AWARD NUMBER: W81XWH-16-1-0370

TITLE: Targeting Increased Polyamine Transport of Resistant Melanomas

PRINCIPAL INVESTIGATOR: Susan Gilmour

CONTRACTING ORGANIZATION: Lankenau Institute for Medical Research
Wynnewood, PA 19096

REPORT DATE: November 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
November 2019	Final	01 August 2016 - 31 July 2019

4. TITLE AND SUBTITLE

Targeting Increased Polyamine Transport of Resistant Melanomas

5a. CONTRACT NUMBER
W81XWH-16-1-0370

5b. GRANT NUMBER
CA150356

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Susan Gilmour

E-Mail: GilmourS@mlhs.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Lankenau Institute for Medical Research
100 East Lancaster Avenue
Wynnewood, PA 19096-3434

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Although BRAF inhibitors (BRAFi) significantly improve survival of patients with metastatic melanoma, recurrences occur within several months. Development of BRAFi-resistance enriches for metastatic cancer stem cells (CSC) and increases tumor-promoting macrophages, both of which require polyamines. The objective of this study was to exploit the oncogene-induced polyamine transport system (PTS) activity in melanoma cells by targeting the PTS with a novel arylmethyl-polyamine (***AP***) compound that is cytotoxic upon cell entry. We hypothesized that ***AP*** 1) would kill BRAFi-resistant melanoma CSCs as a result of induced PTS activity and 2) would block CXCR4 signaling in metastatic tumor cells and stromal macrophages, thus inhibiting melanoma progression and metastasis. We proposed the following specific aims:

1) to compare effects of ***AP*** on mutant and wildtype BRAF melanoma cells and on melanoma tumor cell survival in macrophage co-culture assays with BRAFi

2) to evaluate whether ***AP*** increases the anti-tumor effect of BRAFi in mice

We found that BRAF mutant melanoma cells have higher PTS activity and are more sensitive to ***AP*** compared to BRAF wild type cells. Although cancer stem cell-like subpopulations of BRAF mutant melanoma cells were resistant to BRAFi, they were more susceptible to cytotoxic activity of ***AP*** compared to proliferating populations of the same cells. Moreover, ***AP*** inhibited M2 polarization and VEGF production by macrophages that provides a survival advantage for melanoma tumor cells treated with BRAFi. Co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** decreased tumor-promoting macrophages and significantly delayed the recurrence of PLX4720-resistant melanoma tumors that occurred in animals treated with PLX4720 alone. Our work has greatly added to our understanding of the PTS in tumors and offers a novel therapy (***AP***) that can harness this transport system to better treat resistant forms of metastatic melanoma.

15. SUBJECT TERMS

Melanoma, polyamine transport system, BRAF mutations, CXCR4, cancer stem cell populations, BRAF inhibitor resistance, macrophages

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	57	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. INTRODUCTION:

Melanoma is a highly aggressive tumor with poor prognosis in the metastatic stage. Multiple oncogenic mutations (including BRAF, NRAS, KIT) drive this highly heterogeneous disease, with BRAF mutations detected in half of all melanoma tumors. Although targeting the V600E-mutant BRAF kinase with BRAF inhibitors significantly improves survival of patients with metastatic melanoma, recurrences often occur within several months. Development of BRAFi-resistance enriches for highly tumorigenic and metastatic melanoma initiating cells (MIC) or cancer stem cells (CSC) and increases tumor-promoting macrophages. CSC survival requires increased cellular uptake of polyamines via activation of the polyamine transport system (PTS). Our **objective** is to exploit the oncogene-induced PTS activity in metastatic melanoma cells by targeting the PTS with a novel arylmethyl-polyamine (***AP***) compound which is cytotoxic upon cell entry. Both exogenous polyamines and polyamine-based drugs are imported into tumors via this specific polyamine uptake system. However, normal cells are predicted to be significantly less sensitive to ***AP*** since they have low PTS activity. Recently we observed that another polyamine-containing compound, AMD3100 (an inhibitor of chemokine receptor CXCR4 and tumor invasiveness), weakly inhibits PTS activity in tumor cells. CXCR4 signaling in CSCs is necessary for tumor metastasis. The rationale for our studies is that ***AP*** will not only inhibit uptake of polyamines but will also inhibit CXCR4/SDF-1 signaling that is a critical regulator of melanoma-stromal interactions driving metastasis. **We hypothesize that *AP* 1) will kill BRAFi-resistant melanoma CSCs as a result of induced PTS activity and 2) will block CXCR4 signaling in metastatic tumor cells and stromal macrophages, thus inhibiting melanoma progression and metastasis.** The proposed research is novel in that it explores the association between polyamine transport and CXCR4/SDF-1 signaling in the survival of CSC subpopulations, metastasis, and BRAFi resistance. Another important novel aspect is that ***AP*** may also target M2 macrophages that highly express CXCR4 and have been found to contribute to BRAFi resistance and to indirectly facilitate melanoma progression and metastasis. To test our hypothesis, we have proposed the following specific aims:

1) to compare human BRAFi-sensitive and BRAFi-resistant melanoma tumor cells for effects of ***AP*** on spheroid-forming capacity; cell death and autophagy markers; invasiveness thru collagen-coated filters towards SDF-1; PTS activity (measuring the V_{max} of ^{3}H-spermidine); and melanoma tumor cell survival in macrophage co-culture assays ± PLX4720; and

2) to evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth and metastasis in mice following orthotopic injection of mutant BRAF melanoma cells.

2. KEYWORDS:

Melanoma, polyamine transport system, BRAF mutations, CXCR4, cancer stem cell populations, BRAF inhibitor resistance, macrophages

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1: To compare the effect of increasing concentrations of ***AP*** with long-term and pulse BRAFi (PLX4720) co-treatment a) on the enrichment of BRAFi-resistant slow cycling CSC melanoma subpopulations and b) in melanoma tumor cell survival in macrophage co-culture assays ± PLX4720.

Major Task 1: To investigate the extent to which PTS activity and ***AP*** cytotoxicity correlate with sensitivity to BRAFi in human melanoma cell lines that have been treated chronically with PLX4720 (Months 1-14)
Milestone Achieved: Determination of the effect of BRAFi resistance on PTS activity and ***AP*** cytotoxicity in melanoma cells (Month 14)

We have obtained a series of human melanoma cell lines from Dr. Meenhard Herlyn (The Wistar Institute) that are $BRAF^{WT}$ or $BRAF^{V600E}$. We expanded the cells, made frozen stocks, and verified their sensitivity (or resistance) to the BRAF inhibitor (BRAFi), PLX4720 via proliferation assays and determinations of IC_{50}s. BRAFi-sensitive and BRAFi-resistant cells were assayed in the presence of varying doses of ***AP*** with and without 1 mM DFMO to determine the IC_{50} for ***AP*** and the uptake of ^{3}H-spermidine as a measure of their

polyamine transport system (PTS) activity. We accomplished 100% of this major task, with our results published in the following manuscript.

Peters, M., Minton, A., Phanstiel, O., Gilmour, S. (2018) A novel polyamine targeted therapy for BRAF mutant melanoma tumors, Med. Sci. (Basel), 6(1), pii:E3:10.3390/medsci6010003.
Highlighted on journal cover.

Major Task 2: To evaluate the effect of increasing concentrations of ***AP*** on CSC subpopulations that are enriched following chronic BRAF inhibition (Months 5-16)

Milestone Achieved: Determine to what extent ***AP*** can eliminate CSC-like subpopulations that are enriched following chronic BRAF inhibition (Month 16)

We took advantage of WM983B melanoma cells that are BRAF mutant and PLX4720 sensitive and a WM983B-R subline that was generated by culturing in increasing concentrations of PLX4720 for several weeks to achieve a PLX4720 resistant subline. Although we found that PTS activity is significantly elevated in WM983B-R cells compared to parental WM983B cells, we did not find any evidence that CSC subpopulations are enriched in WM983B-R cells following chronic BRAF inhibition with PLX4720 compared to that in parental WM983B cells. For that reason, we focused on experiments outlined for major task 3 with about 25% completion of major task 2. Experiments in task 3 use a different approach using a shorter high dose treatment with the BRAF inhibitor PLX4720 to enrich for a CSC subpopulation that can be identified by a GFP reporter gene driven by the promoter of the CSC marker gene, JARID1B.

Major Task 3: To evaluate the effect of increasing concentrations of ***AP*** on the slow cycling JARID1B^{+}/EGFP^{high} subpopulation that is enriched following a 24-96 hr pulse PLX4720 treatment of mutant BRAF melanoma cells transduced with a JARID1B-promoter-EGFP-reporter construct (Months 8-18)

Milestone Achieved: Determine to what extent ***AP*** can overcome the enrichment of slow cycling, invasive J/EGFP^{high} BRAF inhibitor-resistant melanoma cells and its correlation with PTS activity and CXCR4 signaling. (Month 18)

We have characterized two human BRAF^{V600E} melanoma cell lines that have been transfected with a GFP reporter gene that is driven by the promoter of the JARID1B gene, a CSC marker. WM3734$^{JARID1Bprom\text{-}EGFP}$ cells and 1205Lu$^{JARID1Bprom\text{-}EGFP}$ cells have been treated with a BRAFi (using a high concentration of 25 μM PLX4720) without or with increasing concentrations of ***AP*** for up to 72 hr. These cells have been cultured and analyzed using a 3-dimensional spheroid model and also as spheroid cultures grown on low-adherent plates to enrich for stem cell population. These spheroid cultures have been compared with the same cells grown in a standard 2-dimensional monolayer culture. In similar manner, we found that spheroid cultures of murine YUMM1.7 melanoma cells that are enriched for stem cell markers (CXCR4 and CD304) are dramatically more sensitive to ***AP*** cytotoxic activity than monolayer cultures. Furthermore, invasiveness of YUMM1.7 cells through a Matrigel-coated filter using the CXCR4 ligand, SDF-1α, can be blocked at noncytotoxic ***AP*** concentrations that have no effect on MMP-2, MMP-9, or CXCR4 expression. We have completed 100% of our proposed experiments for task 3.

Major Task 4: To evaluate to what extent ***AP*** will block macrophage-mediated resistance of melanoma cells to treatment with BRAF-inhibitors (Months 8-18)

Milestone Achieved: Determine to what extent ***AP*** can block the ability of macrophages to confer resistance to BRAF inhibition in melanoma cells. (Month 18)

Monocytes purified from human donor peripheral blood have been differentiated to macrophages using human melanoma conditioned medium or by adding cytokines (GM-CSF or M-CSF). Using a human macrophage and melanoma cell co-culture system, we have demonstrated that pretreatment with ***AP*** blocks M2-macrophage VEFG production and also blocks M2 macrophages from conferring resistance to the BRAFi, PLX4720 in melanoma cells. We completed our proposed task 4 experiments and also used transgenic macrophage cultures in which polyamine biosynthesis is knocked out, resulting in reduced polarization of macrophages to a M2 state. Using murine YUMM1.7 tumor cells co-cultured with murine macrophages, we found that ***AP*** pretreatment blocked M2 macrophages from conferring resistance to PLX4720 in the YUMM1.7 melanoma cells. Less protection was conferred by macrophages in which polyamine biosynthesis is knocked out. We have completed 100% of our proposed experiments for task 4.

Aim 2. To evaluate whether ***AP*** increases the anti-tumor effect of the BRAF inhibitor PLX4720 in mice following orthotopic injection of mutant BRAF melanoma cells.

Major Task 1*:* To determine the dosing scheme of ***AP*** to be used in murine tumor studies (Months 1-8)
Milestone Achieved: Determine an ***AP*** dosing protocol for testing in mouse models of melanoma (Month 8)

IACUC and ACURO approval for our proposed mouse experiments with ***AP*** was obtained in the first couple months of this grant. We have completed task 1 to determine an ***AP*** dosing protocol in mice bearing either B16F10 tumors or YUMM1.7 melanoma tumors.

Major Task 2: To determine to what extent ***AP*** will increase the anti-tumor activity of PLX4720 following s.c. injection of BRAF mutant melanoma tumors in mice (Months 6-24)

Milestone Achieved: Determination of anti-tumor effect of ***AP*** treatment using melanoma xenografts *in vivo*, its correlation with sensitivity to BRAF inhibitors, and the extent to which combination ***AP*** and PXL4720 may overcome BRAFi-resistance and promote tumor killing. (Month 24)

IACUC and ACURO approval for our proposed mouse tumor experiments with ***AP*** was obtained. Our initial animal tumor studies in mice subcutaneously injected with YUMM1.7 melanoma cells have revealed that co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** plus DFMO delays the recurrence of $BRAF^{V600E}$ melanoma tumors that occurs in animals treated with PLX4720 alone. We have completed 75% of task 2.

Major Task 3: Statistical analysis of data; prepare figures and manuscript(s) to report results of the study (Gilmour and Phanstiel, Sites 1 and 2, Months 13-24)

Milestone Achieved: Manuscript(s) submitted for publication. (Month 24)

Collection of data, preparation of figures, and statistical analyses continues to be ongoing. One manuscript has been published, and 2 additional manuscripts have been submitted.

What was accomplished under these goals?

Summary of Significant Findings: PTS activity (assayed via spermidine uptake following a 60 min pulse with 1 μM [^{3}H] spermidine) was significantly elevated in BRAFi-resistant melanoma cells compared to the parental BRAFi-sensitive cells. In addition, $BRAF^{V600E}$ melanoma cells are significantly more sensitive to ***AP*** (lower IC50) compared to $BRAF^{WT}$melanoma cells. Treatment with DFMO upregulates PTS activity in $BRAF^{V600E}$ melanoma cells and further increases their sensitivity to ***AP*** (lower IC50). Spheroid-forming cancer stem cell-like subpopulations of $BRAF^{V600E}$ melanoma cells are more susceptible to cytotoxic activity of ***AP*** and less sensitive to BRAFi compared to proliferating populations of the same cells. Pretreatment of human M2 macrophages with ***AP*** blocks their VEGF secretion induced by the BRAF inhibitor, PLX4720. Macrophage VEGF production has been shown to contribute to melanoma resistance to BRAF inhibitors. ***AP*** pretreatment

also blocks the M2 macrophage-mediated resistance of melanoma cells to PLX4720. This concurs with animal tumor studies showing that co-treatment with the BRAF inhibitor, PLX4720, and ***AP*** plus DFMO delays the recurrence of $BRAF^{V600E}$ melanoma tumors that occurs in animals treated with PLX4720 alone. The delay in recurrence of PLX4720-resistant tumors in ***AP***-treated mice is also accompanied by a dramatic decrease in tumor infiltrating M2 macrophages ($CD206^{+}F480^{+}$).

Aim #1, Major Task 1: Effect of BRAFi resistance on PTS activity and ***AP*** cytotoxicity in melanoma cells

We obtained and characterized 18 different melanoma cell lines (15 human melanoma cell lines and 3 murine melanoma cell lines) for their BRAF mutational status; sensitivity (IC50) to the BRAFi, PLX4720; sensitivity to ***AP*** ± DFMO; and their polyamine transport system (PTS) activity as determined via spermidine uptake following a 60 min pulse with 1 µM [^{3}H]spermidine in the absence and presence of DFMO. Human melanoma cells were obtained from Dr. Meenhard Herlyn (The Wistar Institute) and $BRAF^{V600E}$ YUMM1.7 murine melanoma cells from Dr. M. Bosenberg (Yale Medical School). We wanted to determine whether 1) PTS activity is increased in $BRAF^{V600E}$ melanoma cells compared to $BRAF^{WT}$ melanoma cells; 2) to what extent blocking polyamine biosynthesis with difluoromethylornithine (DFMO) would increase PTS activity; and 3) to what extent sensitivity to ***AP*** correlated with changes in PTS activity. In addition, we tested our hypothesis that BRAFi-resistance of melanoma tumors correspond to a high demand for polyamine growth factors and upregulated PTS activity. This was complicated by our observation that the PTS activity is greatly affected by the confluency of the cells and also the highly variable plating efficiency and growth rates of the different cell lines. We standardized our assays so that the cells were 80% confluent within a similar protein range to which the [^{3}H] spermidine uptake was normalized. We found that $BRAF^{V600E}$ melanoma cells are significantly more sensitive to ***AP*** (lower IC50) compared to $BRAF^{WT}$ melanoma cells, reflecting the higher PTS activity in $BRAF^{V600E}$ melanoma cells. Cells with higher PTS activity not only transport in more polyamines but also the polyamine-containing drug ***AP***. Treatment with DFMO not only blocks polyamine biosynthesis but also further upregulates polyamine uptake in many tumors via the PTS. As a result, DFMO treatment further increases the sensitivity to ***AP*** (lower IC50). Our findings are summarized in the following 2 figures and were published in a special issue for Polyamines in the journal *Medical Sciences* (Basel).

Figure 1

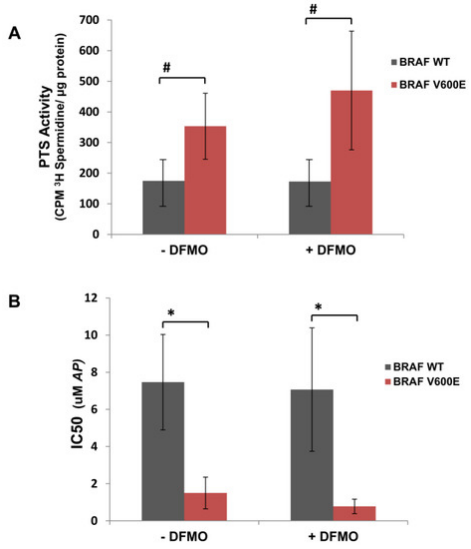


Figure 1. Greater PTS activity and increased sensitivity to ***AP*** in mutant $BRAF^{V600E}$ human melanoma cells compared to wild type (WT) $BRAF^{WT}$ melanoma cells. (**A**) $BRAF^{V600E}$ human melanoma cells (WM983B, WM3734, 1205Lu, WM989, and WM88) and $BRAF^{WT}$ human melanoma cells (WM3451, WM3743, and WM3211) were cultured with and without 1 mM DFMO for 40 h and then pulsed with 0.5 µM ^{3}H-spermidine for 60 min at 37 °C. Cell lysates were assayed for CPM ^{3}H-spermidine per mg protein by scintillation counting. The mean PTS activity ± SD for $BRAF^{WT}$ melanoma cells is compared with that of $BRAF^{V600E}$ melanoma cells

under conditions where cells were cultured without added DFMO or with 1 mM DFMO. (**B**) $BRAF^{V600E}$ melanoma cells (WM983B, WM3734, 1205Lu, WM989, and WM88) and $BRAF^{WT}$ human melanoma cells (WM3451, WM3743, and WM3211) were treated with increasing doses of ***AP*** with or without 1 mM DFMO, using 5–6 samples per dose of ***AP***. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay (Alstem, Richmond, CA, USA). ***AP*** IC50 values were calculated by GraphPad Prism 6. The mean ***AP*** IC50 values ± SD for $BRAF^{WT}$ melanoma cells is compared with that of $BRAF^{V600E}$ melanoma cells under conditions where cells were cultured without added DFMO or with 1 mM DFMO; # $p \leq 0.05$; * $p < 0.01$.

