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The Demethylating Agent 5-Aza Reduces the Growth, Invasiveness, and Clonogenicity of Uveal and Cutaneous Melanoma

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PURPOSE. Uveal melanoma is the most common primary intraocular malignancy in adults. Although local disease can be controlled with radiation therapy or enucleation, many cases are complicated by metastasis, which account for the significant mortality from this disease. To date, no chemotherapeutic regimen effectively treats local or metastatic disease. Epigenetic silencing of tumor suppressor genes has been shown to be an important factor in the growth and metastasis of many cancers. One form of epigenetic alteration is DNA methylation, which often occurs at promoter elements resulting in the silencing of target gene transcription.

METHODS. We used 5-aza-2′-deoxycytidine (5-Aza), a well characterized demethylating agent that is US Food and Drug Administration approved to decrease DNA methylation in multiple uveal and cutaneous melanoma cell lines.

RESULTS. Demethylation of melanoma cell lines using 5-Aza causes significant decreases in growth, invasion, and clonogenicity. Treatment of melanoma cells with combined 5-Aza therapy and irradiation showed an even more pronounced effect on cell viability. In addition, treatment with 5-Aza decreased the number of metastases from the eye to the lung in a murine cutaneous melanoma xenograft model.

CONCLUSIONS. We demonstrate in vitro and in vivo that demethylating agents such as 5-Aza may be promising chemotherapeutic agents for treating melanoma and decreasing progression to metastatic disease. These results provide proof of concept for an exciting potential therapy to reduce mortality from this disease. Future work will focus on identifying pathways that mediate these changes.

Keywords: demethylation, epigenetics, melanoma, metastasis, uveal melanoma

Uveal melanoma is the most common primary intraocular malignancy in adults, with an age-adjusted incidence of 5.1 cases per million.1 Advances in local therapy have allowed preservation of the patient’s eye and vision in many cases; however, the 5-year survival rate of 81.6% has remained unchanged from 1976 to 2008, underscoring the need to develop effective therapies for this deadly disease.1

Epigenetic mechanisms controlling gene expression have long been known to have a role in cancer development.2–4 Changes in DNA methylation serve as early markers of tumor formation, and DNA methylation at CpG islands in promoters has been shown to decrease expression of tumor suppressor genes in many malignancies, including uveal melanoma.5,6 In addition, demethylating agents have been used to decrease tumor formation in animal models of cancer and are US Food and Drug Administration (FDA) approved as treatment of myelodysplastic syndrome.7 However, the functional effects of altered DNA methylation in uveal melanoma are unclear.

To determine the role of DNA methylation in melanoma tumorigenesis and test the possibility that demethylating agents may be potential chemotherapeutic agents for uveal melanoma patients, we used 5-aza-2′-deoxycytidine (5-Aza) to inhibit DNA methylation in uveal and cutaneous melanoma cell lines. We show that treatment with 5-Aza decreased cell growth, clonogenicity, and invasive behavior in uveal and cutaneous melanoma cells. In addition, we used a murine melanoma intraocular xenograft model, which metastasizes hematogenously, to demonstrate that treatment with 5-Aza decreases metastasis in vivo.

MATERIALS AND METHODS

Cell Culture

Human melanoma cell lines (OCM1, OCM3, OMM1, 92.1, Mel 285, and Mel 290), kindly provided by Jerry Niederkorn (University of Texas Southwestern Medical Center, Dallas, TX, USA) or purchased from American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI medium with 10% fetal bovine serum (FBS), HEPEs buffer, sodium pyruvate,
penicillin/streptomycin, and l-glutamine. Descriptions of the isolation and early characterization of the lines can be found elsewhere.6–12 More detailed recent genetic profiles of these lines have been reported by Griewank et al.13 and Folberg et al.14 The cell lines’ identities were authenticated in our laboratory by short tandem repeat (STR) testing, as previously described, with repeat confirmation of identity and mycoplasma testing performed at least once per year.15 Queen’s murine melanoma cells were obtained from American Type Culture Collection and cultured in MEM with 10% FBS, HEPES buffer, sodium pyruvate, penicillin/streptomycin, MEM vitamin solution, and nonessential amino acids mixture.

