DNA Demethylating Activity of Hydralazine in Cancer Cell Lines

Edel de la Cruz-Hernandez, Edgardo Perez-Cardenas, Liliana Taja-Chayeb, Alfredo Chavez-Blanco, César Trejo-Becerril, J. Trujillo, José Luz Medina-Franco, Alfonso Dueñas-González*

1Unidad de Investigación en Cáncer, Instituto de Investigaciones Biomédicas, UNAM, Mexico City, and Instituto Nacional de Cancerología, Mexico City, ISSEMYM Cancer Center, Toluca, Mexico.
2Torrey Pines Institute for Molecular Studies, 11350 SW Village Parkway, Port St. Lucie, FL 34987, USA.

*Correspondence to: Alfonso Dueñas-González, alfonso_duenasg@yahoo.com

Abstract

Hydralazine is a non-nucleoside analog that inhibits DNA methylation and reactivates the expression of tumor suppressor genes. The objective of this study was to determine whether the hydralazine's demethylating effects depend on either inhibition of DNA methyltransferase or by downregulating the expression of DNA methyltransferases. The in vitro and in vivo effects of hydralazine upon DNA methyltransferase activity were investigated as well as its effect upon ERK-regulated DNA methyltransferase expression in Jurkat and cancer cell lines. Hydralazine inhibits the activity of methyltransferase in vitro and in vivo. Hydralazine decreased the gene expression of DNMT1 in Jurkat cells but no in other cancer cell lines. The results of this study demonstrate that hydralazine inhibits DNA methyltransferase activity and has little or no activity in the downregulation of DNA methyltransferases.

Keywords: DNA demethylation; hydralazine; cancer cell lines.

1. Introduction

The reactivation of tumor suppressor genes silenced by epigenetic mechanisms such as DNA promoter methylation and histone deacetylation by using inhibitors of the enzymes DNA methyltransferases (DNMTs) and class I-II histone deacetylases (HDACs) is a promising therapeutic approach for cancer therapy [1]. The clinical development of DNA methylation inhibitors for the treatment of solid tumors has been hampered by the scarcity of clinically suitable agents. So far, the nucleoside analogs 5-azacitidine and decitabine, are approved for the treatment of myelodysplastic syndrome, however, their clinical efficacy for this condition do not entirely depend on their DNA methylation inhibitory activity [2,3]. On the other hand, a number of studies indicate that at least for decitabine, DNA damage induction is key for its antitumor effects as this agent activates classical DNA damage detection and signaling molecules including ATM, CHK1 and H2AX [4-6]. Moreover, it has recently shown that the p53-inducible ribonucleotide reductase is a DNA hypomethylation-independent decitabine gene target [7]. At last, but not the least, these nucleosides are carcinogenic [8-10] and chemically unstable [11,12]. In particular their myelotoxicity even used at low-doses cautions against their use for solid tumor cancer patients in combination with cytotoxics or biological agents as this side effect is a common limiting toxicity [13-16].

These facts led several groups to undertake the preclinical and clinical development of non-nucleosides analog compounds that target DNA methyltransferases without being incorporated into RNA or DNA as more suitable DNA demethylating agents for the treatment of solid tumors. These include the known drugs procarbazine, procaine and hydralazine, which are commonly used as antiarrhythmic, local anesthetic and antihypertensive drugs, respectively [17]. Additional DNMT inhibitors are the polyphenol compound from green tea extract, (-)-epigallocatechin-3-gallate (EGCG) [18] and RG108, which was discovered by a docking-based virtual screening methodology based on a homology model of human DNMT1 [19].

Hydralazine has been shown to demethylate and reactivate the expression of several tumor suppressor genes [20-23] and this activity is synergized when used in combination with valproic acid, a HDAC inhibitor [24-26]. Clinical studies with this combination of epigenetic agents have demonstrated that are well-tolerated when used with chemotherapy and chemoradiation against a number of solid tumors and that lead to re-expression...
of hundreds of genes in the primary tumors of cancer patients [27-29]. To further investigate the demethylating effect of hydralazine, here we report its effects in a number of cancer cell lines.