Figure 2

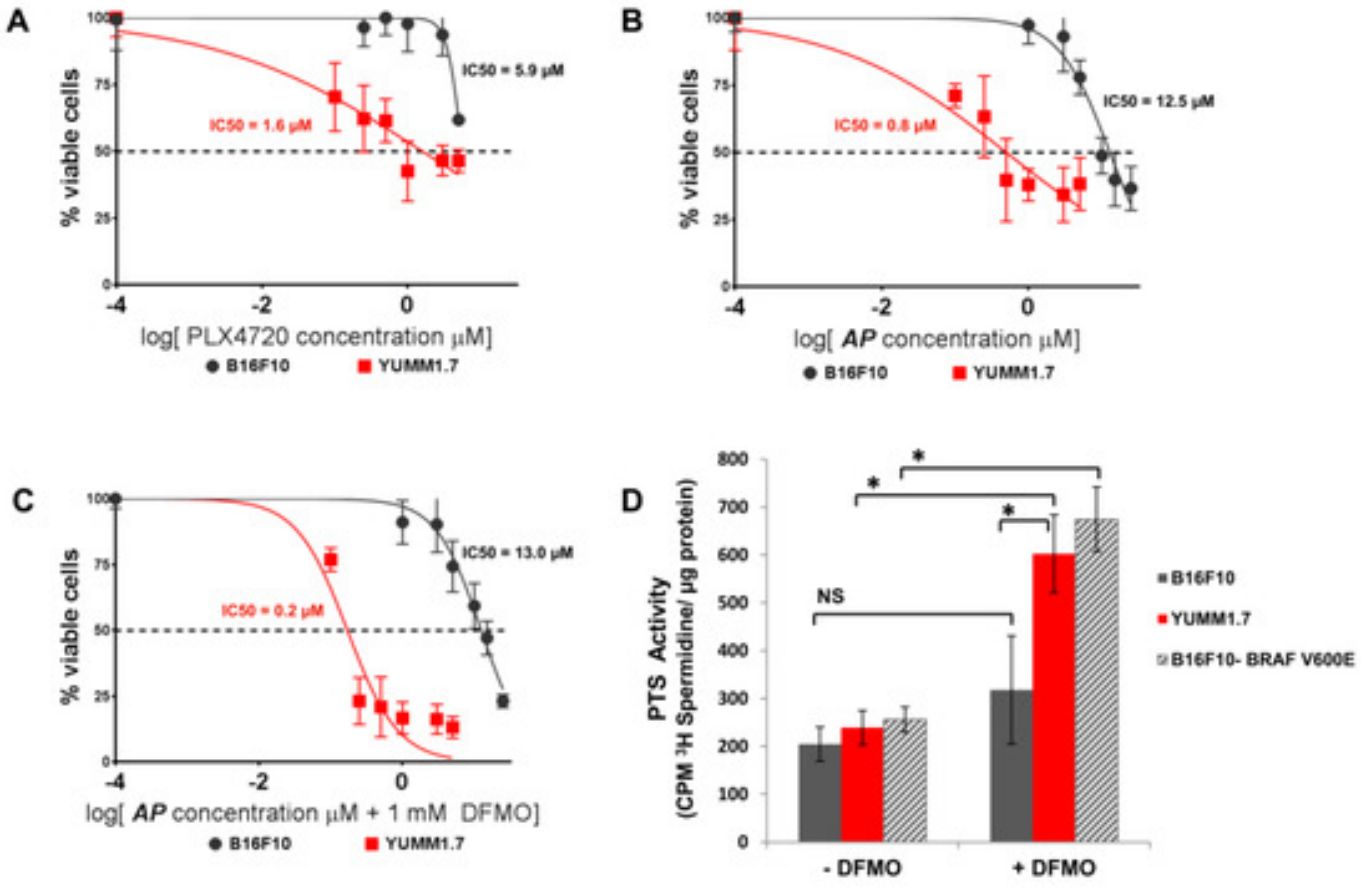
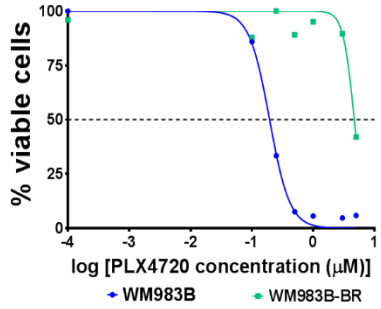


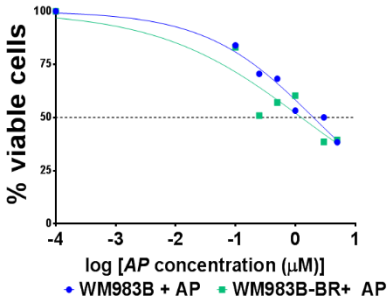
Figure 2. $BRAF^{V600E}$ murine melanoma cells are more sensitive to ***AP*** than $BRAF^{WT}$ melanoma cells. (**A**) Murine $BRAF^{V600E}$ YUMM1.7 melanoma cells and $BRAF^{WT}$ B16F10 melanoma cells were treated with increasing doses of PLX4720. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p = 0.0013$. (**B**) Murine $BRAF^{V600E}$ YUMM1.7 melanoma cells and $BRAF^{WT}$ B16F10 melanoma cells were treated with increasing doses of ***AP***. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p < 0.0001$. (**C**) Murine $BRAF^{V600E}$ YUMM1.7 melanoma cells and $BRAF^{WT}$ B16F10 melanoma cells were treated with increasing doses of ***AP*** ± 1 mM DFMO. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p < 0.0001$. (**D**) YUMM1.7 and B16F10 melanoma cells and B16F10 cells retrovirally infected to express the mutant $BRAF^{V600E}$ protein were cultured with and without 1 mM DFMO for 40 h and then pulsed with 0.5 µM ^{3}H-spermidine for 60 min at 37 °C. Cells were washed with cold PBS containing 50 µM spermidine, and cell lysates were assayed for CPM ^{3}H-spermidine per mg protein by scintillation counting; * $p < 0.0001$; NS: not significant.

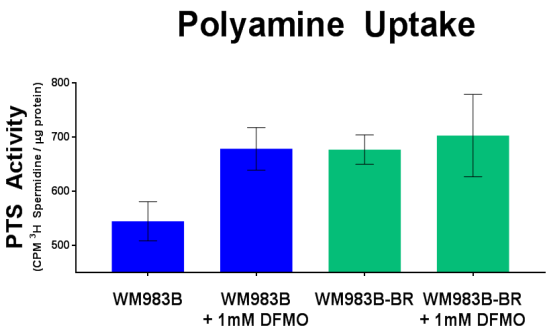
Major Task 2: To determine to what extent ***AP*** can eliminate CSC-like subpopulations that are enriched following chronic BRAF inhibition

We compared WM983B cells with its subline WM983B-BR which has acquired BRAF inhibitor resistance via chronic *in vitro* exposure of WM983B cells to 1 μM PLX4720. The basis for our study was that the oncogene addiction and BRAFi-resistance of melanoma tumors corresponds to a high demand for polyamine growth factors and a greatly upregulated PTS activity. We were encouraged to find that basal PTS activity is indeed greatly increased in the WM983B-BR cells that can grow in 1 μM PLX4720 compared to the parental, PLX4720-sensitive WM983B cells. However, DFMO co-treatment did not further increase PTS activity in the WM983B-BR cells, unlike that in the parental WM983B cells. If polyamine uptake (PTS activity) is increased in resistant $BRAF^{V600E}$ melanoma cells (made resistant by chronic treatment with PLX4720), then we predicted that ***AP*** treatment would increase cell death in BRAFi-resistant $BRAF^{V600E}$ melanoma cells compared to those that are BRAFi-sensitive. However, although PTS activity was increased in BRAFi-resistant WM983B-BR cells, their sensitivity to ***AP*** was not significantly changed compared to that found in parental BRAFi-sensitive WM983B cells.

Figure 3. Human $BRAF^{V600E}$ Melanoma WM983B-BR Subline with Acquired BRAFi Resistance Demonstrates Increased PTS Activity Compared to Parental BRAFi-Sensitive WM983B Cells







Cell Line	PLX4720 IC50 (μM)	*AP* IC50 (μM)	*AP* + 1 mM DFMO IC50 (μM)
WM983B	0.1944	2.004	0.5127
WM983B-BR	4.679	1.258	2.42

Accumulating literature shows that treatment with BRAF inhibitors enriches a slow cycling cancer stem cell-like (CSC) subpopulation in melanomas. We proposed 2 different approaches (major tasks 2 and 3) to test our hypothesis that ***AP*** would accumulate selectively in BRAFi-resistant CSCs as a result of induced PTS activity. In task 2, we proposed to use $BRAF^{V600E}$ melanoma cells that have been chronically exposed to low dose PLX4720 (i.e. WM983B-BR cells) to examine the effect of ***AP*** on BRAFi-resistant CSCs. However, analyses of WM983B-BR cells by flow cytometry did not revealed increased expression of melanoma CSC markers such as JARID1B, CXCR4, CD271, or CD34 compared to that seen in parental WM983B cells. *It is likely that chronic treatment with PLX4720 leads to differentiation of the CSC subpopulation and loss of the enrichment for this stem cell-like subpopulation.* In this case, we predicted that PLX4720 pulse treatment of BRAFi-sensitive melanoma cells (such as WM983B cells) would lead to the greatest enrichment of stem-cell-like subpopulations with greater formation of spheroids with increased expression of CSC markers and perhaps sensitivity to ***AP***. Indeed, as mentioned previously, the IC50 to ***AP*** was not significantly changed in WM983B-BR cells compared to WM983B cells. For these reasons, we focused more on accomplishing goals

in major task 3 where $BRAF^{V600E}$ melanoma cells are not chronically treated with low dose PLX4720 but are treated for a much shorter time with higher concentrations of PLX4720 or using $BRAF^{V600E}$ melanoma cells cultured using a 3-dimensional spheroid model and also as spheroid cultures grown on low-adherent plates to enrich for stem cell subpopulations.

Major Task 3: To determine to what extent ***AP*** can overcome the enrichment of slow cycling, invasive $J/EGFP^{high}$ BRAF inhibitor-resistant melanoma cells and its correlation with PTS activity and CXCR4 signaling.

Since it has been shown that treatment with various chemotherapeutic drugs including PLX4720 enriches for a slow-cycling melanoma cell subpopulation that expresses the H3K4-demethylase JARID1B, we used a cell-based reporter system to test whether this resistant phenotype can be eradicated with ***AP***. For isolation of live $JARID1B^{+}$ cells, we used two $BRAF^{V600E}$ mutant melanoma cell lines (WM3734 and 1205Lu) that were stably transduced with a JARID1B-promoter-EGFP-reporter construct (cells obtained from Dr. Meenhard Herlyn, The Wistar Institute). For 3D cell culture we used NanoCulture Plates (NCP, from SCIVAX Life Sciences) that are part of a scaffold-based 3D culture system in which cells can easily form spheroids. The spheroid microenvironment closely resembles that in tumors and is more relevant for drug sensitivity compared to that seen with monolayer cultures. Considerable effort was spent on optimizing the conditions to generate spheroids with $Wm3734^{JARID1Bprom\text{-}EGFP}$ cells, $1205Lu^{JARID1Bprom\text{-}EGFP}$ cells, and YUMM1.7 cells using the NCP culture method and also on ultralow attachment plates. We found that melanoma cells growing as spheroids in NCP culture are more resistant to treatment with 25 μM PLX4720 compared to cells growing in monolayer. Confirming our hypothesis, a short (2 day) exposure to PLX4720 led to a 4-fold enrichment of JARID1B-driven GFP expressing melanoma cells grown as spheroids, which is characteristic of a CSC subpopulation. We were excited to find that 48 hr co-treatment with both 25 μM PLX4720 and 25 μM ***AP*** led to the largest reduction in cell viability in the spheroid cultures (Fig. 4).

Figure 4

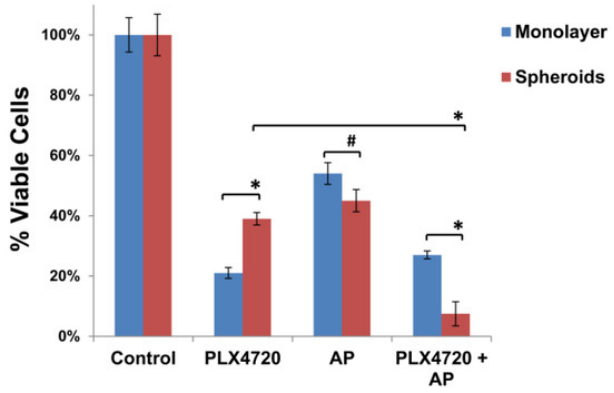


Figure 4. Increased resistance of spheroid melanoma cells to PLX4720 is overcome with ***AP*** co-treatment. $BRAF^{V600E}$ mutant $1205Lu^{JARID1Bprom\text{-}EGFP}$ melanoma cells were seeded at 1×10^4 cells in each well of 24-well NanoCulture plates. When spheroids were formed on day 3, the 3D cultures of spheroids were treated with PLX4720 (25 μM) and/or ***AP*** (25 μM). 2D monolayer cultures of $1205Lu^{JARID1Bprom\text{-}EGFP}$ melanoma cells were also treated with PLX4720 (25 μM) and/or ***AP*** (25 μM). After drug treatment for 48 h, the viability of spheroids and monolayer cultures was assayed using the CellTiter-Glo Luminescent Cell Viability Assay. The percent cell survival in each treatment group was calculated relative to cells treated with medium only under the same conditions. As controls, the growth of cells without drug treatment under each condition was normalized as 100% separately. The means are presented ± SD; * $p < 0.0001$; # $p = 0.0028$.

We also compared the effect of ***AP*** ± PLX4720 on murine YUMM1.7 cells grown as monolayer cultures and as 3D spheroid cultures on NanoCulture plates. In agreement with our studies with the human melanoma cells, we found that YUMM1.7 spheroid cultures (enriched for expression of stem cell markers CXCR4 and

CD304) are sensitive to the cytotoxic activity of ***AP*** unlike that of monolayer YUMM1.7 cultures. Moreover, treatment with both PLX4720 and ***AP*** further decreased the viability of the spheroid cultures (Fig. 5)

Figure 5

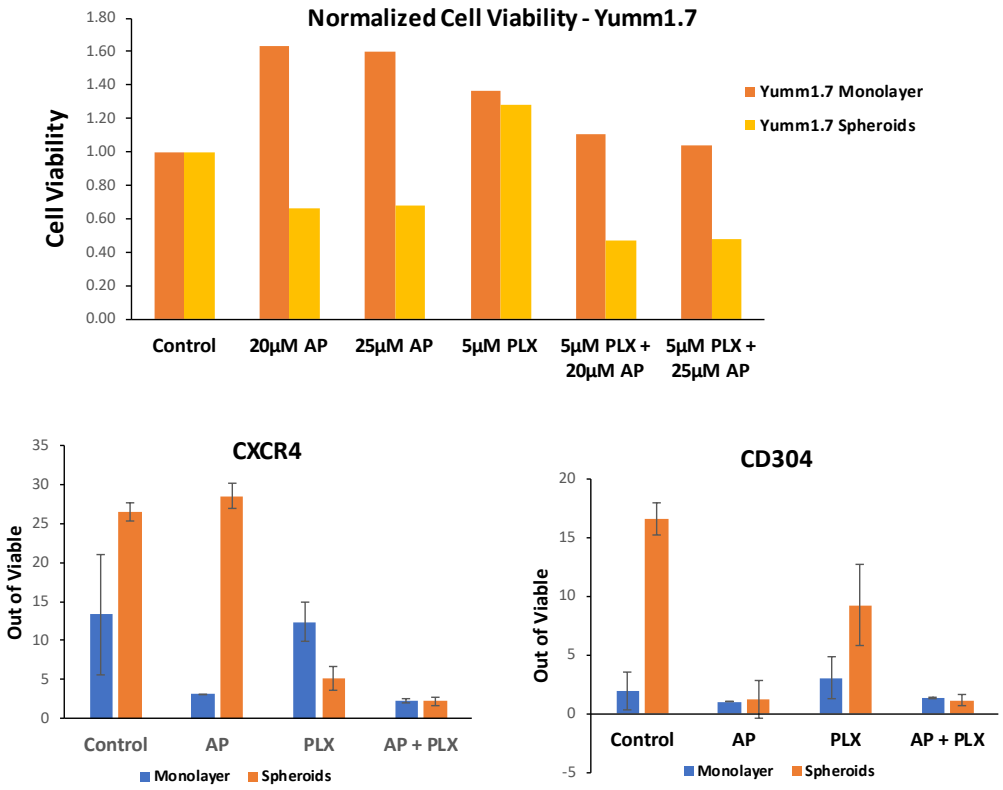


Figure 5. Melanoma spheroid cultures enriched for stem cells are more sensitive to ***AP*** than monolayer cultures. $BRAF^{V600E}$ mutant YUMM1.7 melanoma cells were seeded at 6×10^3 cells in each well of 24-well NanoCulture plates. When spheroids were formed on day 3, the 3D cultures of spheroids were treated with PLX4720 (5 μM) and/or ***AP*** (20 and 25 μM). 2D monolayer cultures of YUMM1.7 melanoma cells were also treated with PLX4720 (5 μM) and/or ***AP*** (20 and 25 μM). After drug treatment for 48 h, the viability of spheroids and monolayer cultures was assayed using the CellTiter-Glo Luminescent Cell Viability Assay. The percent cell survival in each treatment group was calculated relative to cells treated with medium only under the same conditions. As controls, the growth of cells without drug treatment under each condition was normalized as 100% separately. Monolayer and spheroid cultures were also analyzed by flow cytometry for expression of the stem cell markers CXCR4 and CD304.

Since $CXCR4^+$ cancer stem cells are thought to be chemo-resistant and to give rise to more invasive and metastatic tumors, we tested the effect of ***AP*** on invasiveness of YUMM1.7 melanoma cells across a Matrigel-coated filter using the CXCR4 ligand, SDF-1α, as the chemoattractant. Very low, non-cytotoxic concentrations of ***AP*** significantly inhibited the number of YUMM1.7 cells invading through a Matrigel-coated filter (Fig. 6). Whereas SDF-1α-induced invasion was inhibited by ***AP***, there was no effect of ***AP*** on MMP-2, MMP-9, CXCR4, or CXCR7 (another SDF-1α receptor) expression in the melanoma cells suggesting that ***AP*** can also block CXCR4-SDF-1α chemotaxis as does the known CXCR4 inhibitor AMD3100. Interestingly, CXCR4 is also highly expressed on pro-tumorigenic M2-like macrophages. We found that ***AP*** blocked the chemotactic effect of SDF-1α on macrophages as well (Fig. 7). Thus, in addition to targeting the PTS, ***AP*** appears to also block SDF-1-mediated migration of $CXCR4^+$ leukocytes as well as SDF-1-mediated invasiveness of $CXCR4^+$ melanoma cells.

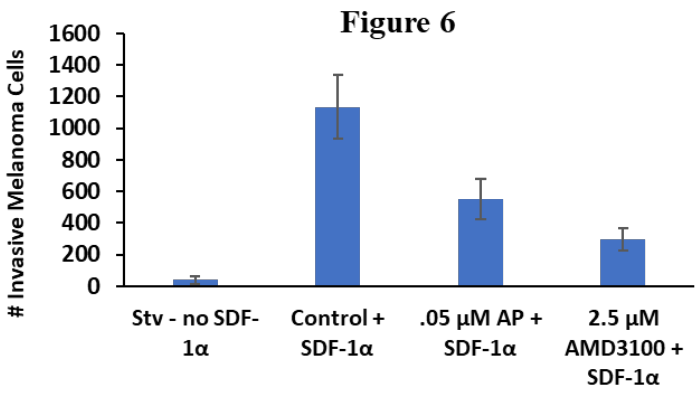


Figure 6. YUMM1.7 melanoma cells (1 x 10^5) were plated with or without 0.05 µM ***AP*** or 2.5 µM AMD3100 on a Matrigel-coated filter of a Boyden chamber with SDF-1α (200 ng/ml) as the chemoattractant in the bottom well. 24 hr later, non-invaded cells in the top chamber were removed with a cotton-tip swab and the invasive cells on the filter underside were fixed with formalin, stained with crystal violet, and counted.