Cell Growth Assay
For each cell line tested, 2500 cells were plated in 96-well plates in medium with the addition of dimethyl sulfoxide (DMSO), and 0.5 μM, 1 μM 5-Aza, or 2 μM 5-Aza. MTS colorimetric assays were performed at days 0, 3, 5, and 7 to establish growth curves. Data are means ± standard error of the mean (SEM) of three separate experiments.

Clonogenicity Assay
Soft agar colony assays were performed by plating 5000 cells per well in medium with 0.5% soft agar (Invitrogen, Grand Island, NY, USA) over a base layer of medium with 1% agar after 48 hours of treatment with DMSO control or the indicated dose of 5-Aza. Plates were incubated at 37°C for 10 days and then stained overnight with 1 mg/ml p-Nitro Blue Tetrazolium Chloride (USB Corp., Cleveland, OH, USA) solution. Colonies greater than 50 micrometers in diameter were scanned and counted using MCID Elite software ( Imaging Research, Inc., Cambridge, England, UK). The means of three independent experiments were compared.

Invasion Assay
Transwell invasion assays were performed using 6.5-mm cell culture inserts with a pore size of 8 μm (Becton-Dickinson, Franklin Lakes, NJ, USA) in 24-well culture plates that were pretreated with 1% Matrigel (Becton-Dickinson) in medium. After 48 hours of pretreatment with 5-Aza or vehicle, 100,000 cells were plated in serum-free medium onto the inserts. Invasion was assayed at 5 PM. Migrated cells were fixed in ethanol and stained with hematoxylin. Eight high-power fields (HPF) were imaged, and cells were counted within the 8 HPF to establish a mean ± SEM value for each of three separate experiments.

Radiation Experiments
A total of 500,000 cells were plated in T-25 flasks and treated with 5-Aza or control for 48 hours prior to exposure to γ irradiation using a Gammacell 3000 Elan unit (MDS Nordion, Ottowa, ON, Canada). After this initial treatment, no additional drug or vehicle control was added to the medium. Five days after irradiation, viable cells were counted using Guava ViaCount, according to the manufacturer’s protocol (EMD Millipore, Billerica, MA, USA).

Genomic DNA Methylation Assay
Cells were plated in T-25 flasks in the medium described above with the addition of DMSO and 0.5 μM or 1 μM 5-Aza for 48 hours. Genomic DNA (500 ng) was extracted from the cells and bisulfite converted using the EZ DNA methylation-gold kit (Zymo, Irvine, CA, USA) to convert unmethylated cytosines in the genome to uracil. DNA was then used as the PCR template to amplify a 150-base-pair sequence of the repetitive LINE-1 (L-1) retrotransposon repeats that are usually densely methylated in the genome. The PCR products were quantitatively sequenced using the PyroMark Q24 pyrosequencing system (Qiagen, Valencia, CA, USA) to determine the percentages of 5-methylcytosine compared to unmethylated cytosines in each copy of the L-1 ampiclon.

In Vivo Assay For Metastases
Queen’s melanoma cells, which are derived from metastases of B16F10 murine skin melanoma cells, have been used in vivo as a model of ocular melanoma metastasis in a number of studies.16–20 They were treated with 2.5 μM 5-Aza or vehicle for 72 hours, and then 2.5 × 10⁶ cells in 2.5 μl were injected into the subretinal space of 15 C57BL6 mice as previously described.18 After 26 days, the surviving mice were sacrificed and necropsy performed. The lungs and livers of the animals were preserved and sectioned at 5 μm. Slides were stained with hematoxylin and cosin, and the metastases were counted by a masked board-certified pathologist (CGE).

Statistical Analysis
All experiments were performed in triplicate, and values are given as means ± SEM. Statistical analyses were performed using Prism4 software (GraphPad, LaJolla, CA, USA). P values, unless otherwise stated, were calculated by the two-tailed Student’s t-test.

