

2015

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Recommended Citation

Green, Lauren A. and Stern, Matthew M. (2015) "Brain-Penetrating Histone Deacetylase Inhibitor RG2833: A Potential Malignant Melanoma Growth Suppressor," *The Winthrop McNair Research Bulletin*: Vol. 1, Article 6.

Available at: <https://digitalcommons.winthrop.edu/wmrb/vol1/iss1/6>

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Brain-Penetrating Histone Deacetylase Inhibitor RG2833: A Potential Malignant Melanoma Growth Suppressor

Lauren A. Green
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ABSTRACT

Histone deacetylases (HDACs) play an important role in the epigenetic control of gene expression in both normal and cancer cells. Previous studies have demonstrated that pharmaceutical inhibition of HDACs can kill and/or suppress the growth of cancer cells. RG2833 is a HDAC inhibitor that targets specific HDACs known to be active in cancer cells. Melanoma cells have previously been shown to respond to HDAC inhibitors that are structurally similar to RG2833. We hypothesized that the inhibition of HDAC activity by RG2833 would result in the reduced growth and/or death of cells from the malignant melanoma cell lines SK-MEL-5 and SK-MEL-28. To test our hypothesis, we exposed SK-MEL-5 and SKMEL-28 cells to increasing concentrations of RG2833. We found that concentrations of RG2833 that effectively inhibited HDAC activity also resulted in reduced melanoma cell growth and viability. These results demonstrate the effectiveness of RG2833 in reducing the growth and viability of malignant melanoma cells in vitro and warrant further investigation of the potential therapeutic use of RG2833 and related compounds in the battle against cancer.

INTRODUCTION

Approximately 3.5 million people are diagnosed with skin cancer each year, of which, melanoma has the lowest survival rate [7]. Over fifty percent of people with melanoma develop metastatic tumors in the brain [6,7]. Essential functions governed by the brain such as sensory perception and breathing can be tremendously affected or impaired depending on the location of metastatic tumor development in the brain. Anticancer therapeutic studies targeting histone deacetylase inhibitors have shown promising activity in the suppression of cancer cell growth [2,3,10,11].

Histone deacetylase (HDAC) prevents acetylation of lysine receptors on histones, tightening DNA around histones [9]. When lysine receptors are acetylated, DNA is loosened around the histone - nucleosome complex and therefore, becomes more accessible. Accessibility to DNA promotes gene expression because RNA polymerase is allowed to facilitate the transcription process. HDAC inhibitors promote acetylation by blocking HDAC activity; this keeps the lysine receptors acetylated, promoting transcription. HDAC inhibitors have a unique ability to make changes in gene expression, which has been shown in previous studies to suppress cancer cell growth. Cancer cells generally display abnormal gene expression that allows them to undergo rapid metastasis and proliferation [11,12]. RG2833 is an HDAC inhibitor that penetrates the blood-barrier of the brain. RG2833 is an effective and efficient acetylation promoter [12]. It is structurally similar to Vorinostat, which is an HDAC inhibitor that is currently used clinically.

In this study, we provide a link between two malignant melanoma cell lines (SK-MEL-5 and SK-MEL-28) and RG2833 activity. We predict that as the concentrations of RG2833 increase, the growth rates and overall HDAC activity of the malignant melanoma cells will decrease.

MATERIALS & METHODS

Cell Lines and Cell Culture

The two human malignant melanoma cell lines, SK-MEL-5 and SK-MEL-28 (Figure 1), were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in high

glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and were incubated at 37°C, 5% CO₂, and 99% humidity.

HDAC Inhibitor

Brain-penetrating HDAC1 and HDAC3 inhibitor RG2833 (Figure 2) was obtained from Selleck Chemicals (Houston, TX). 40.0mM stock solution of DMSO and RG2833 was serially diluted for varied concentration amounts.

HDAC Assay

The HDAC Cell-Based Activity Assay Kit was obtained from Cayman Chemical (Ann Arbor, MI) and used according to manufacturer's instructions. Briefly, the assay determines the effect of an HDAC inhibitor on the enzyme provided in the kit. Results gathered from various tests within the kit will create a dose-response curve. A fluorescence plate reader was used to obtain the results of varying concentrations of RG2833 in 96-well plates. An ANOVA test was used to assess all data.

