could make patients with ER-negative tumors responsive to endocrine management, and in the same way, the efficacy of retinoid acid treatment of some tumor types could be increased by re-expressing the RARβ through its demethylation.

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