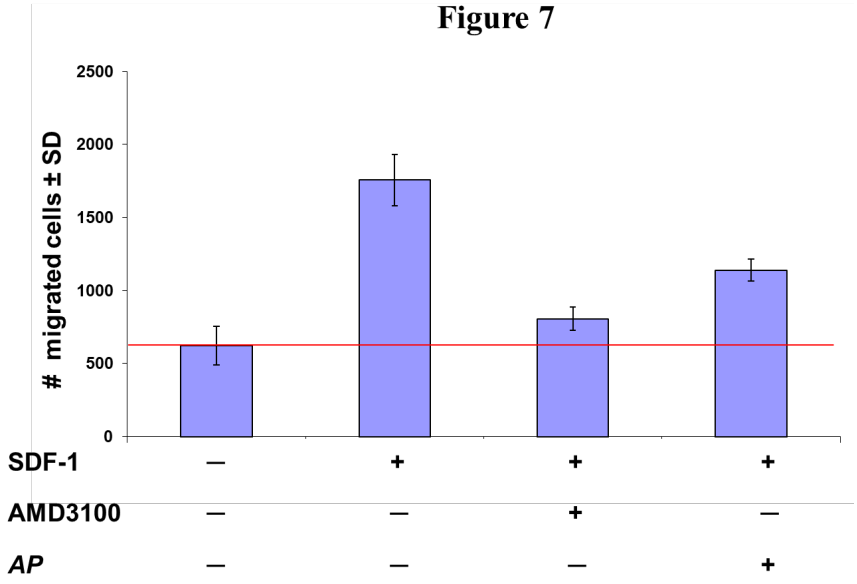


Figure 7. Macrophages were plated with or without 5 µM ***AP*** or 5 µM AMD3100 in the top chamber of a fibronectin-coated 5 µm filter with SDF-1α (200 ng/ml) as the chemoattractant in the bottom well. Three hr later, remaining cells in the top chamber were removed with a cotton-tip swab and the migrated macrophages on the filter underside were fixed with formalin, stained with crystal violet, and counted.

Major Task 4: To evaluate to what extent ***AP*** will block macrophage-mediated resistance of melanoma cells to treatment with BRAF-inhibitors

It has been reported that factors produced by macrophages confer melanoma cell resistance to BRAF inhibition. We have obtained monocytes purified from human donor peripheral blood, and differentiated them to macrophages using human melanoma conditioned medium or by adding cytokines (GM-CSF or M-CSF). We found that PLX4720 induces M2-macrophages (not M1 macrophages) to produce VEGF (Fig. 8), and ***AP*** blocks this PLX4720-induction of VEGF in macrophages (Fig. 9). VEGF stimulates cell growth in melanoma cells by reactivating the MAPK pathway. We then co-cultured BRAF-inhibitor-sensitive WM983B human melanoma cells with differentiated human macrophages seeded on a collagen-coated Transwell filter. Using this macrophage and melanoma cell co-culture system, we confirmed that human M2 macrophages contribute to melanoma tumor cell resistance to the BRAFi, PLX4720, and demonstrated that ***AP*** pre-treatment blocks this macrophage-mediated resistance to PLX4720 in human melanoma cells (Fig. 10).

Fig. 8. PLX4720 Induction of VEGF in Human M2 Macrophages

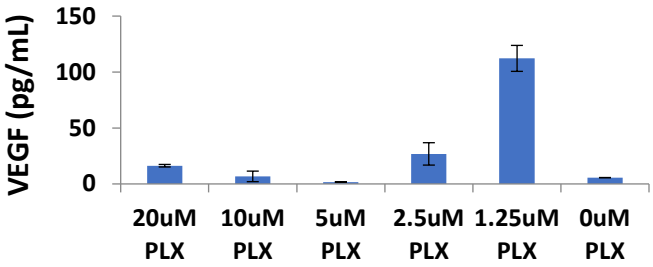


Fig. 9. *AP* Blocks PLX4720 Induction of VEGF in Human M2 Macrophages

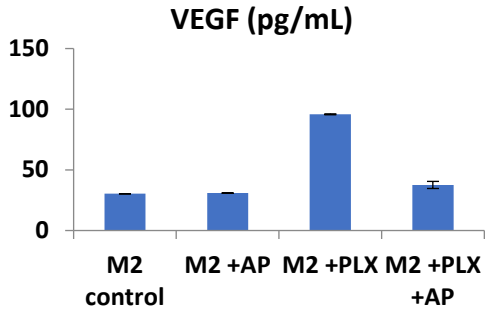


Figure 10

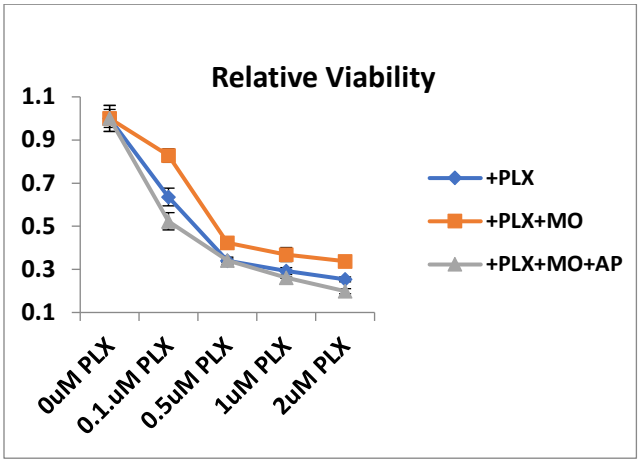


Fig. 10. Macrophage-Mediated Resistance in PLX4720-Treated Melanoma Cells is Blocked with *AP* Pretreatment. BRAF-inhibitor-sensitive WM983B human melanoma cells were co-cultured with differentiated human macrophages (**MO**) seeded on a collagen-coated Transwell filter (pore size: 0.4 µm). Some macrophages were pretreated with 0.1 µM ***AP*** for 16 hr and then refed with medium lacking ***AP*** prior to co-culture with melanoma cells and treating both melanoma and macrophages with increasing concentrations of PLX4720 (**PLX**) for 72 hr. Cell viability of the melanoma cell layer was assessed using an MTS Assay.

Similar results were obtained when co-culturing murine YUMM1.7 melanoma cells with murine bone marrow-derived macrophages (Fig. 11). Interestingly, the macrophage-protective effect on melanoma cells treated with PLX4720 was diminished using macrophages derived from bone marrow of ODCΔMye transgenic mice in which polyamine biosynthesis (ornithine decarboxylase [ODC] is knocked out) is impaired specifically in monocyte/macrophage populations compared to macrophages derived from wildtype mice. With both wildtype and ODCΔMye macrophages, non-cytotoxic ***AP*** pre-treatment of only the macrophages prior to melanoma/macrophage co-culture blocked the macrophage-mediated resistance to PLX4720 cytotoxicity in human melanoma cells. This finding indicates that ***AP*** affects the function of pro-tumorigenic macrophages so they no longer can provide survival factors to the melanoma cells. Interestingly, we found that M2 polarization of macrophages with IL-4 or conditioned medium from YUMM1.7 tumor cells increased their expression of CXCR4. Moreover, M2 polarization of bone marrow derived macrophages (indicated by increased CXCR4 expression and induced arginase activity) was inhibited by ***AP*** (Fig. 12). Our studies with wildtype and ODCΔMye transgenic macrophages showed that both polyamine biosynthesis and uptake are essential for M2 polarization in macrophages (Fig. 13). ODCΔMye macrophages that lack ODC activity are more dependent on polyamine uptake (Fig. 13, bottom panel), and IL-4 treatment leads to less arginase induction in ODCΔMye macrophages compared to wildtype macrophages. Others have found that CXCR4-SDF-1α signaling in macrophages controls not only macrophage recruitment but also upregulates their expression of VEGF and CCL1 (Blood, 117:88-97, 2011). Thus, ***AP*** blocks not only M2 polarization of macrophages but also blocks VEGF secretion from these macrophages, thus decreasing the VEGF survival factor needed to provide PLX4720 resistance in melanoma cells.

Figure 11

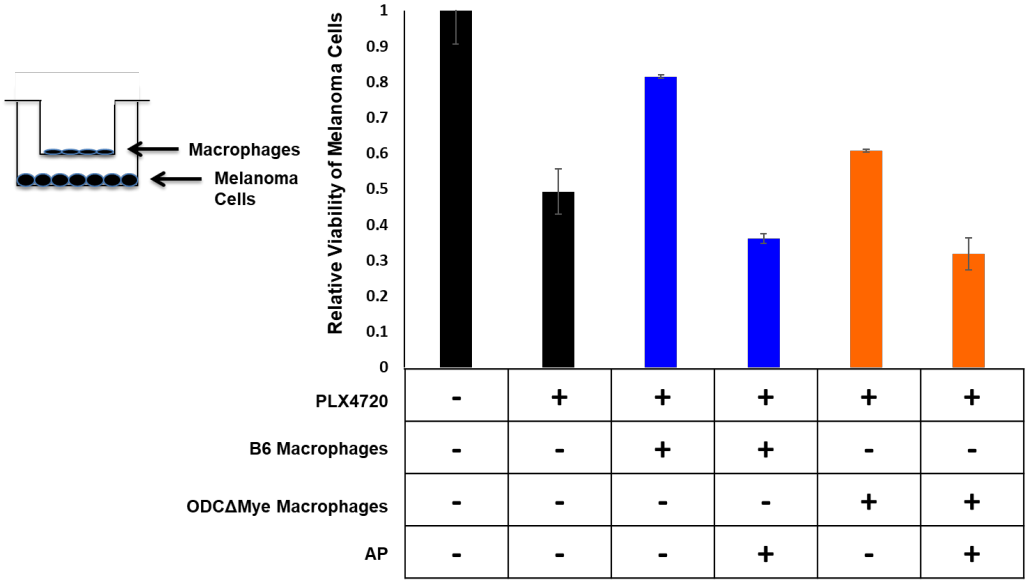


Figure 11. ***AP*** blocks the macrophage pro-survival effect on YUMM1.7 melanoma cells treated with PLX4720. Murine YUMM1.7 melanoma cells (5 x 10^3) were co-cultured with wildtype C57B6 (B6) or ODCΔMye bone marrow derived macrophages (2 x 10^5) that were seeded on a collagen-coated Transwell filter (pore size: 0.4 μm). Some macrophages were pretreated with 0.1 μM ***AP*** for 16 hr and then refed with medium lacking ***AP*** prior to co-culturing the YUMM1.7 melanoma cells in the bottom well of a Boyden chamber and with the macrophages in the insert using medium ± 3 μM PLX4720 for 72 hr. Cell viability of the melanoma cell layer was assessed using a MTS assay.

Figure 12

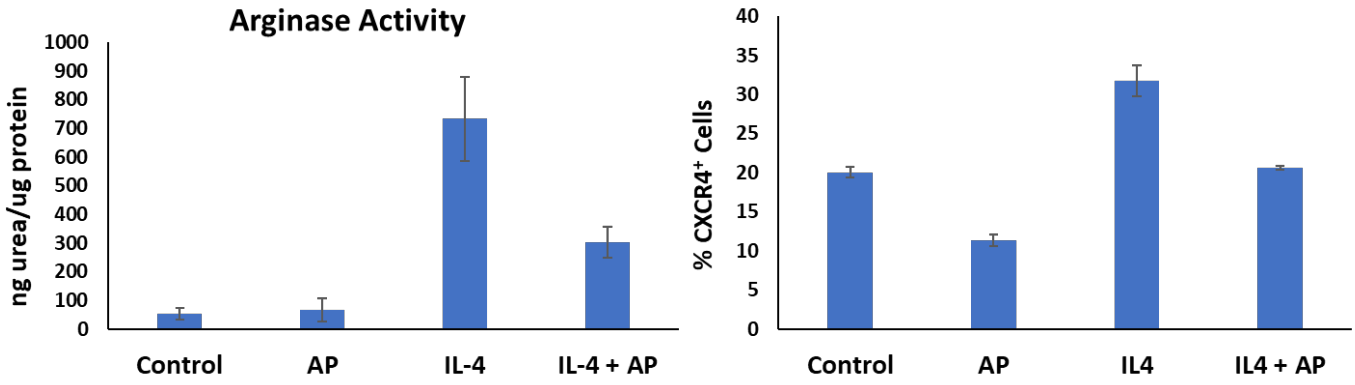


Figure 12. ***AP*** inhibits IL-4 induced M2 polarization of macrophages. Bone marrow derived macrophages from C57Bl/6 mice were polarized with IL-4 ± 0.5 μM ***AP***. As a measure of M2 polarization, macrophages were analyzed for arginase activity and for CXCR4 expression by flow cytometry.

Figure 13

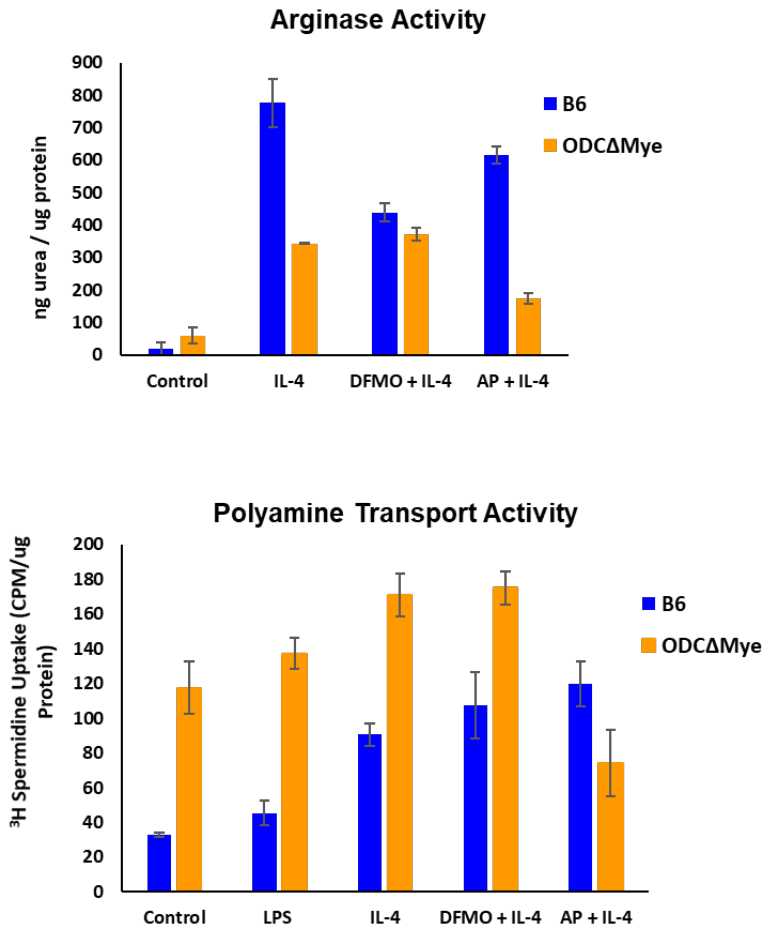


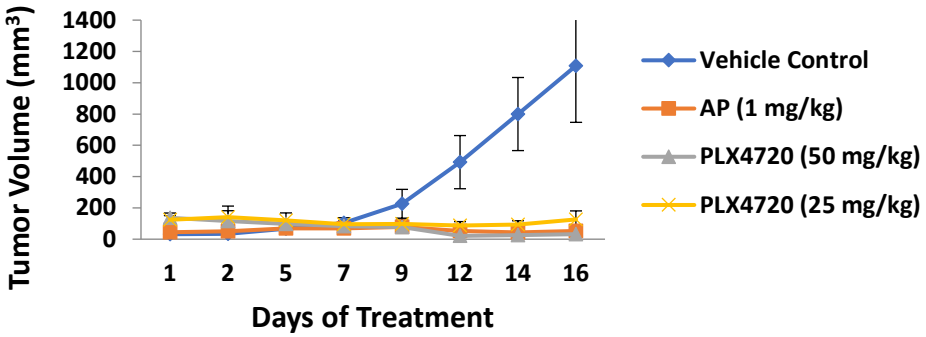
Figure 13. Bone marrow derived macrophages from wildtype C57Bl/6 (B6) and ODCΔMye mice were polarized with IL-4 ± 0.5 μM ***AP*** or DFMO. As a measure of M2 polarization, macrophages were analyzed for arginase activity (top panel). Macrophages were also analyzed for polyamine transport activity (bottom panel). Following 24 hr treatment with IL-4, macrophages were pulsed with 0.5 μM ^{3}H-spermidine for 60 min at 37 °C. Cells were washed with cold PBS containing 50 μM spermidine, and cell lysates were assayed for CPM ^{3}H-spermidine per mg protein by scintillation counting.

Aim #2: To evaluate whether ***AP*** increases the anti-tumor effect of PLX4720 on tumor growth in mice following orthotopic injection of mutant BRAF melanoma cells

The *in vivo* dosing scheme of ***AP*** (also called Me44Nap44Me by my collaborator, Dr. Otto Phanstiel) was optimized for use in murine tumor studies. A similar evaluation was made using another competitive inhibitor of polyamine transport, Trimer44NMe, (see UCF consortium report at the end of this section). The Me44Nap44Me compound (***AP***) is a cytotoxic polyamine compound that enters and kills cells via the polyamine transport system. In contrast, the Trimer44NMe is much less toxic and effectively inhibits the uptake of native polyamines. We have continued to measure blood and tissue levels of these compounds in mice administered ***AP*** or the Trimer44NMe compounds. To summarize work done collaboratively between the Gilmour lab and Phanstiel lab, we observed rapid clearance of ***AP*** (Me44Nap44Me) from the blood after 1 hour when mice, with likely clearance through the kidneys. We found that mice demonstrated no adverse effects (no weight loss and no change in activity or outward appearance) with 2 weeks of daily i.p. injections of ***AP*** (1 mg/kg body weight).