2. Methods

2.1. Cell lines and treatment

Hydralazine (Sigma-Aldrich, Co.) was dissolved in medium DMEM-F12 (Gibco) as a stock solution and diluted to the required concentration with medium DMEM-F12. The human cancer cells lines HeLa, MCF-7, HEP-2, D54, SW480 and Jurkat, were resuspended at a density of 3x10^5 cells/mL in DMEM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine at 2 mM, 100 U/ml Penicillin G, and 100 mg/ml Streptomycin (Gibco). All cultures were maintained under a fully humidified atmosphere of 95% air 5% CO2 at 37°C. All cell lines were cultured in FBS-free DMEM-F12 for 24 h, and then FBS was added to medium and just before adding hydralazine and U0126. Hydralazine was used at 10µM for 5 days (fresh hydralazine every day); U0126 was used at 10µM for 2 days.

2.2. Hydralazine mediated inhibition of methylation by SsSI

For the in vitro assay an 1112 bp fragment of the Herpes Simplex Virus type-1 (HSV-1) TK gene rich in CG dinucleotide was used as methylation target. 500ng of the DNA fragment were incubated with the following concentrations of hydralazine: 0 µM, 10 µM, 20 µM and 30 µM, along with 4 units of the CpG methyltransferase SsSI (New England Biolabs Inc.). 1X NEBuffer 2 and 160 µM S-adenosyl-L-methionine. Methylation was achieved by incubating reaction at 37°C for 1 hour. After this incubation the samples were purified with a PCR purification system (Marligen Bioscience, Ijamsville, MD). Each sample was split in two eppendorf tubes, and incubated with the restriction endonucleases HpaII and MspI respectively (New England Biolabs Inc.), according to the manufacturer instructions. The reactions were incubated at 37°C for 1 hour and then agarose-gel electrophoresed. Assays were performed in triplicate.

2.3. DNA methyltransferase assay

The in vivo DNA methyltransferase assay was performed as follows: MCF-7 cells were treated with increasing concentrations of hydralazine (2.5-20 µM) for 5 days and then DNA methyltransferase activity measured employing 500 ng of a 1112-bp fragment of the HSV-1 TK gene, which has a high GC content, in presence of 50 mg cellular protein obtained with lysis buffer (50 mM Tris-HCl pH 8.0, EDTA 1 mM, 0.001% Na azide, 10 % glycerol, 1 mM DTT, 0.06 mg/mL) (Sigma). Final reaction volume (50 µL) was completed with 3 µL of S-adenosyl-L-[methyl-3H]methionine (1 mCi/mL) (Amersham, Buckinghamshire, UK). Reactions were conducted at 37°C for 2 h and stopped with 350 µL of stop solution (1% SDS, 2 mM EDTA, 0.04 g/mL 4-aminosalicylate, 125 mM NaCl, 0.25 mg/mL salmon testis DNA, 1 mg/mL proteinase K). DNA was purified using phenol-chloroform and precipitated with cold ethanol. DNA was resuspended in 30 µL of 0.3 M NaOH and spotted on Whatman DE81 filter paper disc (Whatman, Maidstone, UK). The disc was washed three times in 5% trichloroacetic acid (J.T. Baker, Xalostoc, Edo. de México, Mexico) with Bovine Serum Albumin (Research Organics, Cleveland, OH, USA) and stored in 70% ethanol, and dried. Radioactivity was measured in a Beckman liquid scintillation counter (LS 6000TA). A blank control reaction was carried out simultaneously using only lysis buffer. The results, expressed in dpm, were adjusted by subtracting the background level and turned to percentage. Each assay was performed in triplicate.