RESULTS
5-Aza Causes a Dose-Dependent Decrease in Cell Growth in Melanoma Cell Lines
To evaluate the effects of treatment with 5-Aza on melanoma growth, cells were treated with 5-Aza, and rates of cell growth were determined using MTS assays. We used three primary uveal melanoma cell lines, 92.1, Mel 285, and MEL 290, as well as the OMM1 cell line, which was derived from a uveal melanoma metastasis, to test the effect of 5-Aza on cell growth.6,21 In addition, we used the OCM3 and OCM1 cell lines, which were initially described as uveal melanoma but were recently found to lack GNAQ or GNA11 mutations and instead contain BRAFV600E mutations which are generally found in cutaneous rather than uveal melanoma.22–25

At day 7, a statistically significant decrease in cell growth was observed in all six cell lines treated with 1 μM or 2 μM 5-Aza compared to vehicle-treated controls (Fig. 1A). Representative growth curves from two cell lines highlight the somewhat delayed effects of the demethylating treatment. For OCM3 cells, the decrease in cell growth in cells treated with 1 μM and 2 μM 5-Aza was not seen until day 7 (Fig. 1B), although a slight but statistically significant change at the higher dose was first noted at day 5 in the 92.1 cell line (Fig. 1C). Treatment of OCM3, 92.1, and OCM1 cells with 0.5 μM 5-Aza did not cause a significant change in cell proliferation compared to controls (Figs. 1B, 1C, and data not shown).

Treatment With 5-Aza Decreases Clonogenicity in Melanoma Cell Lines
To determine the effects of demethylation on clonogenicity of melanoma cells, we performed soft agar colony-forming assays. Because maximal effects on cell growth were achieved with 1 μM 5-Aza, we used this dose and a lower drug level in experiments examining clonogenicity and migration. Cells...
FIGURE 1. Treatment with 5-Aza decreased proliferation, as measured by MTS assays, in uveal and cutaneous melanoma cells. (A) Cell growth was decreased in OCM3, 92.1 OCM1, OMM1, Mel 285, and Mel 290 cells on day 7 after treatment with 1 μM and 2 μM 5-Aza. (B) Growth curve for OCM3 cells shows that treatment with 0.5 μM 5-Aza did not cause a significant change in cell growth compared to controls; treatment with 1 μM and 2 μM 5-Aza decreased growth at day 7. (C) Growth curve for 92.1 cells demonstrates a significant change in growth by day 7 in cells treated with 1 μM 5-Aza and decreased growth by day 5 in cells treated with 2 μM 5-Aza. *P < 0.05 by Student’s t-test, compared to DMSO controls. **P < 0.01 compared to DMSO controls. ***P < 0.001 compared to DMSO controls.
were treated with 5-Aza for 48 hours prior to plating in low density cultures in soft agar and were stained and counted after 10 days. Treatment of melanoma cell lines with 0.5 or 1 μM 5-Aza caused a profound decrease in colony formation that was dose dependent, with over 90% suppression at the higher level of drug in all three lines examined, compared to that in vehicle-treated controls (Figs. 2A, 2B). In control OCM3 cells, a mean of 533 colonies formed, compared with 59.8 colonies in cells treated with 0.5 μM 5-Aza and 28.9 colonies in cells treated with 1 μM 5-Aza. Vehicle-treated 92.1 cells formed 706.8 colonies, whereas 0.5 μM 5-Aza-treated cells formed 184.4 colonies, and 1 μM 5-Aza-treated 92.1 cells formed 43.4 colonies. OCM1 cells treated with vehicle formed a mean of 666.7 colonies, whereas those treated with 0.5 μM and 1 μM 5-Aza formed 105.3 and 10 colonies, respectively. Control-treated Mel290 cells formed 153.3 colonies, compared with 72.7 colonies in cells treated with 0.5 μM 5-Aza and 71.3 colonies in cells treated with 1 μM 5-Aza. In the OMM1 cells line, treatment with vehicle control resulted in 392.8 colonies forming, compared to 125.4 and 34.7 colonies forming after treatment with 0.5 μM and 1 μM 5-Aza, respectively. The decrease in numbers of colonies formed was statistically significant (P < 0.001) in all cell lines at both doses (Fig. 2B).