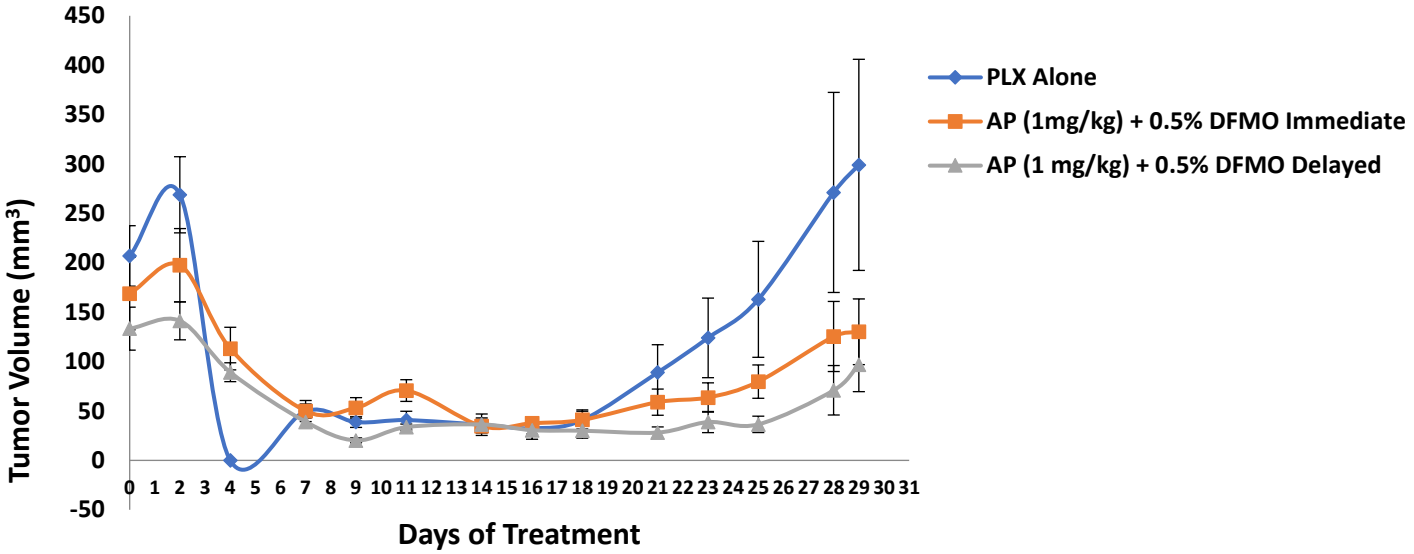
C57Bl/6 mice were then injected s.c. with syngeneic murine mutant BRAF, PTEN-null melanoma cells (YUMM1.7), and mice with established tumor xenografts were treated with ***AP*** (1 mg/kg, i.p., daily) or PLX4720 (25 or 50 mg/kg, oral gavage, bid). As expected, YUMM1.7 tumors rapidly regressed in mice treated with PLX4720. We were excited to see that daily ***AP*** treatment also suppressed tumor growth as effectively as the BRAFi, PLX4720 (Fig. 14). In addition, we observed that tumor-bearing mice that were co-treated with ***AP*** and DFMO did not lose weight as did the mice that were treated with ***AP*** alone. Thus, in the past year, we have treated tumor-bearing mice with both ***AP*** and DFMO in order to maximize the anti-tumor efficacy of ***AP*** and also to alleviate adverse effects from ***AP***. That DFMO co-treatment increases the anti-tumor efficacy and safety of ***AP*** in tumor-bearing mice agrees with our cell culture experiments (Major Task 1) that demonstrated that DFMO upregulates PTS activity in mutant BRAF melanoma tumor cells, thus further increasing uptake of ***AP*** into tumor cells, greater accumulation of ***AP*** in tumor cells, and increased tumor cell death.

Fig. 14. *AP* Treatment Retards Yumm1.7 Tumor Growth in Syngeneic Immunocompetent C57Bl/6 Mice



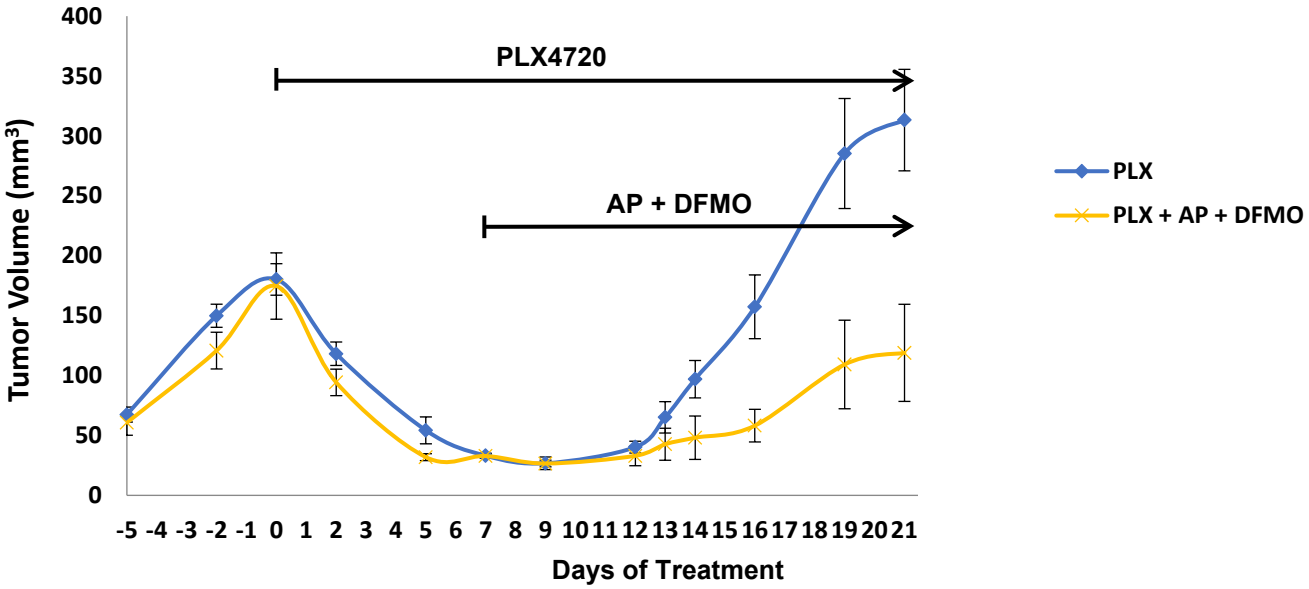
In a follow-up experiment, mice were s.c. injected with $BRAF^{V600E}$ YUMM1.7 melanoma cells, and treatment was initiated with PLX4720 (supplemented in the chow at 417 mg/kg chow) in <u>*all*</u> mice having established tumors. One third of the mice were co-treated with ***AP*** (1 mg/kg, i.p., daily) + 0.5% (w/v) DFMO in the drinking water, *beginning at the same time as initiation with PLX4720*, and one third of the mice were co-treated with ***AP*** (1 mg/kg, i.p., daily) + 0.5% (w/v) DFMO in the drinking water, *beginning 7 days after initiation with PLX4720*. Figure 15 shows that tumors in mice treated with PLX4720 rapidly shrunk in size initially, only to develop resistance to PLX4720 after 2 weeks of treatment and continue to grow. We were able to co-treat mice with both ***AP*** and DFMO without any adverse effects (weight loss) in the mice and significantly slow down the recurrence of PLX4720-resistant tumors. Interestingly, treatment with ***AP*** and DFMO *begun 7 days after initiation with PLX4720* (point at which tumors had regressed the most) delayed the recurrence of tumors better than ***AP*** + DFMO treatment *initiated same time as PLX4720* treatment (Fig. 15).

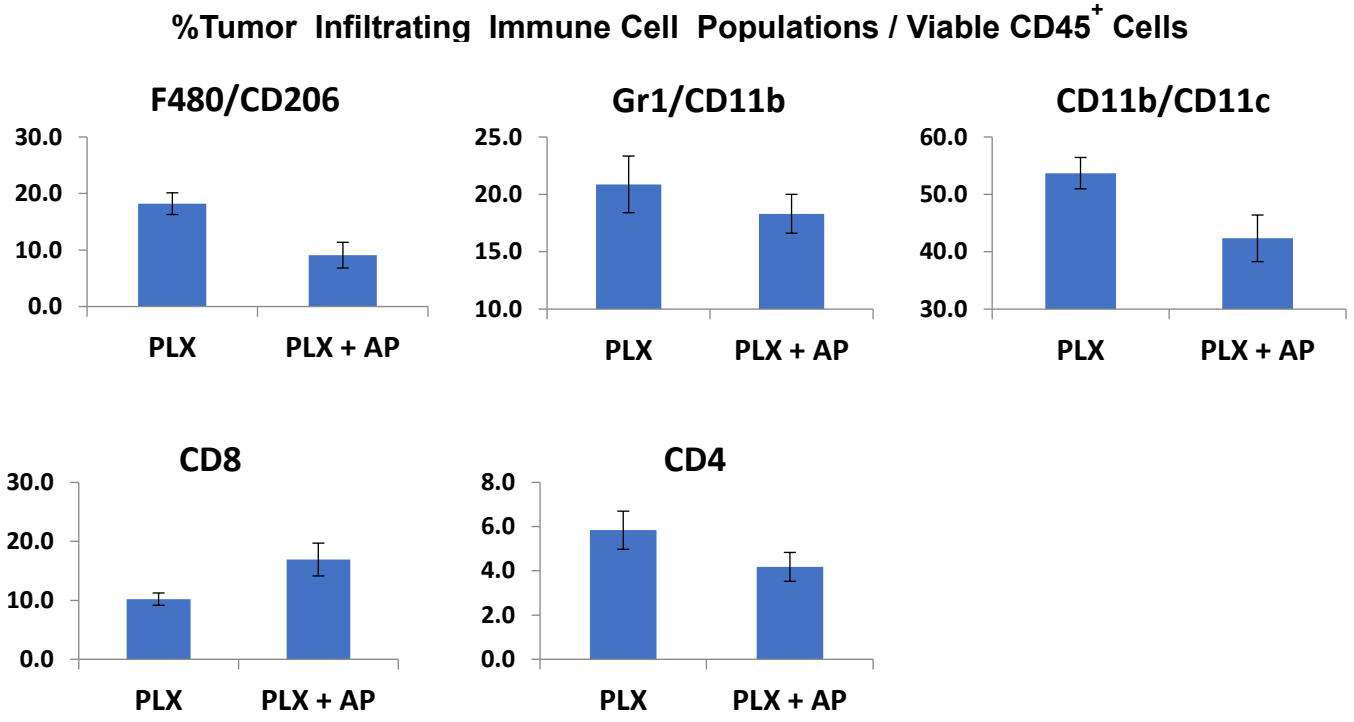
Fig. 15. *AP* ± DFMO Treatment Delays the Recurrence of BRAF^{V600E} Melanoma Tumors That Occurs in Animals Treated with PLX4720 Alone



It has been reported that the initial period of remission produced by BRAFi (PLX4720) treatment provides an opportunity to activate an immune attack that can prevent the recurrence of BRAFi-resistant melanoma. Because we recently showed that combination treatment of DFMO + Trimer44NMe PTI enhances anti-tumor immune response, we predicted that a polyamine blocking therapy with ***AP*** + DFMO may also activate an immune response that would suppress recurrence of aggressive malignant melanoma following treatment with a BRAF inhibitor. We investigated to what extent co-treatment with ***AP*** + DFMO will not only increase the anti-tumor effects of PLX4720 but also diminish infiltrating tumor associated myeloid cells. Figure 16 shows that treatment with ***AP*** and DFMO *begun 7 days after initiation with PLX4720* (point at which Yumm1.7 tumors had regressed the most) significantly delayed the recurrence of the tumors. Flow cytometry analyses of viable

Figure 16. *AP* ± DFMO-Delayed Recurrence of PLX4720-Resistant Melanoma Tumors is Accompanied by a Sustained Tumor Immune Attack

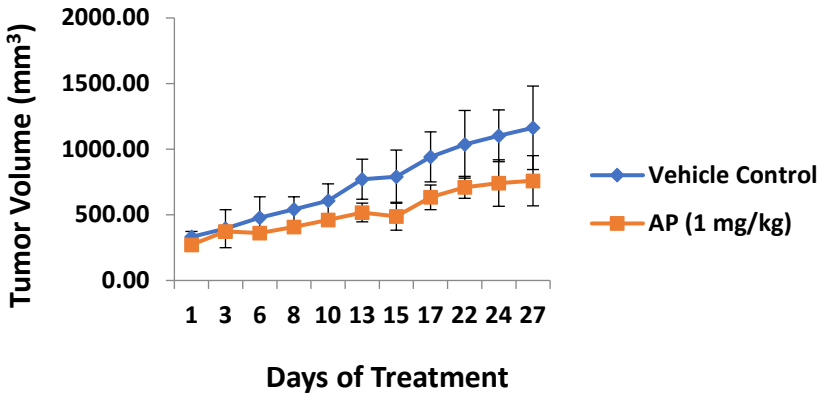




tumor infiltrating immune cell subpopulations revealed that treatment with ***AP*** + DFMO reduced immunosuppressive myeloid populations including $CD206^{+}F480^{+}$ macrophages, $Gr\text{-}1^{+}CD11b^{+}$ myeloid derived suppressor cells, and $CD11b^{+}CD11c^{+}$ dendritic cells, and increased $CD8^{+}$ lymphocytes. These data suggest that treatment with ***AP*** + DFMO in the initial period of remission produced by PLX4720 sustains the initial period of immune attack to delay the recurrence of $BRAF^{V600E}$ Yumm1.7 tumors that occurs in mice treated with PLX4720 alone. The dramatic decrease in tumor infiltrating M2 macrophages ($CD206^{+}F480^{+}$) and delay in recurrence of PLX4720-resistant tumors in ***AP*** + DFMO-treated mice correlates with our previously described finding that M2 macrophage-mediated resistance in PLX4720-treated melanoma cells can be reversed with ***AP*** treatment. These *in vivo* studies suggest that treatment with ***AP*** + DFMO may enhance new immunotherapeutic approaches in treating patients with melanoma resistant to BRAF inhibitors.

We have also tested the anti-tumor efficacy of ***AP*** treatment in athymic nude NCI mice s.c. injected with human WM983B melanoma cells (2×10^{6} cells in 50% Matrigel). We observed that ***AP*** treatment retarded tumor growth in these mice.

Figure 17. *AP* Treatment Retards Growth of Human WM938B Melanoma Xenografts in Athymic Nude Mice



Final report– Activity in Phanstiel Laboratory at University of Central Florida

The Phanstiel lab processed tissue samples for Dr. Gilmour and quantified the levels of the native polyamines as well as the UCF polyamine compounds, trimer44NMe (**1a**, Fig 18) trimer44 (**1b**) MeN44Nap44NMe (referred to as ***AP*** in this report) (**2a**), and 44Nap44 (**2b**). The Phanstiel lab also generated these compounds to support Dr Gilmour's *in vivo* studies at Lankenau.

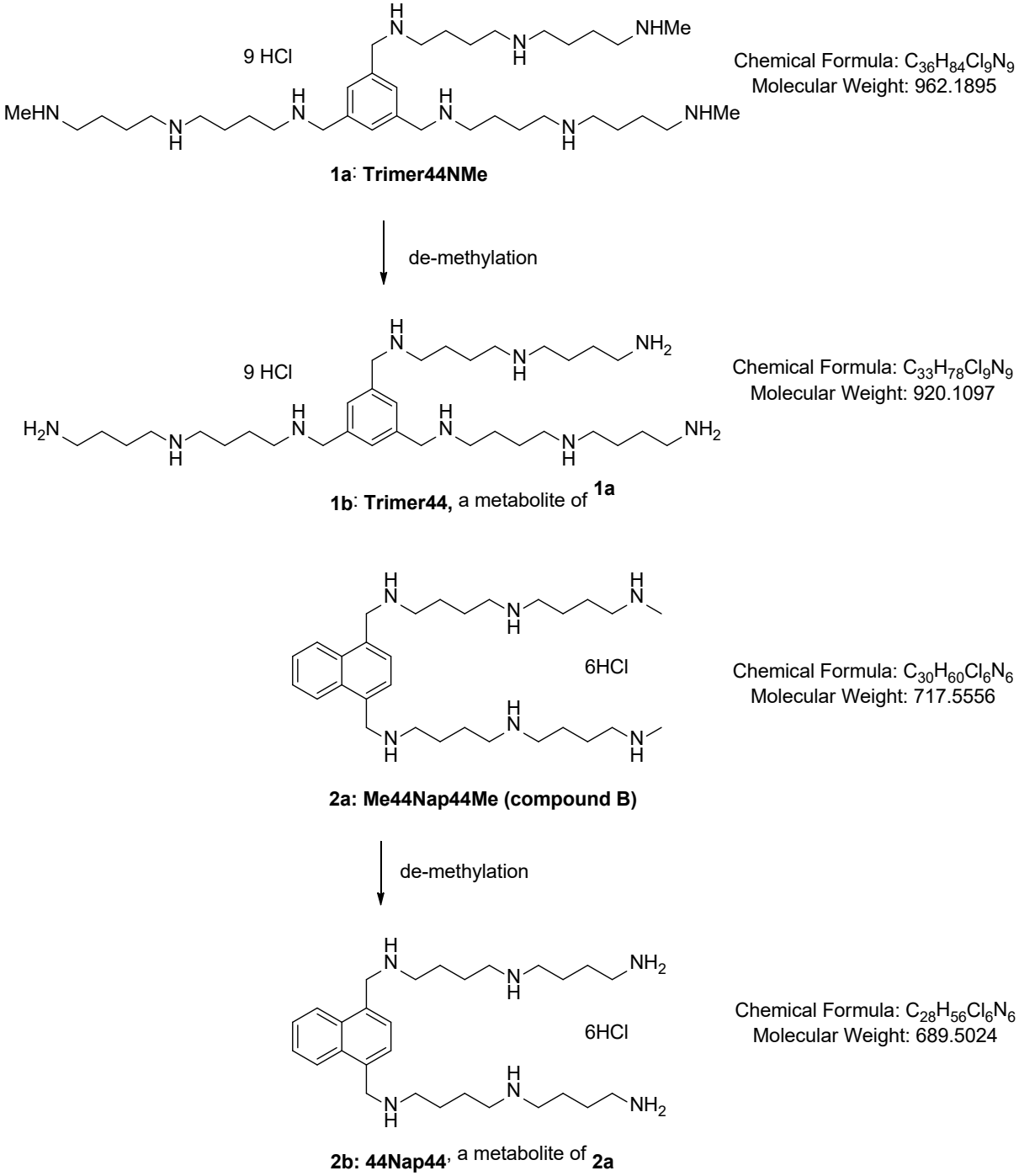


Figure 18. Structures of Trimer44NMe (**1a**) and Me44Nap44Me (***AP***) (**2a**) and their de-methylated metabolites **1b** and **2b**, respectively.

Several polyamine-targeting compounds developed at UCF were tested for their ability to target melanomas in mice at Lankenau Institute for Medical Research (LIMR). Two lead compounds, trimer44NMe (**1a**) and Me44Nap44Me **2a** (Figure 18) were evaluated for their ability to target tumor tissues over other mouse tissues (e.g., spleen, kidney, lung, liver) and for their clearance rates from mouse tissues in order to inform future dosing

regimens. Both compounds are competitive inhibitors of polyamine transport. The trimer44NMe (**1a**, Fig 18) is much less toxic than **2a** and effectively inhibits the uptake of native polyamines. The Me44Nap44Me compound (**2a**) is a cytotoxic polyamine compound that enters and kills cells via the polyamine transport system.

The Phanstiel lab received numerous tissue samples (tumors, spleens, skin, lung, kidney, and liver) from Dr. Gilmour at the Lankenau Institute for Medical Research (LIMR) over the past 3 years. The levels of the native polyamines and the polyamine derivatives **1a, 1b, 2a** and **2b** (Fig 18) were determined using a LC-MS technique and then normalized to protein in the samples. None of the UCF compounds (MeN44Nap44NMe (**2a**) trimer44NMe (**1a**) nor their metabolites **1b** or **2b**) were detected in these tissue samples suggesting that the UCF compounds are efficiently cleared *in vivo*.

Most recently the Phanstiel lab developed a synthetic approach to make extended version of ***AP*** (compound 2a in Scheme 2 of Dr. Phanstiel's report). This was an effort to reduce the toxicity of ***AP*** by removing the nearest nitrogen of the polyamine chain farther away from the naphthalene ring system. Earlier studies with monosubstituted model systems indicated that this strategy will significantly reduce the toxicity of the naphthyl compound.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
I gave a lay presentation concerning my research to members of the Lankenau Women's Board, a dedicated group of women who have raised impressive funds to support research efforts at the Lankenau Medical Center.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The polyamine transport system (PTS) is not well characterized even though we know that it is often upregulated in tumors. We have discovered that PTS activity is increased in melanoma tumor cells possessing a BRAF mutation. We have created a novel drug ***AP*** that is disguised as a polyamine to enter tumor cells that have increased PTS activity, and then kills the tumor cells. In contrast, normal cells have limited PTS activity and will remain unharmed at the low doses of ***AP*** needed to kill the tumor cells. Our work has greatly added to our understanding of the PTS in tumors and how we can harness this transport system to better treat resistant forms of melanoma.