2.4. Western blot analysis

Total cellular proteins were extracted from cells harvested from a 75 cm² plate flask. Cells were pelleted and disrupted with 300 µL of a lysis buffer (100mM Tris, pH 8, 100 mM NaCl, 0.5% Nonidet P-40, 1% aprotinin, 1 mM PMSF). Proteins were boiled in sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 2% β-mercaptoethanol and 0.01% bromphenol blue) for 5 minutes and then loaded onto 10-18% SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane Hybond-C extra (Amersham) in a wet chamber during 1 h at 100V. Membranes were then blocked with TBS 1x containing 1% skimmed milk and 0.1% Tween-20, washed and incubated with the corresponding antibody (Actin, ERK, pERK, DNMT1 and DNMT3a) (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibody was used for protein-primary
antibody complex detection. The levels of the corresponding proteins were visualized using the ECL system (Amersham).

3. Results

In this work, we have tested the inhibitory activity of hydralazine in an in vitro system. The results showed that in the restriction enzyme assay, hydralazine at concentrations between 10µM to 30µM inhibits the activity of DNA methyltransferase. This is shown in Figure 1, where the methylation sensitive enzyme HpaII was unable to cut the HSV-1 TK gene rich in CG dinucleotide in the absence of hydralazine, but partial digestion occurred when the reaction contained hydralazine demonstrating that the tested drug partly inhibited the in vitro methylation reaction by SssI.

![Figure 1](http://astonjournals.com/lsmr)

See figure legend changes: White rectangles indicate that methylation-sensitive HpaII enzyme partially digests the DNA when this was incubated in the presence of HpaII (H) at different hydralazine (H) concentrations, but not in the absence of H. MspI (M) cuts regardless of H as is a methylation-insensitive enzyme. A dose-effect relationship is not seen in this range of H concentration.

Further, we demonstrated in an in vivo inhibition of DNA methyltransferase-catalyzed transfer of methyl groups from S-adenosylmethionine to DNA as shown in Figure 2. The breast cancer MCF-7 cells treated for five days with hydralazine at concentrations between 2.5 µM to 20 µM showed a reduction in the activity of methyltransferase from 23%, 29% and 35% with hydralazine at 2.5 µM, 10 µM and 20 µM respectively.
It has been proposed that hydralazine inhibits DNMT1 and DNMT3a expression through the ERK pathway. We treated HeLa, MCF-7, Hep-2, D54, SW480 and Jurkat cell lines with hydralazine at 10µM for five days. The results showed that in Jurkat cells, hydralazine decreased the phosphorylation of ERK without modifying its total level, and reduced DNMT1 expression as reported. This effect seems to be cell line dependent as this change was not observed in HeLa, Hep-2, D54 or SW480 cells. Only MCF-7 cells showed a slight decrease in ERK activation but not changes in DNMT1 (Figure 3).

Figure 2. In vivo methyltransferase assay.

In vivo inhibition of DNA methyltransferase-catalyzed transfer of methyl groups from S-adenosylmethionine to DNA. MCF-7 cells were treated for five days with hydralazine at concentrations between 5µM to 20µM. A 23% inhibition was shown at 2.5µM further increasing to 29% and 35% at 10µM and 20 µM of hydralazine treatment.