SRB-Proliferation Assay

The National Cancer Institute (NCI) 60-cell line screen tested the effects of thousands of varying compounds on cancer cells. Two separate plates containing the two cell lines were fixed in Trichloroacetic acid (TCA). 1nM, 10nM, 100nM, 1µM, and 10µM of RG2833 were placed into a 96-well plate. 100µL of Sulforhodamine B (SRB) solution in 1% acetic acid was added to each well after a series of incubation and rinsing patterns. A 10mM trizma base and the absorbance was read using a colorimetric plate reader. Other plating protocol can be found on the NCI website. An ANOVA was used to analyze all data.

AlamarBlue® Viability Assay

AlamarBlue® was obtained from Life Technologies (Carlsbad, CA) and is used to determine cell viability and proliferation. SK-MEL-5 and SK-MEL-28 cells were plated and allowed 24 hours before addition of RG2833 and AlamarBlue® reagent. Cells were then incubated for 24, 48, and 72 hours. Protocol from the manufacturer was followed in order to analyze results. An ANOVA test was used to analyze all obtained data.

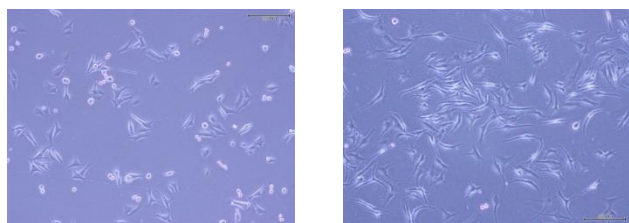


Fig. 1: SK-MEL-5 (left) and SK-MEL-28 (right) cell lines. Note: Two cell lines experience varying proliferation rates despite being plated at the same density. Also, the two cell lines have a different morphology.

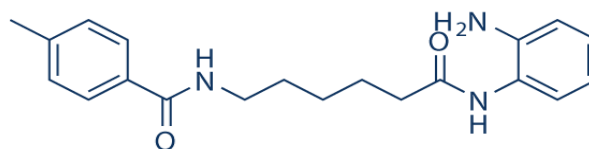


Fig. 2: Histone Deacetylase Inhibitor RG2833

RESULTS

HDAC activity was monitored in the SK-MEL-5 and SK-MEL-28 cell lineages. The two cell lines were not compared to one another; the effect of the inhibitor concentration was compared to percent HDAC activity relative to control (Figure 3). Statistical differences are present in accordance with higher RG2833 concentrations.

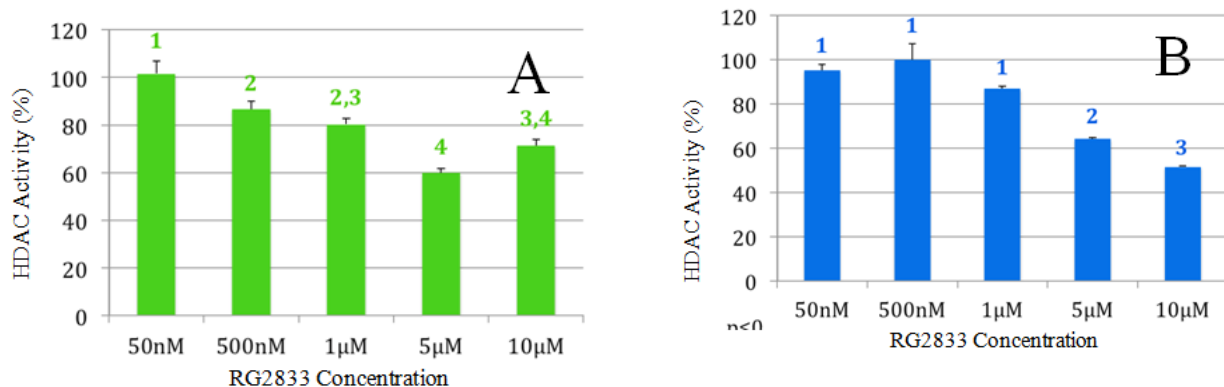


Fig. 3: (A) refers to the HDAC activity in SK-MEL-5 cells. (B) refers to the HDAC activity in SK-MEL-28 cells. All HDAC activity is relative to a control of 0nM. Numbers above bars indicate statistically independent groups.