What was the impact on other disciplines?
We have been optimizing techniques to identify how a polyamine-targeting drug (***AP***) can selectively target and kill cancer stem cell populations in melanomas and also possibly reverse the ability of macrophages in the melanoma tumor microenvironment to make melanoma tumor cells resistant to certain chemotherapy drugs. So our work impacts other disciplines such as cancer stem cells and the immune response in tumors.

What was the impact on technology transfer?
We have invented novel polyamine-derived drugs (including ***AP***) that inhibit the uptake of polyamines by virtue of their polyamine tails that compete with normal polyamines for entry into tumor cells via the polyamine transport system (PTS). We have filed a provisional patent that claims that these novel polyamine-derived drugs (including ***AP***) can modulate the immune response to tumors.

What was the impact on society beyond science and technology?

The impact of this project will be a new therapy for melanoma patients that will prevent tumor relapse and improve survival. New medicines provide physicians with new options for patient care.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report.

Changes that had a significant impact on expenditures
Nothing to report.

Significant changes in used or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report.

Significant changes in use or care of human subjects
Nothing to report.

Significant changes in use or care of vertebrate animals
Nothing to report.

Significant changes in use of biohazards and/or select agents
Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations

Journal publications
Peters, M., Minton, A., Phanstiel, O., Gilmour, S. (2018) A novel polyamine targeted therapy for BRAF mutant melanoma tumors, Med. Sci. (Basel), 6(1), pii:E3:10.3390/medsci6010003.

Alexander, E., Mariner K., Donnelly, J., Phanstiel, O., and Gilmour, S., Use of polyamine blocking therapy to decrease survival of tumor-infiltrating immunosuppressive myeloid cells and to enhance the anti-tumor efficacy of PD-1 blockade, Submitted for publication.
Alexander, E., El Naggar, O., Fahey, E., Mariner K., Donnelly, J., Wolfgang, K., Phanstiel, O., and Gilmour, S., Harnessing the polyamine transport system to treat BRAF inhibitor-resistant melanoma, Submitted for publication.

Books or other non-periodical, one-time publications
Nothing to report.

Other publications, conference papers, and presentations

Our work was presented in an invited oral presentation at the following:

- The Immunology/Oncology group at Thomas Jefferson University (2/20/18)
- Roswell Park Cancer Institute, Buffalo, NY (3/29/18)
- Abcam Immunometabolic Adjuvants for Cancer Immunotherapy conference in Baltimore, MD (3/26/18)
- International Skin Carcinogenesis Conference, Austin, TX (10/29/18)

- International Conference on Polyamines: Biochemical, Physiological, and Clinical Perspectives, Taipei, Taiwan (9/6/18)
- Polyamines Gordon Research Conference, Waterville Valley, NH (6/25/19)

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques
Nothing to report.

Inventions, patent applications, and/or licenses
We have invented and characterized novel polyamine-derived drugs (including ***AP***) that inhibit the uptake of polyamines by virtue of their polyamine tails that compete with normal polyamines for entry into tumor cells via the polyamine transport system (PTS). We have filed a provisional patent (joint patent by UCF and LIMR) that claims that these novel polyamine-derived drugs (including ***AP***) can modulate the immune response to tumors.

Other products
We are developing a novel new therapy for melanoma patients that will prevent tumor relapse and improve survival.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Susan Gilmour
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0001-6228-860X
Nearest person month worked:	1 calendar month
Contribution to Project:	Dr. Gilmour developed experimental designs and worked out technical problems, was responsible for all histopathologic analyses, analyzed and interpreted data, wrote all IACUC protocols, directly supervised Eric Alexander in her laboratory, coordinated sharing of resources with the lab of Meenhard Herlyn at the Wistar Institute, coordinated sending blood and tissue samples to the lab of Dr. Otto Phanstiel at the University of Central Florida for analyses of ***AP*** and polyamine levels, wrote progress reports and manuscripts, and prepared meeting presentations.
Funding Support:	No other support provided

Name:	Eric Alexander
Project Role:	Research Assistant Professor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1 calendar month
Contribution to Project:	Dr. Alexander refined the dosing schedule for *in vivo* experiments with ***AP*** and DFMO, performed tumor experiments in mice with ***AP*** + DFMO ± PLX4720, and analyzed tumors for immune infiltrates. He also evaluated melanoma tumor cell survival in macrophage co-culture assays ± PLX4720.
Funding Support:	No other support provided

Name:	Otto Phanstiel

Project Role:	Subrecipient PI
Researcher Identifier:	0000-0001-7101-1311
Nearest person month worked:	0.1 calendar month
Contribution to Project:	Provided training and supervision of the technician
Funding Support:	No other support provided

Name:	Aiste Dobrovolsakite
Project Role:	Technician
Nearest person month worked:	12 calendar months
Contribution to Project:	Processed biological samples received from Lankenau Institute and determined both polyamine levels and levels of the polyamine transport targeting compound
Funding Support:	No other support provided

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes.

Susan Gilmour changes in other support

RECENTLY COMPLETED

1. Title: Polyamine Transport Inhibitors (PTI) as Immunomodulatory Tools to Deplete MDSC
   PI: Gilmour, S.K.
   Time Commitment: 4%
   Supporting Agency: Biostrategy Partners
   Contracting/Grants Officer: Stephen Nappi
   Performance Period: 7/1/18-12/31/18
   Level of Funding: $30,000
   Project's Goals: To starve tumors of polyamines that are essential for their growth and survival, we have developed a new polyamine-blocking therapy (PBT) that includes a combination of 1) α-difluoromethylornithine (DFMO), an FDA-approved drug that specifically inhibits ornithine decarboxylase and 2) a novel polyamine transport inhibitor. A successful outcome of our planned experiments would be if PBT enhances the anti-tumor efficacy of immune checkpoint blockade or chemotherapeutic agent to increase survival of tumor-bearing animals.
   Overlap**:** None - this project investigated the use of a polyamine targeted therapy that is not ***AP***

2. Title: Development of a Novel Therapy for Acute Pancreatitis
   PI: Gilmour, S.K.
   Time Commitment: As needed (No salary is requested for Dr. Gilmour)
   Supporting Agency: The Women's Board of Lankenau Medical Center
   Contracting/Grants Officer: Alice Chase
   Performance Period: 7/1/18-6/30/19
   Level of Funding: $35,000 (direct costs for total grant period)
   Project's Goals: The major goal of this project was to evaluate to what extent inhibitors of polyamine synthesis or hypusine formation can provide protective effects in two models of acute pancreatitis in mice.
   Overlap: None

3. Title: Development of a Novel Therapy for Ovarian Cancer.

PI: Gilmour, S.K.
Time Commitment: 5%
Supporting Agency: Sharpe-Strumia Research Foundation of the Bryn Mawr Hospital
Contracting/Grants Officer: Louise Gethers
Performance Period: 7/1/18-6/30/19
Level of Funding: $37,500 (direct costs for total grant period)
Project's Goals: The major goal of this project was to evaluate to what extent polyamine inhibition can enhance the anti-tumor efficacy of platinum and PARP inhibitors in ovarian cancer.
Overlap: None

PENDING

1. Title: Exploiting Polyamine Dependencies to Treat Resistant Triple Negative Breast Cancer (PI: Gilmour, S.K.)
Time Commitment: 10% effort
Supporting Agency: NIH
Contracting/Grants Officer: N/A
Performance Period: 04/1/2020 – 03/31/2025
Level of Funding: $1,250,000 (direct costs for total grant period, inclusive of consortium costs)
Project Goals: To exploit changes in polyamine metabolism that are downstream mediators of MYC to identify more effective therapeutic approaches for patients with resistant triple negative breast cancer.
Overlap: None

Otto Phanstiel changes in other support

RECENTLY COMPLETED:

1. Title: Development of Antimetastatic Drugs for Pancreatic Cancers (PI: Phanstiel, Otto)
Time Commitment: 8% effort
Supporting Agency: UCF College of Medicine Internal Research Award
Contracting/Grants Officer: Elise Dantuma
Performance Period: 1/1//2018 – 12/31/2018
Level of Funding: $35,000 Total Costs
Project Goals: To develop new antimetastatic medicines for treating metastatic pancreatic cancers.
Overlap: None

PENDING

2. Title: Evaluating novel combinations of polyamine targeting therapeutics in pancreatic ductal adenocarcinoma (Fellowship / Co-Mentor)
Time Commitment: As needed effort
Supporting Agency: U.S. Department of Defense
Contracting/Grants Officer: N/A
Performance Period: 10/1/2020 – 09/30/2022
Level of Funding: $220,895 Total Costs
Project Goals: To elucidate underlying molecular effects of polyamine blockade therapy on the tumor microenvironment and anti-tumor immune response, and to test strategies of immunotherapy to increase survival of pancreatic cancer patients.
Overlap: None

3. Title: Exploiting Polyamine Dependencies to Treat Resistant Triple Negative Breast Cancer (Co-I: Phanstiel, Otto)
Time Commitment: 5% effort

Supporting Agency: Lankenau Institute for Medical Research/NIH Flowthru
Contracting/Grants Officer: N/A
Performance Period: 04/1/2020 – 03/31/2025
Level of Funding: $1,983,050 Total Costs (inclusive of prime costs)
Project Goals of Consortium: To synthesize the two-arm arylpolyamine (***AP***) and to resynthesize more of the **trimer44NMe** polyamine transport inhibitor (**PTI**).
Overlap: None

What other organizations were involved as partners?

Organization Name: University of Central Florida
Location of Organization: Orlando, FL
Partner's contribution to the project: Synthesis of ***AP*** and quantification of levels of native polyamines and ***AP*** in biological samples

8. Special Reporting Requirements
   Annual Report from Otto Phanstiel, Ph.D., UCF Professor of Medicine

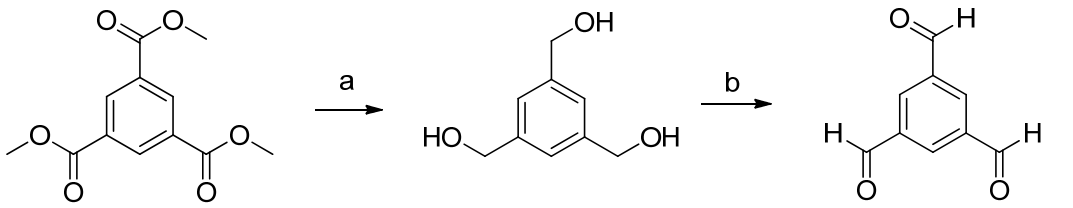
Project 25068008

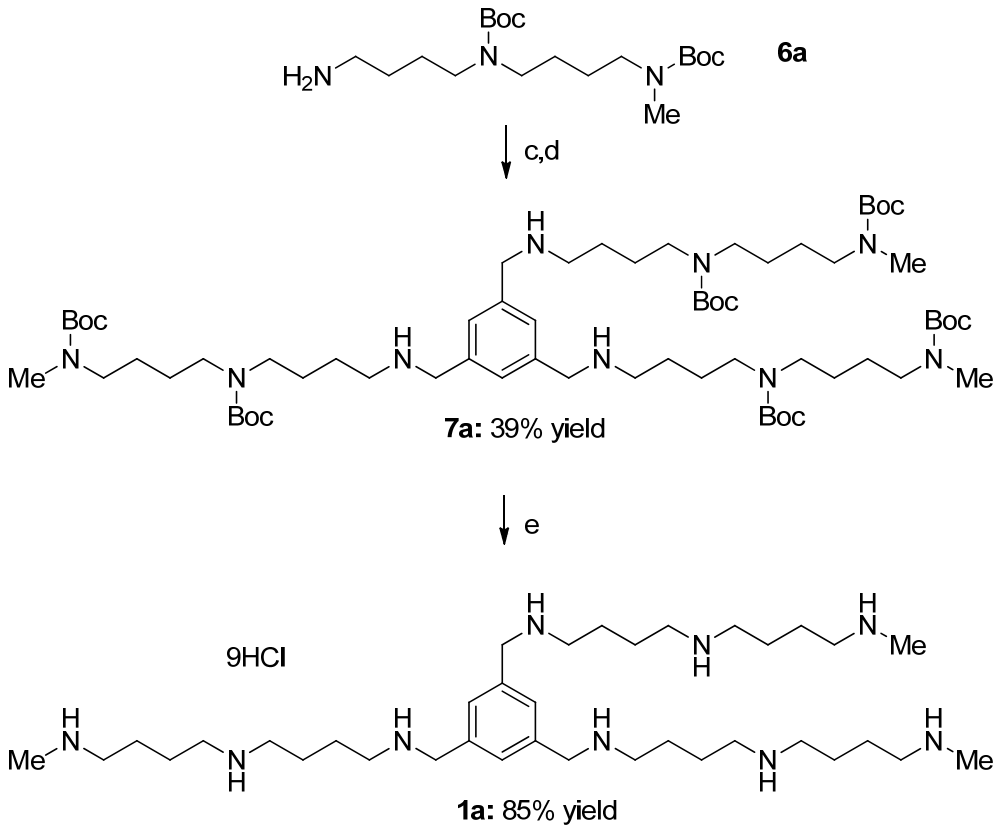
Annual report for 2019 (1 year no cost extension).

This past year we supported the Lankenau team by synthesizing and mailing more of the lead compounds (**1a** and **2a**) for the planned mouse studies.

The trimer44NMe compound (**1a**) was synthesized by the following route in Scheme 1 and sent to Dr. Gilmour to further assist her biological investigations.

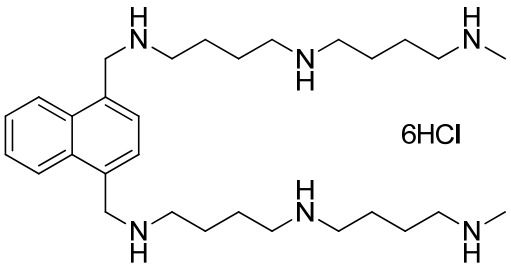
Scheme 1[a]





[a]*Reagents*: a) $LiAlH_4$/THF, b) IBX/*t*-butanol, c) **5** in 25% MeOH/CH_2Cl_2, d) 50% MeOH/CH_2Cl_2/$NaBH_4$, e) 4 *M* HCl/EtOH

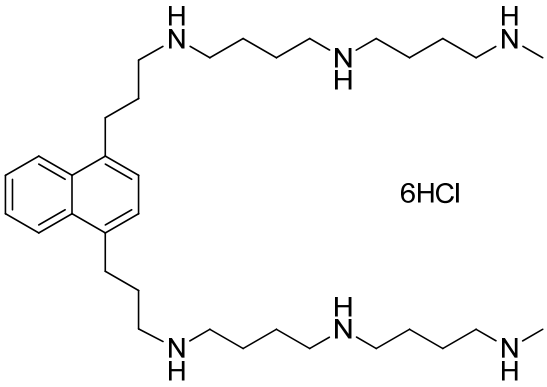
We also prepared and mailed more of the Me44Nap44NMe compound by our published methods (Ref: Muth, A.; Kamel, J.; Kaur, N.; Shicora, A. C.; Ayene, I. S.; Gilmour, S. K.; Phanstiel, O., Development of polyamine transport ligands with improved metabolic stability and selectivity against specific human cancers. *J Med Chem* **2013,** *56* (14), 5819-5828.)



2a: Me44Nap44Me (compound B)

Chemical Formula: $C_{30}H_{60}Cl_6N_6$
Molecular Weight: 717.5556

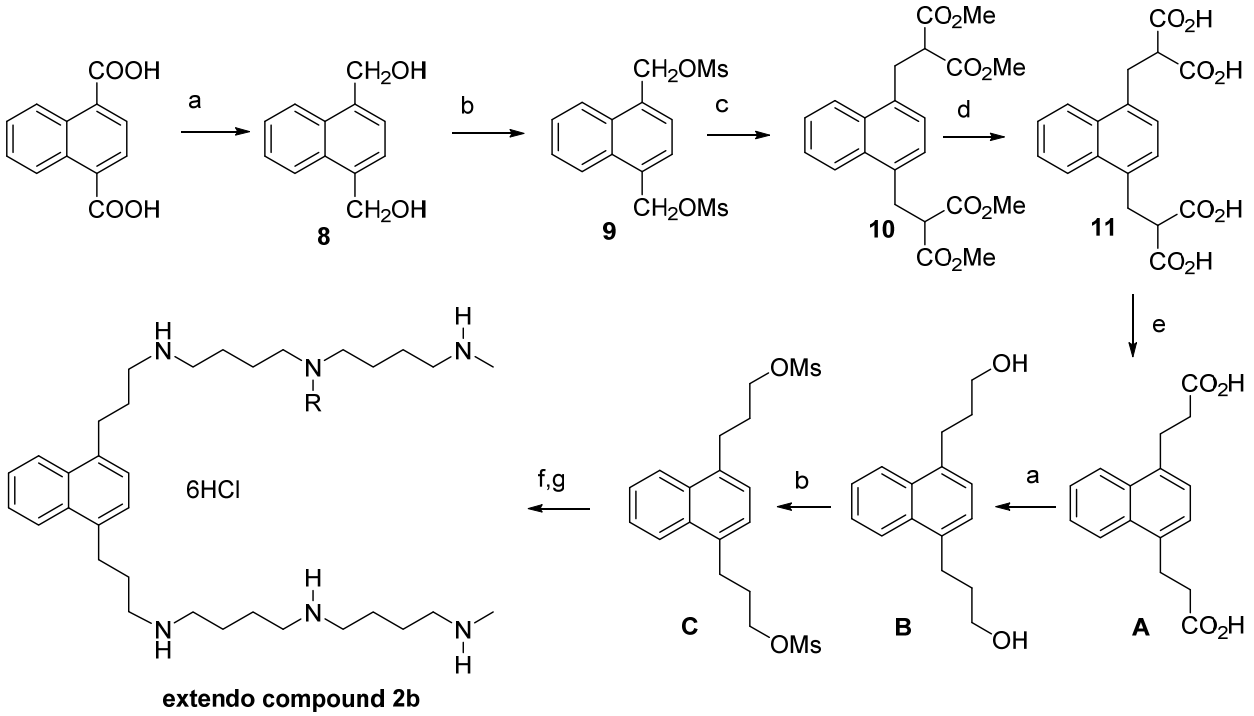
Most recently we also developed a synthetic approach to make extended versions of compound **2a** (Scheme 2). This was an effort to reduce the toxicity of compound **2a** by moving the nearest nitrogen of the polyamine chain farther away from the naphthalene ring system. Earlier studies with mono-substituted model systems indicated that this strategy would significantly reduce the toxicity of the naphthyl compound.