Interestingly, the colonies which formed were significantly smaller than those in controls in both 0.5 μM and 1 μM 5-Aza-treated cell lines (Figs. 2A, 2C). The median diameter of colonies formed by vehicle-treated OCM3 cells was 152 μm, which was significantly larger than that of 0.5 μM (99.1 μm) or 1 μM (86.9 μm) 5-Aza-treated cells. In 92.1 cells, control-treated cells formed colonies with a median diameter of 169.2 μm, and 0.5 μM and 1 μM 5-Aza-treated cells formed colonies with median diameters of 89.8 μm and 79.9 μm, respectively. OCM1 cells treated with vehicle formed colonies with median diameters of 181.8 μm, compared to 91.4 μm in 0.5 μM 5-Aza treated cells and 97.1 μm in 1 μM 5-Aza-treated cells. Vehicle-treated Mel290 cells formed colonies with a median diameter of 165.1 μm, compared to 79.2 μm in 0.5 μM 5-Aza treated cells and 72.4 μm in 1 μM 5-Aza treated cells. In OMM1 cells, control-treated cells formed colonies with a median diameter of 137.6 μm, and 0.5 μM and 1 μM 5-Aza-treated cells formed colonies with median diameters of 67.1 μm and 60.9 μm, respectively. The differences in median colony diameters in cells treated with vehicle compared to those treated with 5-Aza were statistically significant in all cell lines (Fig. 2C). Colony size in soft agar has been linked to both proliferation in cells treated with vehicle compared to those treated with 5-Aza and 52% after treatment with 1 μM 5-Aza (Figs. 3C, 3D). In the OMM1 cell line, 0.5 μM 5-Aza caused a 56% reduction, and 1 μM 5-Aza caused a 78% reduction. In addition, differences between the 0.5 μM 5-Aza treatment and the 1 μM 5-Aza treatment was also statistically significant in OCM3 and OMM1 cells (P < 0.001).

Effects of 5-Aza in Response to Radiation

In order to test whether 5-Aza was able to sensitize melanoma to γ irradiation, cells were subjected to 4 Gy of γ irradiation or sham therapy after 48 hours of pretreatment with 0.5 μM or 1 μM 5-Aza or control and then grown for 5 more days with no additional therapy. Control-treated OCM3 cells did not have a consistent response to radiation treatment in terms of the effects on growth and were therefore not further assayed. Treatment of the OCM1 (Fig. 4A) and 92.1 (Fig. 4B) cell lines with both 5-Aza and a single 4-Gy dose of γ radiation decreased the number of viable cells as measured by flow cytometry by 79% to 96% in a dose-dependent manner compared to treatment with vehicle and no radiation. This effect was statistically significant when OCM1 cells were treated with 1 μM of 5-Aza and when 92.1 cells were treated with either 0.5 μM or 1 μM of 5-Aza. This decrease in viable cell number may be more pronounced than noted in Figure 1C, using MTS, because of the different assay used. The decrease in viable cells following combined treatment was 51% to 92% greater than that seen when cells were treated with either 5-Aza or radiation alone, and this additive interaction between the effect of 5-Aza and radiation was statistically significant by ANOVA (P < 0.01).

Treatment With 5-Aza Causes Global DNA Hypomethylation in Melanoma Cell Lines

To demonstrate that treatment of human melanoma lines with 5-Aza results in decreased DNA methylation, global genomic methylation was analyzed using a quantitative PCR-based pyrosequencing assay of repetitive L1 retrotransposon methylation. Treatment of the OCM1, OCM3, and 92.1 cell lines with 0.5 μM or 1 μM 5-Aza for 48 hours led to a decrease of 32% to 38% in genome-wide L1 methylation compared to DMSO-treated controls (Fig. 5). The decreases in cell growth, clonogenicity, and invasion seen in cells treated with 5-Aza along with a reduction in global genomic methylation suggest that, as in other tumor types, this agent acts by modulating epigenetic methylation marks.

Treatment With 5-Aza Decreases Metastases in an Animal Model

To test whether 5-Aza can also modulate melanoma biology in the eye, we used a murine cutaneous melanoma intraocular xenograft model which hematogenously metastasizes and has been used to examine melanoma dissemination from the globe. First, we demonstrated that treatment with 0.5 μM, 1 μM, or 2.5 μM 5-Aza caused genomic hypomethylation, decreased growth, and invasive behavior of Queen’s melanoma cells in vitro compared to vehicle-treated control cells (Supplementary Figs. S1, S2, and data not shown). We further observed that Queen’s melanoma cells treated with 2.5 μM 5-Aza were at the middle of the logarithmic growth phase at 72 hours and that these cells were viable for injection (data not shown).