Staining total protein in the cells assessed proliferation via an SRB Proliferation assay. After 48 hours, the growth rate was received based upon absorbance readings (Figure 4). Percent proliferation relative to control was compared to increasing RG2833 concentrations.

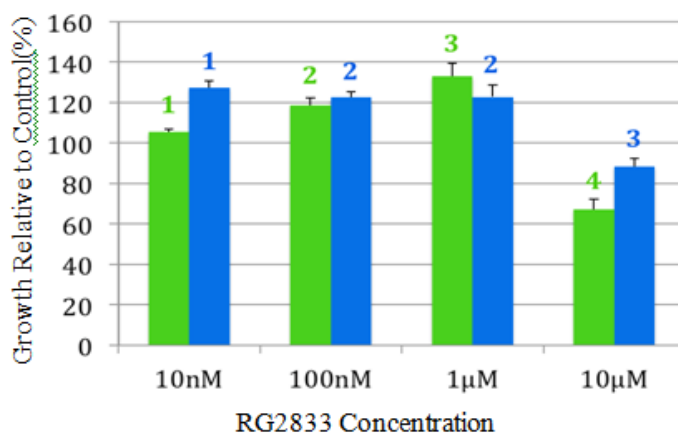


Fig. 4: Refers to the proliferation rate in SK-MEL-5 cells (green) and SK-MEL-28 cells (blue). The percent growth rate is relative to a control of 0nM. Numbers above bars indicate statistically independent groups within the individual cell types.

AlamarBlue® reagent metabolizes to colored product in cells. It assessed cell viability at multiple time points of 24 hours, 48 hours, and 72 hours. Cell viability was determined by comparing the percent viability to increasing RG2833 concentrations.