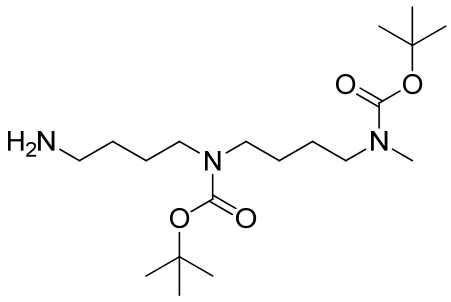
extendo compound 2b

Chemical Formula: $C_{34}H_{68}Cl_6N_6$
Molecular Weight: 773.6619

Scheme 2[a]



[a]*Reagents:* a) $LiAlH_4$, 93% yield (60% yield for reduction of **A**); b) mesyl chloride, 81%,; c) dimethylmalonate, NaH, 75%; d) NaOH, H_2O, MeOH, 90°C, 93%; e) 200° C, 94%; f) *tert*-butyl-(4-aminobutyl)(4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)carbamate (2 equiv), g) 4M HCl



tert-butyl (4-aminobutyl)(4-((*tert*-butoxycarbonyl)(methyl)amino)butyl)carbamate

As shown in Scheme 2, the acid **A** (ref: Costa, D., Fernandes, E.; Santos, J.L.M., Pinto, D.C.G.A., Silva, A.M.S., Lima, J.L.F.C. New noncellular fluorescence microplate screening assay for scavenging activity against singlet oxygen. *Anal. Bioanal. Chem* **2007**, *387*, 2071-2081.) was prepared in several steps and converted to the alcohol **B** in good yield. This diol will next be converted to its corresponding mesylate **C.** We have prepared the polyamine component (i.e., *tert*-butyl(4-aminobutyl)(4-((*tert*-butoxycarbonyl)(methyl)-amino)butyl)-carbamate) needed for the penultimate step in the synthesis of **2b**. Our goal is to have compound **2b** completed in August 2019 for future investigations.

Experimental procedure for the synthesis of 2b (see Scheme 2):

Synthesis of diol 8:

Lithium aluminum hydride (81.0 mmole) was added in portions to ice cooled dry THF (75 mL). A solution of 1,4-naphthalenedicarboxylic acid (23 mmole in 25 mL) in dry THF was added dropwise through syringe. The resulting reaction mixture was refluxed for 15 h. Upon consuming the starting material, the reaction mixture was cooled to 5°C. Cold water (4.0 mL) was added slowly to this ice cooled mixture and stirring was continued for 15 min. Next, 15% aq. NaOH (4.0 mL) was added to form a colorless suspension. The suspension was filtered through a bed of Celite, and the filter cake washed with ethyl acetate (100 mL). The filtrate was evaporated to dryness to form a residue. The residue was re-suspended in water (30 mL) and extracted using ethyl acetate (2 × 70 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated in *vacuo* to give the colorless diol **8** in 93% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.11 (dd, J = 6.5, 3.3 Hz, 1H), 7.55 (dd, J = 6.5, 3.3 Hz, 1H), 7.52 (s, 1H), 4.96 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 137.5, 131.3, 126.0, 124.7, 124.2, 61.7.

Synthesis of mesylate 9:

To an ice cooled solution of diol **8** (5.3 mmole) in THF (20 mL) was added *N,N*-diisopropylethylamine (21.1 mmole) and the solution was stirred for 20 min. Next a solution of methanesulfonyl chloride (15.9 mmole) in THF (5 mL) was charged slowly to the reaction mixture and allowed to stir at room temperature for overnight. After completion of the reaction as evidenced by TLC, water (30 mL) was added and the product extracted using ethyl acetate (2 × 50 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to give mesylate **9** in 81% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.27 – 8.22 (m, 2H), 7.72 – 7.68 (m, 2H), 7.65 (s, 2H), 5.27 (s, 4H), 3.55 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 135.3, 131.5, 128.0, 127.2, 125.2, 70.3, 44.8.

Synthesis of compound 10:

A mixture of dimethylmalonate (33.6 mmole) and sodium hydride (33.6 mmole) in dry THF (25 mL) was refluxed for 3 h. The solution of mesylate **9** (4.0 mmole) in dry THF (10 mL) was added to the reaction mixture over a 15 min period through syringe. The mixture was kept at reflux temperature with stirring for an additional 15 h period. The reaction was quenched by the addition of crushed ice (50 g), water (50 mL) and 1N hydrochloric acid (to adjust to pH ~4). The reaction mixture was extracted with DCM (2 ×100 mL) and dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness to yield a crude solid. The obtained crude compound was triturated in acetonitrile led to pure dimethyl 2,2′-carbmethoxy-3,3′-(1,4-naphthalene) bispropionate **10** (75%). ^{1}H NMR (500 MHz,DMSO-d_6) δ 8.11 (dd, J = 6.5, 3.3 Hz, 2H), 7.60 (dd, J = 6.5, 3.3 Hz, 2H), 7.21 (s, 2H), 3.92 (t, J = 7.9 Hz, 2H), 3.58 (s, 12H), 3.56 (d, J = 7.9 Hz, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.3, 133.3, 131.9, 126.8, 126.6, 124.7, 52.8, 52.4, 31.7.

Synthesis of acid 11.

To a solution of dimethyl 2,2′-carbmethoxy-3,3′-(1,4- naphthalene)bispropionate **10** (1.5 mmole) in methanol (20 mL) was added 12.5% aq. NaOH (8 mL) and the resulting mixture was heated at 90 °C for 1 h. The reaction mixture was then cooled to room temperature and methanol was evaporated to give a residue. Water (40 mL) was added to this obtained residue and the mixture washed with DCM (30 mL). The pH of water layer was adjusted to pH 2 and the product extracted using ethyl acetate (2 × 100 mL). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and evaporated to

dryness to give the pure 2,2'-carboxy-3,3'-(1,4-naphthalene) ispropionic acid **11** (93%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.76 (s, 4H), 8.10 (dd, *J* = 6.5, 3.3 Hz, 2H), 7.60 (dd, *J* = 6.5, 3.3 Hz, 2H), 7.26 (s, 2H), 3.67 (t, *J* = 7.6 Hz, 2H), 3.49 (d, *J* = 7.6 Hz, 4H), 3.25 (s, 1H), 3.17 (s, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 170.7, 133.8, 132.0, 126.7, 126.5, 124.6, 53.0, 31.7.

Synthesis of 3-[4-(2-carboxy-methyl)-naphthalen-1-yl]-propionic acid A:

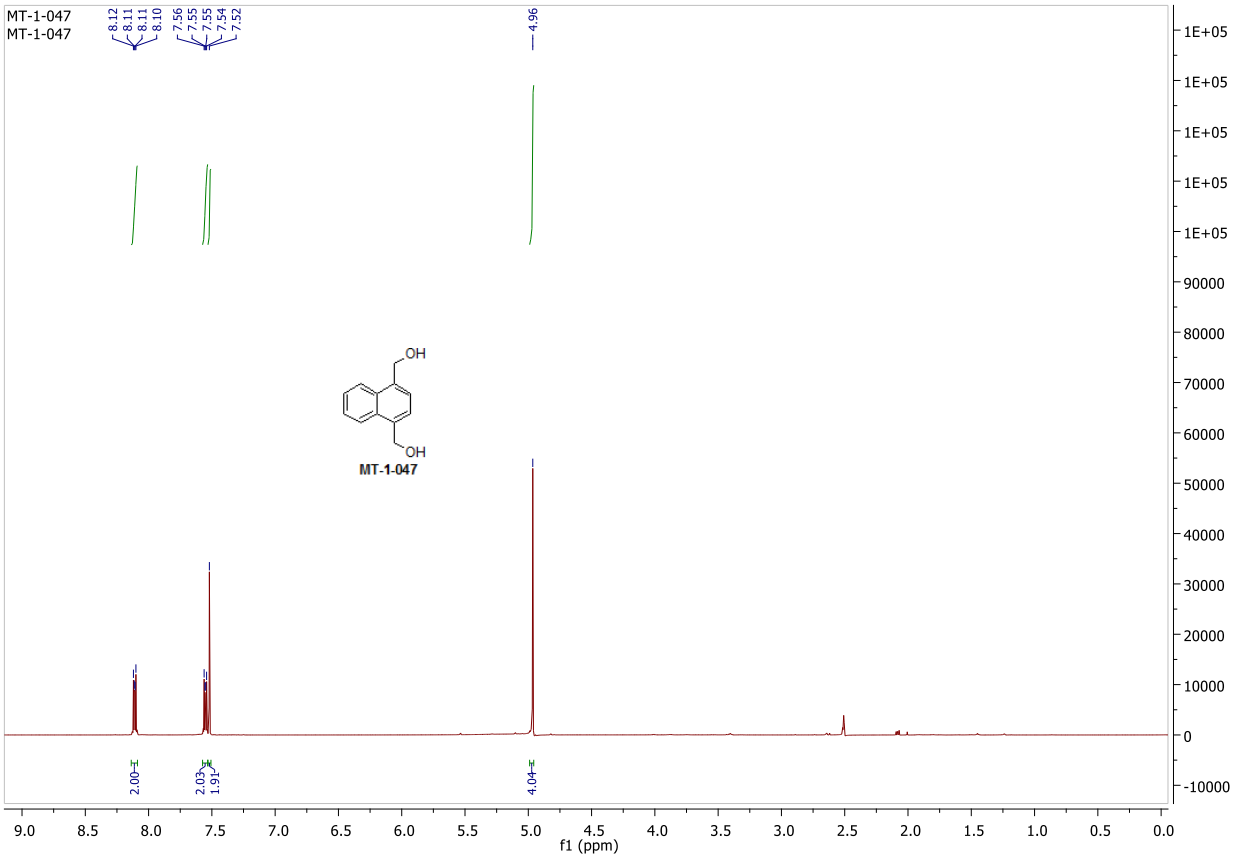
Compound **11** (1.25 mmole) was heated at 200 °C in an oil bath until the weight of the reaction mixture remained constant. An off white solid compound **A** (94%) was obtained and used further without purification. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.24 (s, 2H), 8.17 – 8.12 (m, 2H), 7.66 – 7.61 (m, 2H), 7.35 (s, 2H), 3.33 (t, *J* = 7.7 Hz, 4H), 2.68 (t, *J* = 7.7 Hz, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 174.3, 135.8, 132.0, 126.2, 125.9, 124.7, 35.1, 27.9.

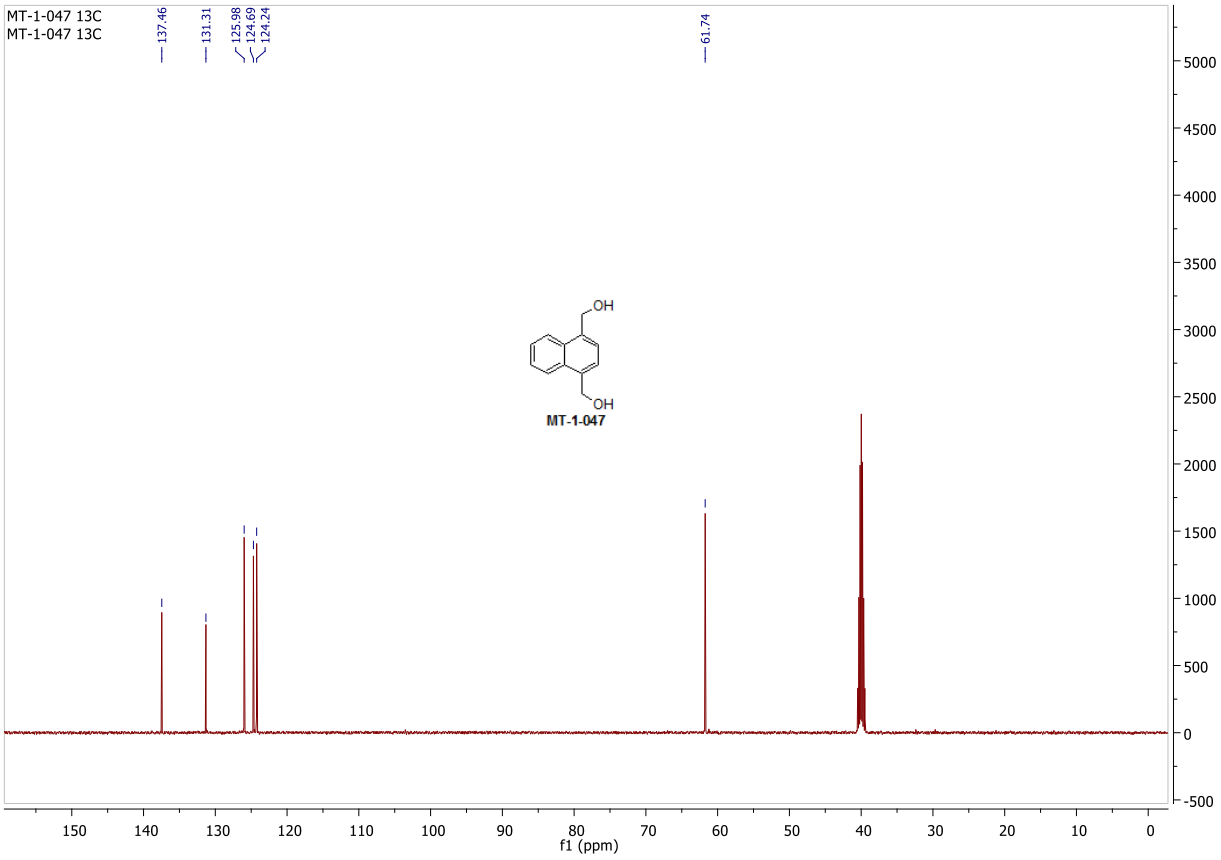
Synthesis of diol B:

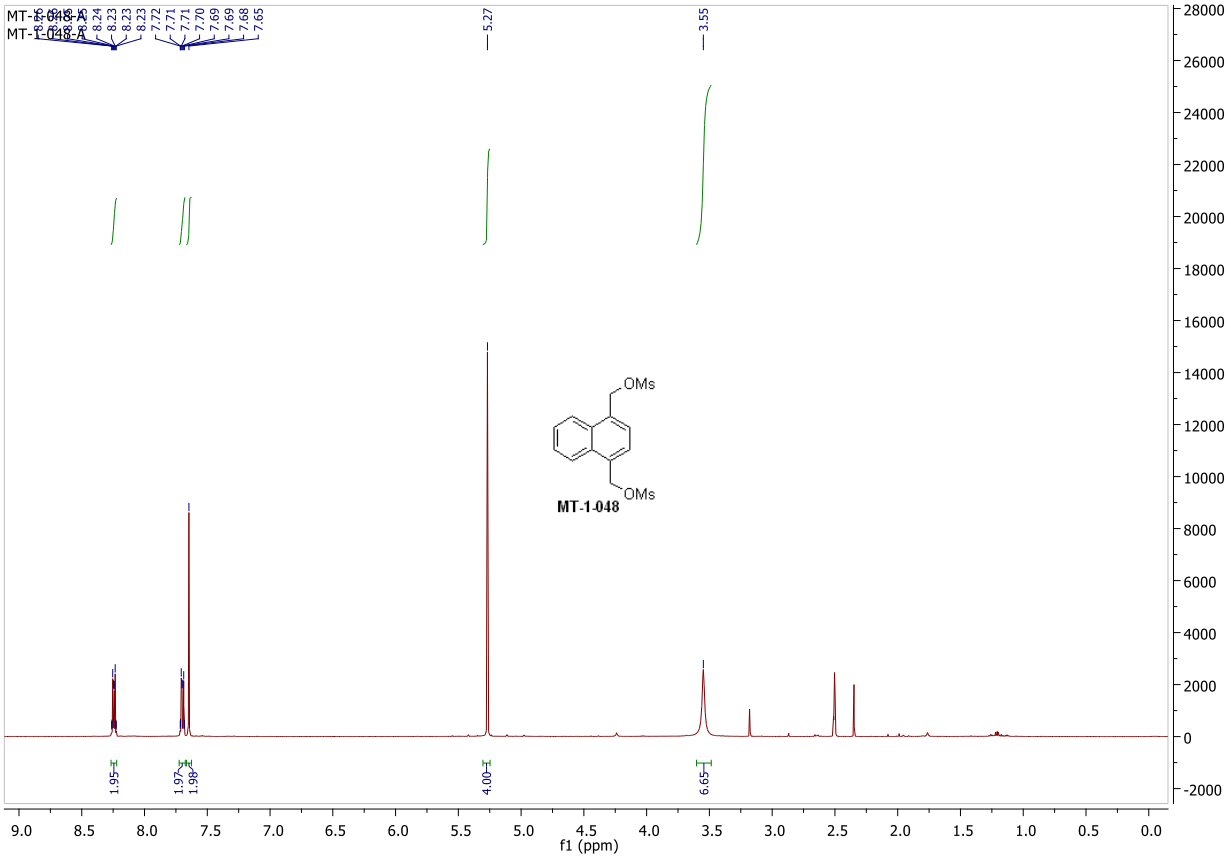
Lithium aluminum hydride (3.8 mmole) was added in portions to ice cooled dry THF (30 mL) followed by the slow addition of acid **A** (1.0 mmole). The reaction mixture was refluxed overnight. Upon completion of the reaction, the mixture was cooled ~5° C, and cold water (0.2 mL) added slowly. The mixture was stirred for 15 min and 15% aq. NaOH solution (0.2 mL) was added to form a colorless suspension. The suspension was filtered through a bed of Celite, and the filter cake washed with ethyl acetate (30 mL). The filtrate was evaporated to give a residue which was re-suspended water (15 mL) and extracted using ethyl acetate (2 × 25 mL). The combined ethyl acetate layer was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to give a crude compound. The crude product was purified through column chromatography using ethyl acetate and hexanes as eluent to give the diol **B** in 60% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.09 (dd, *J* = 6.5, 3.3 Hz, 2H), 7.53 (dd, *J* = 6.5, 3.3 Hz, 2H), 7.26 (s, 2H), 4.55 (s, 2H), 3.50 (t, *J* = 6.3 Hz, 4H), 3.14 – 2.96 (m, 4H), 1.83 –1.77 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 136.9, 132.2, 126.0, 125.8, 124.9, 60.9, 34.3, 29.3.

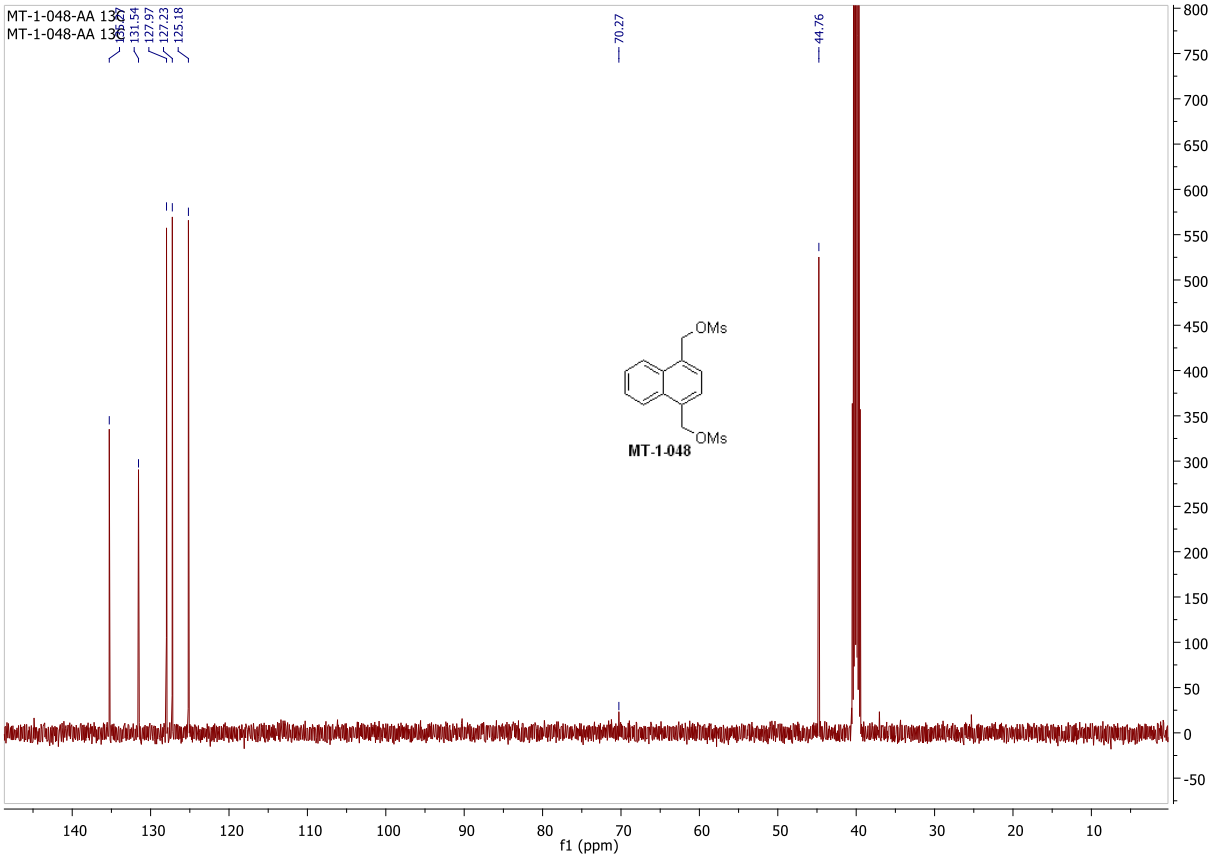
Synthesis of compound C.