Twenty-six days after subretinal injections of Queen’s melanoma cells, the animals were sacrificed and necropsied, and liver and lung were examined for metastases. Only lung lesions were identified, with 7 of 13 vehicle-treated animals showing metastases, whereas just 1 of 12 mice injected with 5-Aza-treated cells contained metastatic tumors. Metastases that formed from control and 5-Aza-treated cells were histologically indistinguishable in terms of cellular morphology (Figs. 6A, 6B). However, fewer lung metastases were identified in tumors previously exposed to the demethylating agent than in controls. In the 13 surviving animals that received xenograft transplants with vehicle-treated cells, the mean number of lung...
**Figure 2.** 5-Aza treatment decreased clonogenicity in uveal and cutaneous melanoma cells. (A) Treatment of OCM3, 92.1, OCM1, OMM1, and Mel290 cells with 0.5 μM or 1 μM 5-Aza decreased colony formation compared to DMSO-treated controls. (B) Treatment with 0.5 μM or 1 μM 5-Aza caused a significant dose-dependent decrease in the number of colonies formed in all cell lines compared to those of controls. (C) Size of the colonies formed by uveal melanoma cells was significantly decreased by treatment with 0.5 μM or 1 μM 5-Aza. **P < 0.01 by Student’s t-test, compared to DMSO controls. ***P < 0.001 compared to DMSO controls.
metastases was equal to 26 ± 8, compared to a mean of 2 ± 2 in the 12 surviving animals injected with 5-Aza-treated cells (Fig. 6C). This decrease was statistically significant ($P < 0.05$).

**DISCUSSION**

Melanoma is a deadly malignancy, and when metastatic disease occurs from tumors arising in the uvea, there are no well-established therapies. In many types of cancer, epigenetic mechanisms controlling gene expression have become therapeutic targets. Other investigators have explored the use of epigenetic mechanisms of disease control in uveal melanoma through inhibiting histone deacetylation using FDA-approved agents with promising results, and vorinostat, a histone deacetylation inhibitor, is in phase 2 studies. The potential for targeting DNA methylation in uveal melanoma, however, is not clear. We used 5-Aza, a drug that is FDA approved for...

**Figure 3.** 5-Aza decreased invasion in uveal and cutaneous melanoma cells. Treatment of cells with 0.5 μM or 1 μM 5-Aza decreased trans-well migration of OCM3 (A, B), Mel290 (C, D), and OMM1 (E, F) cells as compared to controls. *** indicates $P < 0.001$ by Student’s t-test of cells per high powered field (HPF) compared to DMSO controls.

**Figure 4.** 5-Aza and γ irradiation interacted to decrease the number of viable melanoma cells. (A) Treatment of OCM1 cells with 1 μM 5-Aza interacted with radiation (Rad) to decrease the number of viable cells. * $P < 0.01$. (B) Treatment of 92.1 cells with either 0.5 μM or 1 μM 5-Aza interacted with radiation (Rad) to decrease the number of viable cells. $P < 0.01$ by Student’s t-test for the comparison of each of the above 5-Aza plus radiation treatments to either DMSO plus radiation treatment or the same drug dose without radiation.
Treatment of myelodysplastic syndrome, to determine whether epigenetic changes to DNA might represent potential therapeutic targets in these often fatal ocular tumors. The doses we used were clinically achievable and inhibit DNA methylation in cell lines for several other tumor types.51–53

Recent advances in our genetic understanding of uveal melanoma have made it possible to correlate molecular changes in cell lines with those observed in primary tumors. The 92.1, Mel285, and Mel 290 cell lines have the GNAQ or GNA11 mutations that are present in over 80% of uveal melanoma specimens.13 The OMM1 cell line, derived from a uveal melanoma metastasis, is also considered typical due to the presence of the GNAQ mutation. The OCM1 and OCM3 cell lines, however, lack these changes and contain instead the BRAFV600E mutation commonly seen in cutaneous and conjunctival melanoma. In addition, STR analysis of OCM3 cultures revealed a profile highly similar to the SK-Mel28 cutaneous melanoma line, with 37 of 38 matching markers.13 Thus, OCM3 appears to represent a cutaneous rather than a uveal tumor. The origin of OCM1 is less clear. Although BRAF mutations have not been seen using standard sequencing of uveal melanoma tumors, use of pyrophosphorolysis-activated polymerization or a nested PCR technique has demonstrated that the BRAFV600E mutation is expressed heterogeneously within primary choroidal and ciliary body melanomas.23,34,35 It is therefore possible that OCM1 cultures grew from similar clones of cells in uveal tumors, although like OCM3, it may represent a cutaneous melanoma.