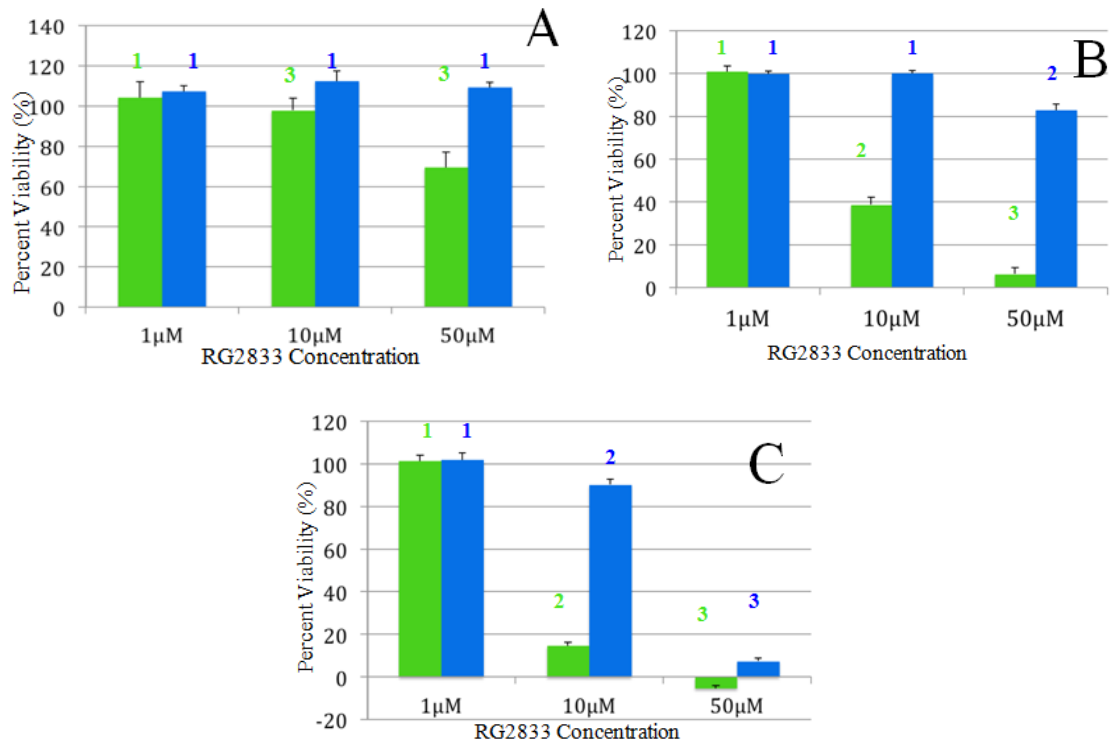


Fig. 5: Refers to the overall percent viability of cancer cells upon treatment with RG2833. The SK-MEL-5 cell line is colored green and the SK-MEL-28 cell line is colored blue. Graph A shows the percent viability after 24 hours. Graphs B and C show the percent viability after 48 and 72 hours respectively. Numbers above bars indicate statistically independent groups when comparing cell lineage viability to RG2833 concentration.

SK-MEL-5 and SK-MEL-28 cells were treated with 10 μM RG2833 for 48 and 72 hours. Effectiveness of RG2833 *in vitro* when added to SK-MEL-5 cells is displayed in Figure 6. Effectiveness in SK-MEL-28 cells is displayed in Figure 7.

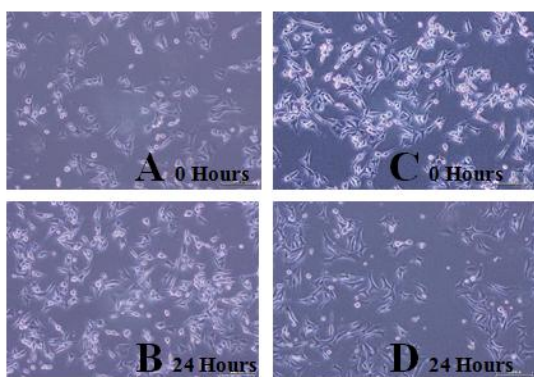


Fig. 6: Depicts 10 μM RG2833 activity *in vitro* after 24 hours on SK-MEL-5 cells. (A) SK-MEL-5 without RG2833 at 0 hours. (B) SK-MEL-5 without RG2833 after 24 hours. (C) SK-MEL-5 with RG2833 at 0 hours. (D) SK-MEL-5 with RG2833 after 24 hours.

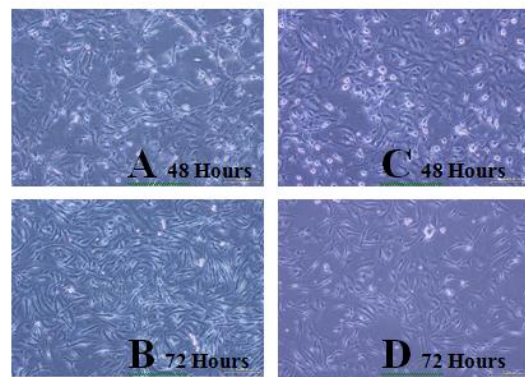


Fig. 7: Depicts 10 μM RG2833 activity *in vitro* after 24 hours on SK-MEL-28 cells. (A) SK-MEL-28 without RG2833 at 48 hours. (B) SK-MEL-28 without RG2833 after 72 hours. (C) SK-MEL-28 with RG2833 at 48 hours. (D) SK-MEL-28 with RG2833 after 72 hours.

DISCUSSION

In this experiment, we strove to inhibit the HDAC activity and suppress the growth rate of malignant melanoma cells. Our data was in accordance with other HDAC inhibitor studies that showed how these inhibitors are contributors to suppression of cancer cell growth [1, 7]. We found that as RG2833 concentrations increase, HDAC activity, cell growth rate, and viability all decrease. This suggests that RG2833 could be of more applicable use in melanoma cancer cell lines.