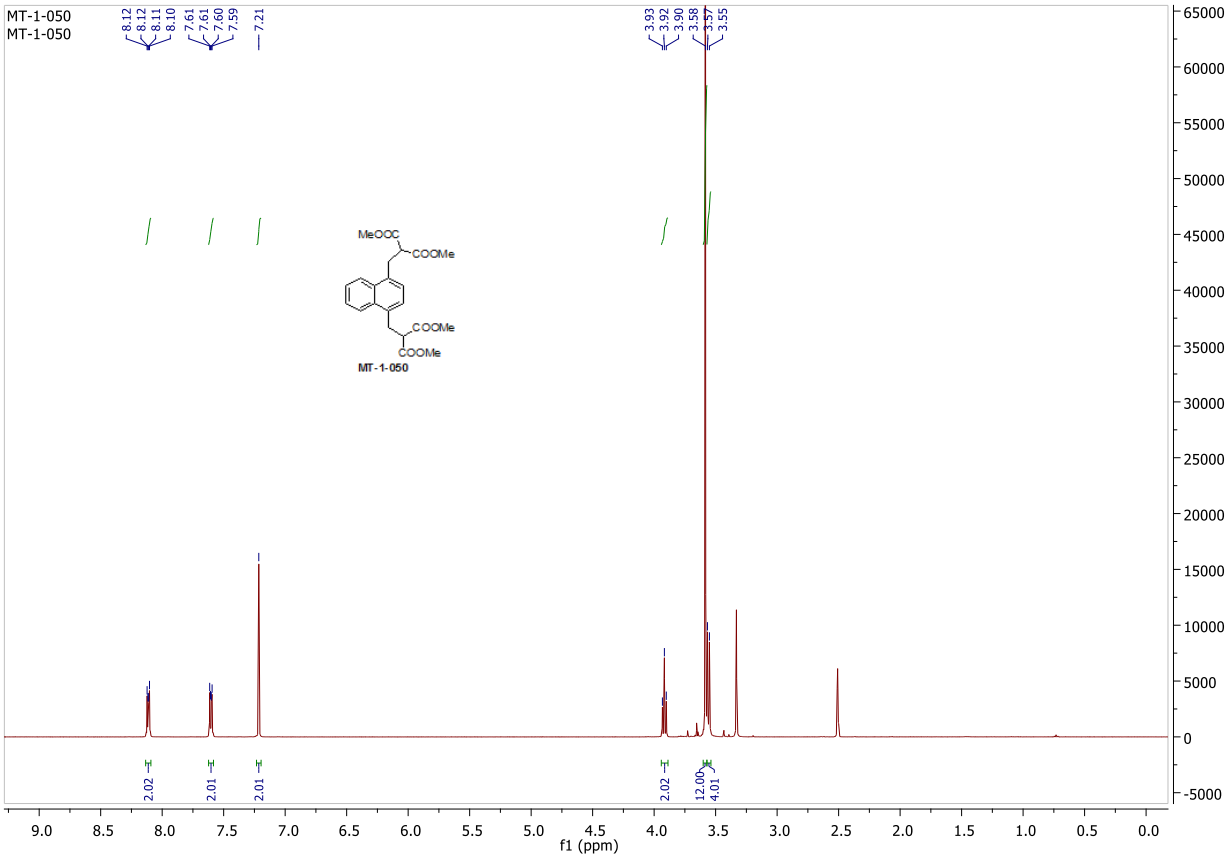
To a solution of diol **B** (0.4 mmole) in THF (10 mL) was added DIPEA (1.6 mmole) at ~5° C and the solution stirred for 20 min. Next, a solution of methanesulfonyl chloride (1.2 mmole) in THF (3 mL) was added dropwise through syringe at ~5° C and allowed to stir overnight at room temperature. After consuming the starting material, water (20 mL) was added and the product extracted using ethyl acetate (2 × 25 mL). The combined ethyl acetate layers were dried over anhydrous sodium sulfate, filtered, and concentrate under reduced pressure to obtain the mesylate compound **C** in 40% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.99 – 7.94 (m, 2H), 7.48 – 7.44 (m, 2H), 7.21 – 7.18 (m, 2H), 4.22 – 4.19 (m, 4H), 3.14 – 3.09 (m, 4H), 2.94 (s, 6H), 2.13 – 2.10 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 135.3, 132.1, 126.1, 125.8, 124.4, 69.5, 37.3, 29.9, 28.7.

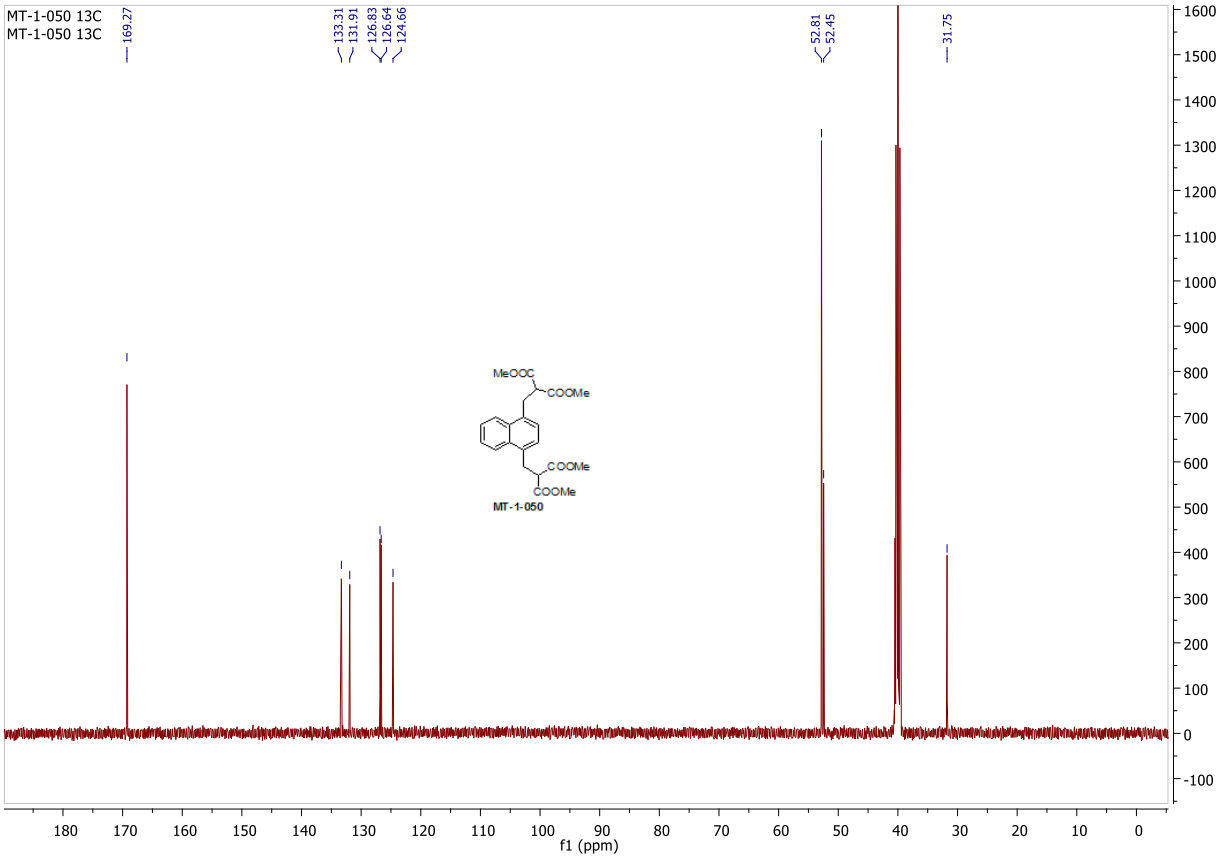

MT-1-047
MT-1-047
8.12
8.11
8.11
8.10
7.56
7.55
7.55
7.54
7.52
4.96
OH
OH
MT-1-047
2.00
2.03
1.91
4.04
f1 (ppm)

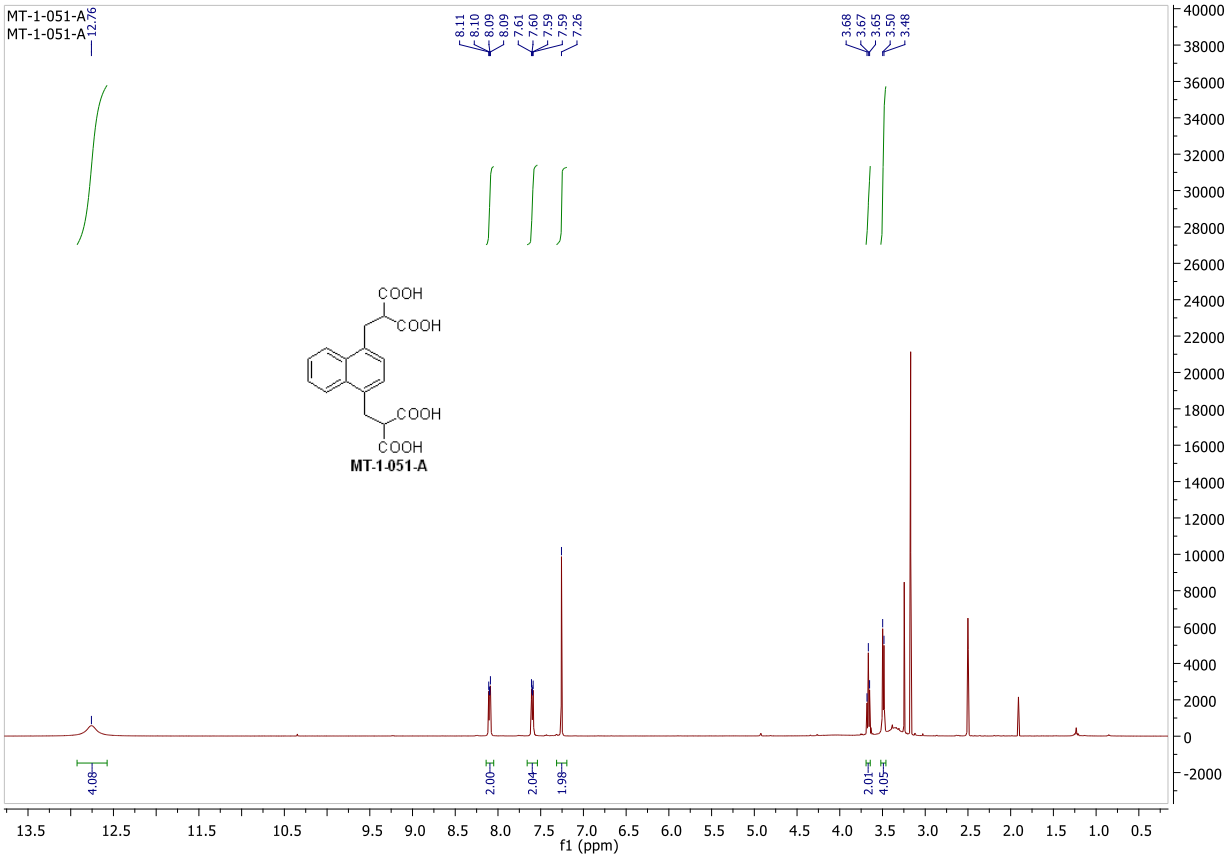

MT-1-047 13C
MT-1-047 13C
137.46
131.31
125.98
124.69
124.24
61.74
OH
OH
MT-1-047
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

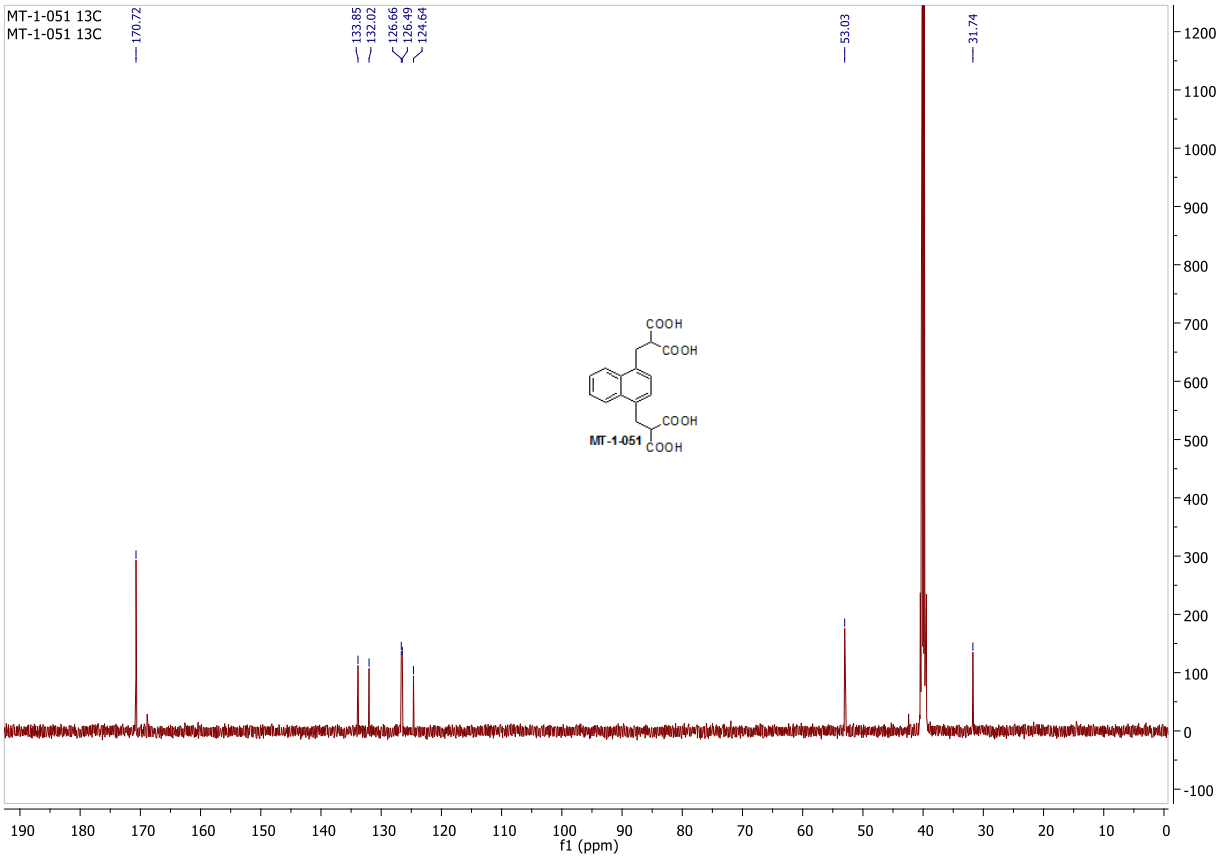

MT-1-048-A
MT-1-048-A
8.24
8.23
8.23
8.23
7.72
7.71
7.71
7.70
7.69
7.69
7.68
7.65
5.27
3.55
OMs
OMs
MT-1-048
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
1.95
1.97
1.98
4.00
6.65
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

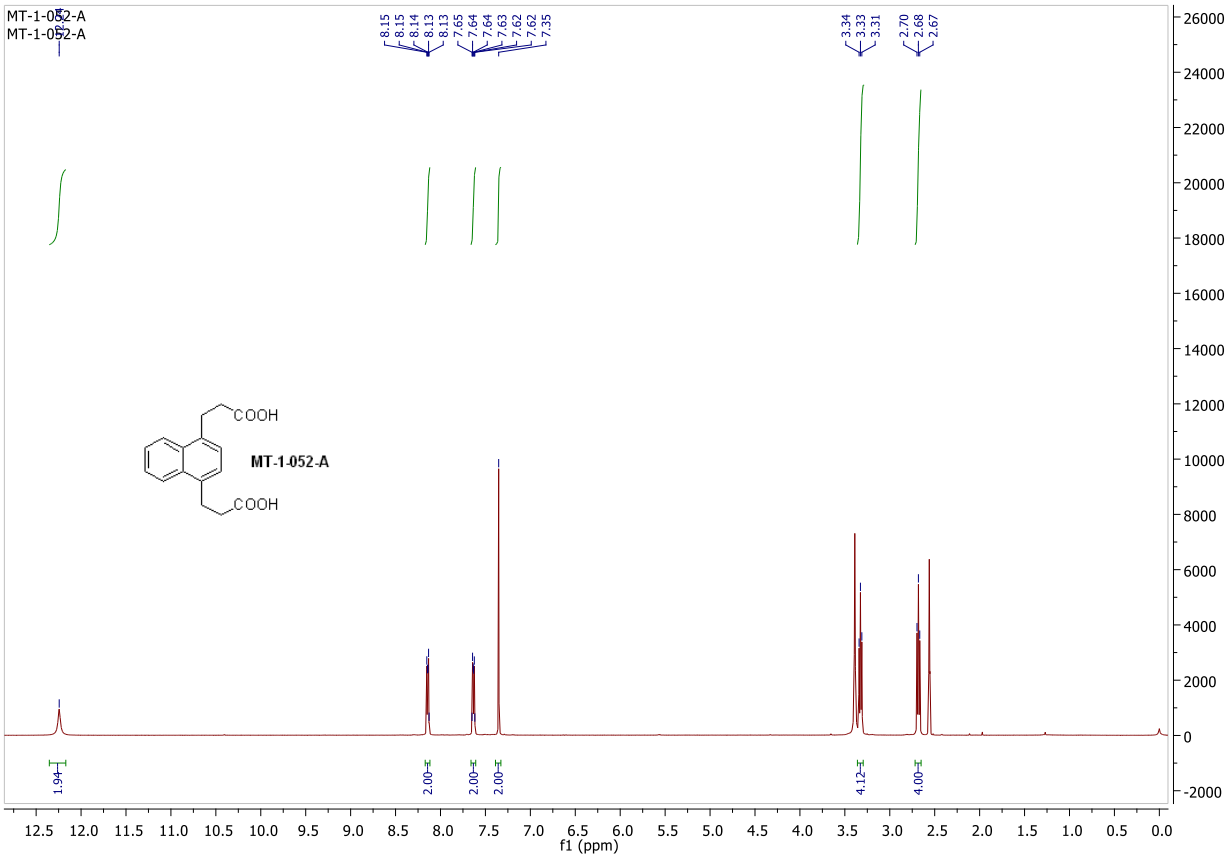

MT-1-048-AA 13C
MT-1-048-AA 13C
131.54
127.97
127.23
125.18
70.27
44.76
OMs
OMs
MT-1-048
f1 (ppm)