See changes at Figure legend: 5µM should be 2.5µM

4. Discussion

We and other groups have demonstrated that hydralazine induces gene promoter and genome-wide demethylation in vitro and in clinical studies [20-24, 27-30], however, it is still controversial whether this effect depend on a direct enzymatic inhibition and/or by decrease in the levels of DNMTs. In a previous study, a binding model of hydralazine with DNMT1 was proposed, nevertheless, it was neither compared with the binding mode of 5-azacytidine and its analogs and other non-nucleoside inhibitors nor did it provide a mechanistic interpretation of the inhibitory ability of hydralazine [31]. Recently, we reported the results of a binding model for hydralazine, with a validated homology model of human DNMT1. The docking protocol was validated by predicting the binding mode of compounds 2’-deoxycytidine, 5-azacytidine and 5-aza-2’-deoxyctydine. The inhibitory DNMT activity of hydralazine may be rationalized at the molecular level by similar interactions within the binding pocket (e.g., similar pharmacophore) as substrate-like deoxycytidine analogs. These interactions involve a complex network of hydrogen bonds with arginine and glutamic acid residues that also play a major role in the mechanism of DNA methylation. Despite the different scaffolds of other non-nucleoside DNMT inhibitors, like procaine and procainamide, the current modeling work reveals that these drugs exhibit similar interactions within the DNMT1 binding site [32].
In our recent work on hydralazine docking, the predicted free-energy of binding hydralazine appeared less favorable than that of nucleoside analogs and other non-nucleoside demethylating drugs which is consistent with the fact that hydralazine is a weaker DNMT1 inhibitor [32]. Deng et al. have reported in Jurkat cells that hydralazine decreases the expression of human DNMT1 and 3a in a similar manner that PD98059, a mitogen-activated protein kinase (MEK) inhibitor, does suggesting that the down-regulation of DNMTs results as consequence of hydralazine-induced MEK inhibition [33].

Our results confirm that hydralazine down-regulates protein expression of DNMT1 by inhibiting MEK activation only in Jurkat cells but not in the malignant cell lines tested. This may reflect that in malignant cell lines the regulation of DNMT1 expression is driven not primarily from alterations in the ERK pathway but from oncogenic alterations through different pathways. For instances, it has been shown that viral oncoproteins such as E1A and E7 from adenovirus and human papilloma virus respectively up-regulates the activity of DNMT [34] and that oncogenic Ras-c-Jun oncogenic signaling pathway as well as c-fos- and SV40 large T antigen regulate the expression of DNMT1 [35,36].

The treatment of cancer with epigenetic agents, particularly with DNA methylation inhibitors is highly promising, however, the nucleoside analogs 5-azacytidine and decitabine which are proven effective against myelodysplastic syndromes, are unlikely to be widely applied for solids tumors in combination with cytotoxic chemotherapy due to their myelotoxicity. The results of this study demonstrate that hydralazine inhibits DNA methyltransferase activity. Because the non-nucleoside analogs 5-azacitidine and decitabine induce secondary demethylation after being incorporated to the DNA and work as antimetabolites, it is imperative to continue the study on non-nucleoside inhibitors of DNA methyltransferases to confirm that inhibition of this process itself has antitumor activities. A recent study identified seven novel small molecule DNMT1 inhibitors with detectable inhibition of DNMT1 by multistep virtual screening with a validated homology model of DNMT1 and experimental evaluation. The seven compounds had more favorable docking scores with DNMT1 than the reference compounds RG108 and hydralazine; however, the observed potency was comparably low for most test compounds [37].
5. Conclusion

In conclusion, the results of this study confirm that hydralazine is a weak demethylating agent and stresses the need of further testing small-molecule compounds that inhibit DNA methylation in order to confirm that methyltransferases are suitable targets for cancer treatment.

Competing Interests

The authors declare that they have no competing interests.

Authors’ Contributions

De la CH, EP-C, LT-C, A C-B, C T-B, JT, and JL performed the experiments; JL M-F critically read the manuscript and AD-G conceived and wrote the manuscript. All authors read and critically contributed to the work.

Acknowledgement

This work was supported by CONACyT grants SALUD-2002-C01-6579 and AVANCE C01-294, Fundación Miguel Alemán México and by Psicofarma, S.A. de C.V., Mexico. Dr. Dueñas-González was partly supported by the Catedra Instituto Científico Pfizer-PUIS Universidad Nacional Autónoma de México.

References

5. Palii SS, Van Emburgh BO, Sankpal UT, et al., 2008. DNA methylation inhibitor 5-Aza-2'-deoxycytidine induces reversible genome-wide DNA damage that is distinctly influenced by DNA methyltransferases 1 and 3B. Molecular and Cellular Biology, 28: 752-771.

http://astonjournals.com/lsmr