The HDAC assay sought to inhibit the traditional cell histone acetyltransferase (HAT) and histone deacetylase (HDAC) mechanistic pathways by keeping an acetyl group on the lysine receptors and promoting gene expression [5]. By successfully inhibiting HDAC activity (shown in Figure 3), the DNA became more loosely associated with the histone (nucleosome) complex. This allowed for RNA polymerase to transcribe the DNA and ultimately suppress the growth rate of the cells. In Figure 3A, the bars portrayed at higher concentrations are statistically more significant from the latter bars. This confirms what we expected to see, a decreasing trend in HDAC activity. Similarly in Figure 3B, in the SK-MEL-28 cells we also see a statistically significant decreased trend in HDAC activity. The HDAC assay also played an important role in our concentration determination. This served as the basis for all other assays.

In the SRB Proliferation assay we stained the total protein in the cells to assess growth rate. The well concentrations were based on the 10 μ M concentration confirmed to inhibit HDAC activity by the previous assay. In Figure 4, the numbers above the green bars show that there is a statistical difference in the growth rate of the SK-MEL-5 cells. The statistical trend is similar but not as prominent in the blue bars (SK-MEL-28 cells). Both cell lines did have a slower proliferation rate at the 10 μ M concentration, which provided a basis for the next assay.

The AlamarBlue® viability assay allowed us to determine cell death. The reagent itself metabolizes to colored product in the cells and emits an absorbance signal that corresponds to either a living or dead cell signal. Figure 5A shows the cell lines after 24 hours of exposure to varying concentrations of RG2833. The focal point of each AlamarBlue® graph is at the 10 μ M concentration. After 24 hours there is no decrease in SK-MEL-28 (blue) viability; these groups are not statistically significant. But, at the 50 μ M concentration, the SK-MEL-5 cells (green) are becoming less viable. There is a statistical difference between the 50 μ M concentration and the other groups. Figure 5B shows the cell lines under the same conditions after 48 hours. The SK-MEL-28 cells are not being killed at the 10 μ M concentration; however, at 50 μ M the cells are becoming less viable. There is a statistical difference present at 50 μ M. After 48 hours, the SK-MEL-5 cells are becoming less viable at the 10 μ M target concentration. This is a statistically independent group. Finally, after 72 hours of RG2833 exposure both cell lines were significantly affected by 10 μ M. There is a statistical difference between each individual cell line and its respected concentration. Cell viability was decreased. Lastly, in Figures 6 and 7, the *in vitro* cell lines are shown before and after the addition of 10 μ M RG2833. SK-MEL-28 cells are not as sensitive to RG2833 whereas the SK-MEL-5 are.

Cancer affects 8 million people per year, many of which die due to some type of metastatic tumor growth [7]. New anticancer agents like HDAC inhibitors are currently being analyzed in clinical trials [6,8]. Many studies have shown how HDAC inhibitors work inside protein complexes, but few studies have shown the effect of these inhibitors on the cells themselves as a whole [4,5]. Mechanistically, HDAC inhibitors prevent the growth of melanoma cells and provide an excellent outlook in the future of anticancer therapeutics [10].

CONCLUSION

Ultimately, we were able to inhibit traditional cell HDAC activity, slow the proliferation rate, and decrease the viability of the SK-MEL-5 and SK-MEL-28 cancer cell lines with increasing concentrations of RG2833.

FUTURE RESEARCH

RG2833 has the potential to be a strong melanoma cell growth suppressor. We would like to assess how the cells are being killed using an apoptosis assay. Then, study changes in gene expression using a Real-Time RT-PCR Array that specifically targets human cancer drugs. Eventually, we would also like to perform a protein analysis and study signaling pathways.

ACKNOWLEDGEMENTS & FUNDING

The Stern lab provided a workbench, assistance in experimental design, and data analysis. Kathryn Steverson, lab mate, aided in experimental protocol and setup. Dr. Sumter's lab allowed access to colorimetric and fluorescence plate reading equipment. Dr. Cheryl Fortner-Wood and the Winthrop McNair Scholars Program provided moral support. SC-INBRE and the Winthrop McNair Scholars Program provided experiment funding.

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