Decreased cell growth in comparison to that of controls was seen in all cell lines treated with 1 μM and 2 μM of 5-Aza. In the three cell lines which were tested further, decreased cell growth was associated with fewer viable cells, but an increase in apoptosis was seen only in OCM3 cells treated with 1 μM 5-Aza (data not shown). Treatment with 0.5 μM and 1 μM 5-Aza caused significant decreases in clonogenicity, affecting both the number and size of colonies formed compared to controls, and decreased migration of uveal melanoma cells in a dose-dependent manner. The fact that treatment with 0.5 μM 5-Aza has significant effects on both invasion and clonogenicity, but not on cell growth, indicates that the mechanism causing the observed changes may be independent from the effects of the drug on cell proliferation.

In addition, we used a murine xenograft model of melanoma that spreads hematogenously from the eye to examine the effects of demethylation on metastatic potential of uveal melanoma, because of the lack of good model systems in which to study uveal melanoma metastasis in vivo. We demonstrated that demethylation of melanoma cells causes a decrease in lung metastases. Interestingly, of the mice injected with 5-Aza-treated cells, only one mouse developed metastases, but in that animal, the number of metastases was approximately equal to the mean number of metastases in the vehicle-treated control group. This suggests that in this animal a clone of cells might have been able to either reverse the methylation changes or to accumulate additional pro-metastatic genetic alterations.

Previous investigations have examined the methylation status of a number of cancer-associated loci in uveal melanoma, including genes involved in suppressing expression of cell cycle regulators and the immunologic response to malignancy. Genes in which methylation has been implicated in uveal melanoma include the tumor suppressors S100A2 and p16INK4a, TIMP3, and the major histocompatibility (MHC) class II gene, as well as other genes involved in cell cycle regulation and immune responses.6,50–56 Those earlier studies suggested specific loci which could be modulated by methylation and proteins that might be viewed as drug targets for uveal melanoma chemotherapy. However, the in vitro and in vivo effects of pharmacological demethylation on ocular melanoma growth and spread has, to our knowledge, not been previously demonstrated.

Our study suggests that 5-Aza or other demethylating agents may form a useful component of new multiagent regimens for treatment of uveal melanoma patients. Importantly, we found that combining this demethylating agent with the current standard of care (radiation) was more effective than either modality alone, in what appeared to be an at least partially additive manner.

Other agents are being developed which might also be combined with 5-Aza. There are currently phase 2 studies of drugs targeting specific proliferation or apoptosis pathways such as the mitogen-activated protein kinase pathway, the phosphatidylinositol-3-kinase-AKT pathway, and vascular endothelial growth factor receptors, as well as a drug targeting the proteasome, which also inhibits adhesion and metastasis.50 Immunotherapies such as ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte-associated antigen-4, which has

**Figure 5.** Treatment of OC1, OCM3, and 92.1 melanoma cell lines with 5-Aza caused global DNA hypomethylation at L1 repeat loci.

**Figure 6.** Treatment with 5-Aza decreased tumor metastases in vivo. (A) Lung metastasis from an animal injected with vehicle-treated melanoma cells. (B) Lung metastasis from an animal injected with 5-Aza-treated melanoma cells. (C) The mean number of lung metastases formed was significantly decreased when cells were treated with 5-Aza. *P < 0.05 by Student's t-test, compared to controls.
been shown to increase overall survival in phase 2 studies of melanoma, are being explored.\textsuperscript{39–41} In addition, recruitment is under way for phase 1 and 2 trials of vaccination of patients with dendritic cells loaded with tumor antigens by mRNA transfection. In summary, our work demonstrates that FDA-approved demethylating agents should also be considered as possible therapies, alone or in combination with radiation and other emerging therapies, for the treatment of uveal melanoma with poor prognosis.

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