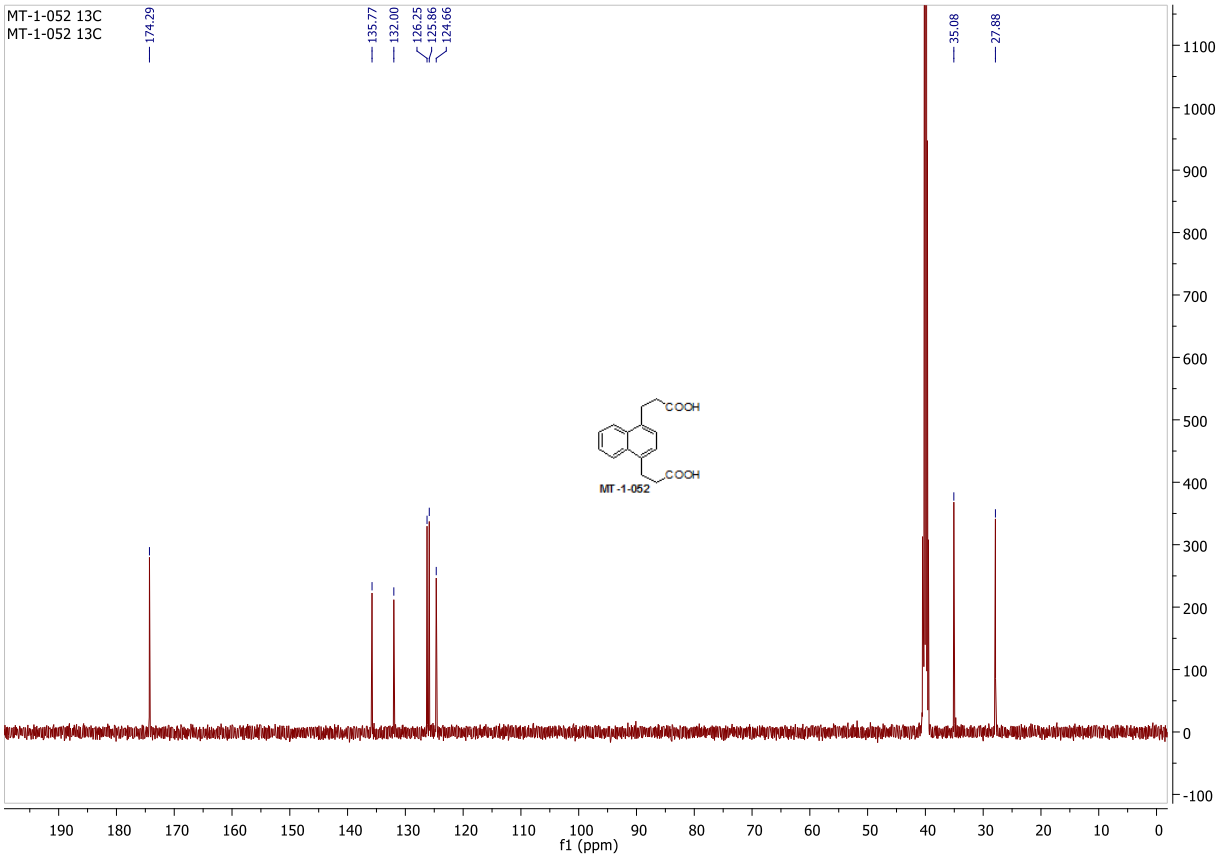

MT-1-050
MT-1-050
8.12
8.12
8.11
8.10
7.61
7.61
7.60
7.59
7.21
3.93
3.92
3.90
3.58
3.57
3.55
MeOOC
COOMe
COOMe
COOMe
MT-1-050
2.02
2.01
2.01
2.02
12.00
4.01
f1 (ppm)

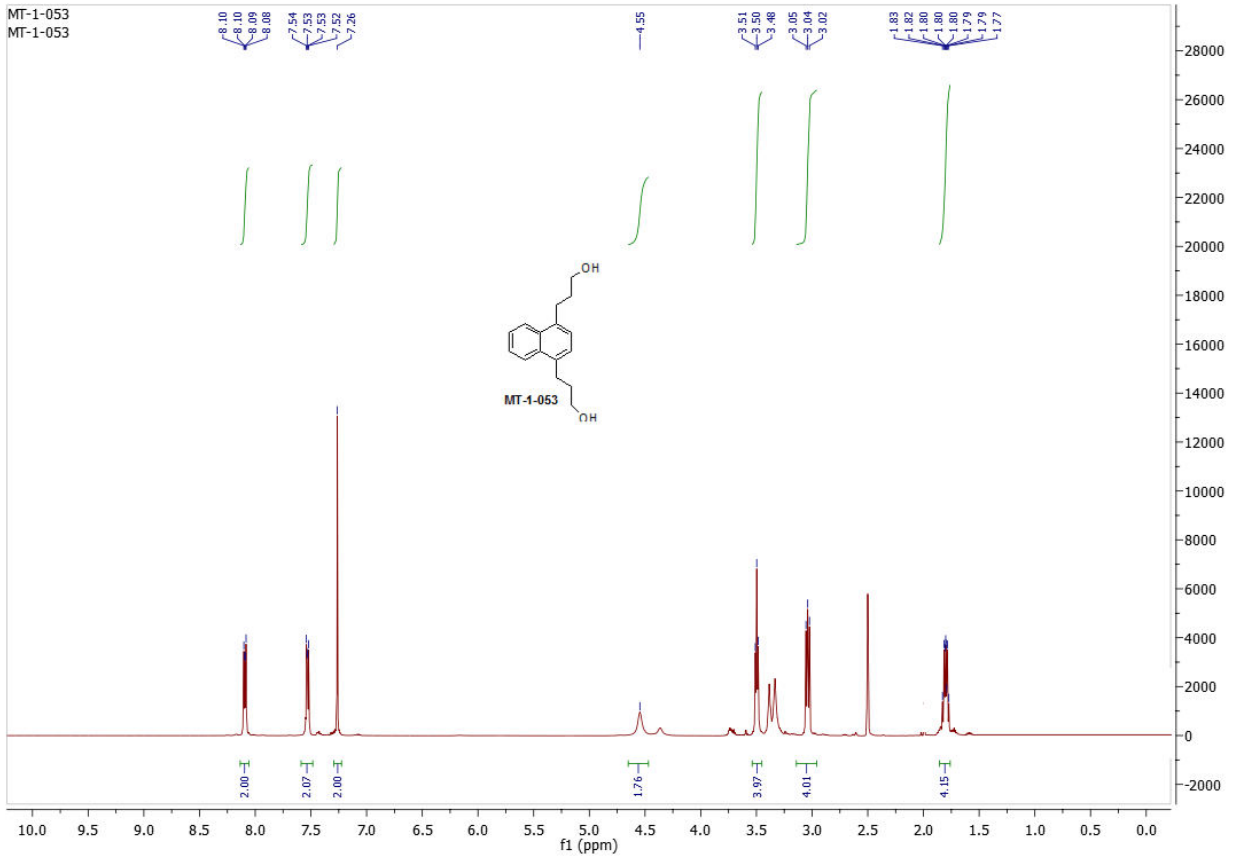

MT-1-050 13C
MT-1-050 13C
169.27
133.31
131.91
126.83
126.64
124.66
52.81
52.45
31.75
MeOOC
COOMe
COOMe
COOMe
MT-1-050
f1 (ppm)

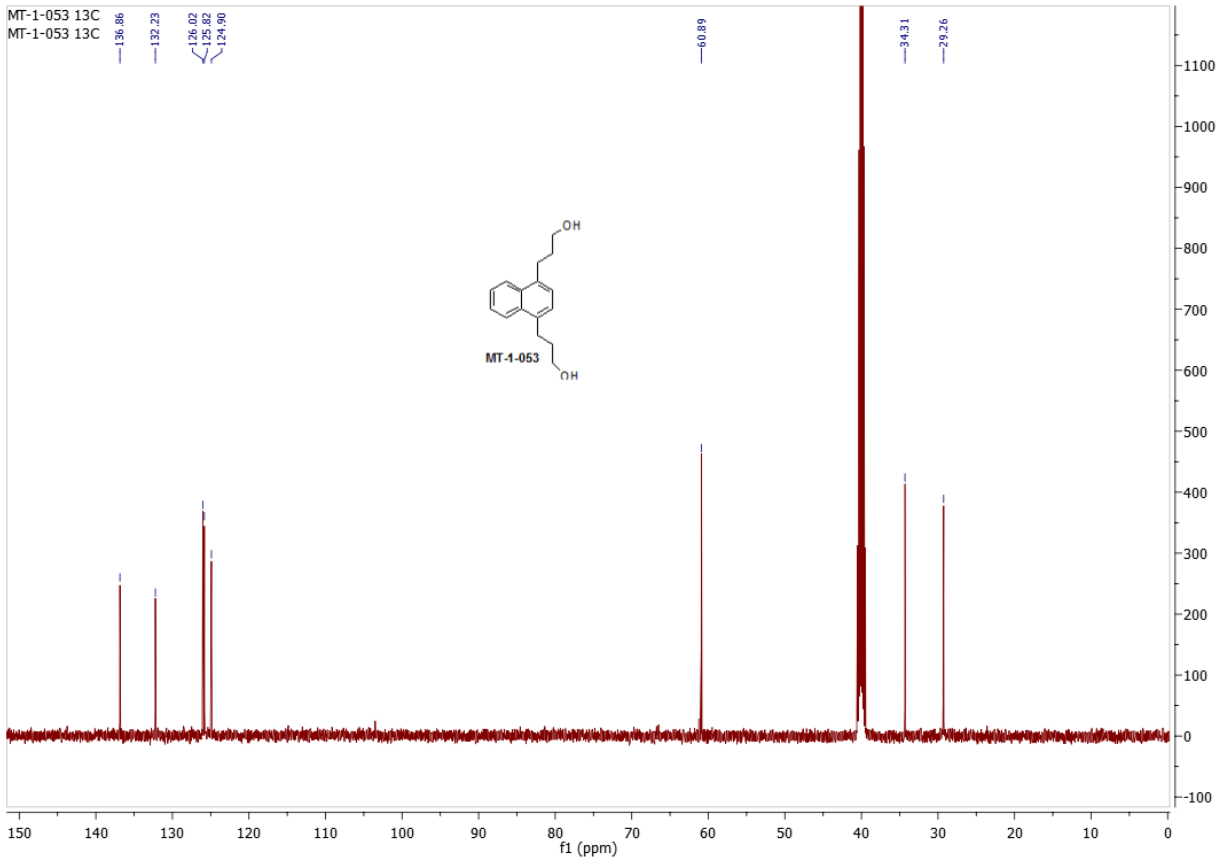

MT-1-051-A
MT-1-051-A
12.76
8.11
8.10
8.09
8.09
7.61
7.60
7.59
7.59
7.26
3.68
3.67
3.65
3.50
3.48
COOH
COOH
COOH
COOH
MT-1-051-A
4.08
2.00
2.04
1.98
2.01
4.05
f1 (ppm)

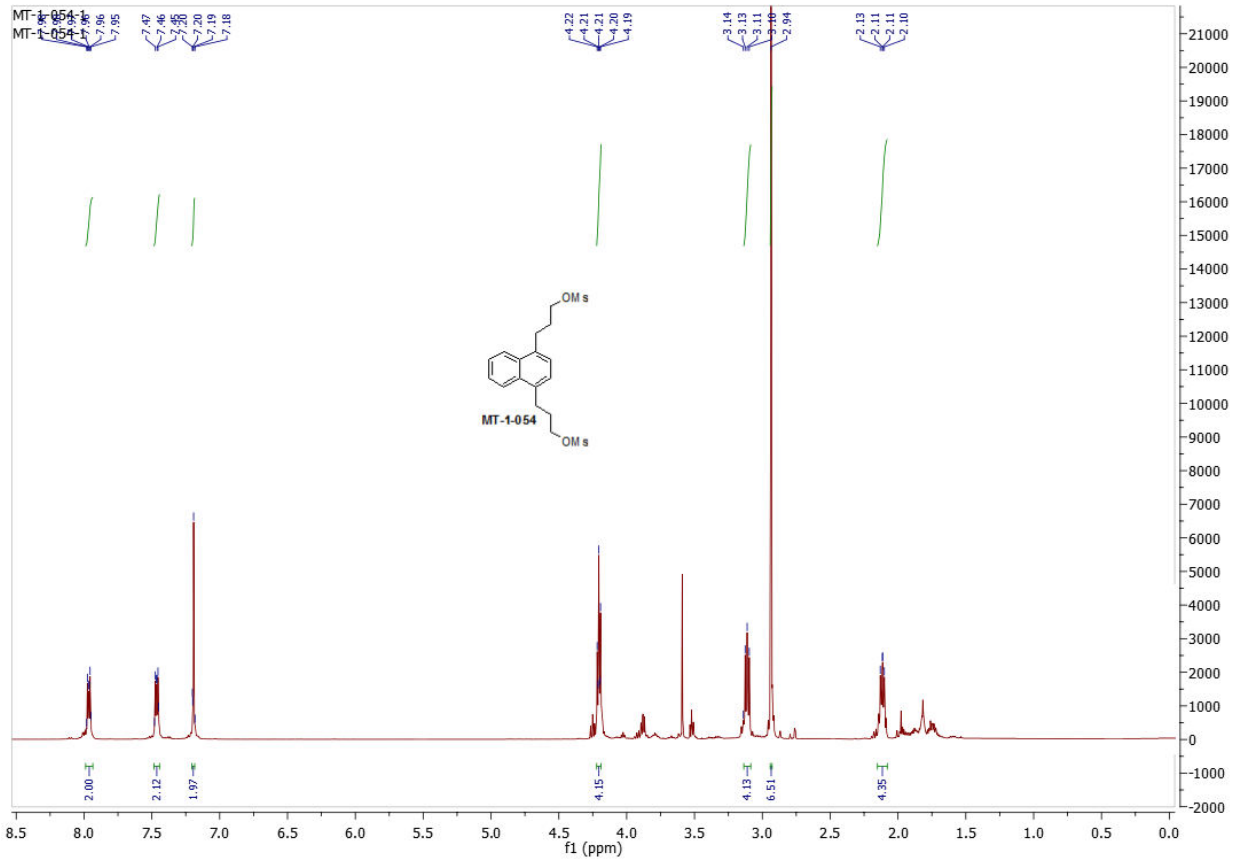

MT-1-051 13C
MT-1-051 13C
170.72
133.85
132.02
126.66
126.49
124.64
53.03
31.74
COOH
COOH
COOH
COOH
MT-1-051
f1 (ppm)

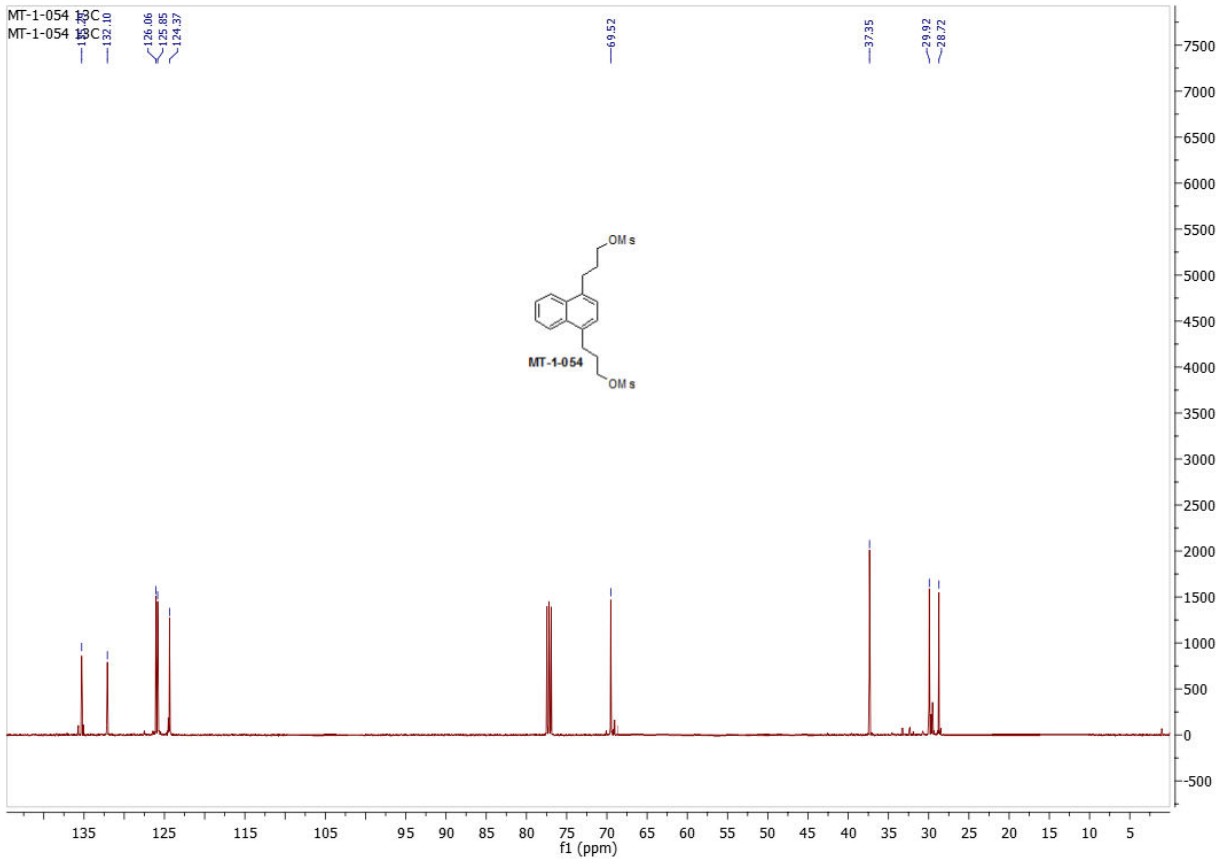

MT-1-052-A
MT-1-052-A
8.15
8.15
8.14
8.13
8.13
7.65
7.64
7.64
7.63
7.62
7.62
7.35
3.34
3.33
3.31
2.70
2.68
2.67
COOH
MT-1-052-A
COOH
1.94
2.00
2.00
2.00
4.12
4.00
f1 (ppm)


MT-1-052 13C
MT-1-052 13C
174.29
135.77
132.00
126.25
125.86
124.66
35.08
27.88
COOH
COOH
MT-1-052
f1 (ppm)


MT-1-053
MT-1-053
8.10
8.10
8.09
8.08
7.54
7.53
7.53
7.52
7.26
4.55
3.51
3.50
3.48
3.05
3.04
3.02
1.83
1.82
1.80
1.80
1.80
1.79
1.79
1.77
OH
MT-1-053
OH
2.00
2.07
2.00
1.76
3.97
4.01
4.15
f1 (ppm)


MT-1-053 13C
MT-1-053 13C
136.86
132.23
126.02
125.82
124.90
60.89
34.31
29.26
OH
MT-1-053
OH
1100
1000
900
800
700
600
500
400
300
200
100
0
-100
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


MT-1-054-1
MT-1-054-1
8.00
7.98
7.96
7.95
7.47
7.46
7.45
7.20
7.19
7.18
4.22
4.21
4.21
4.20
4.19
3.14
3.13
3.11
3.10
2.94
2.13
2.11
2.11
2.10
OMs
MT-1-054
OMs
2.00
2.12
1.97
4.15
4.13
6.51
4.35
f1 (ppm)


MT-1-054 13C
MT-1-054 13C
132.10
126.06
125.85
124.37
69.52
37.35
29.92
28.72
OMs
MT-1-054
OMs
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
135
125
115
105
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
f1 (ppm)

9. **APPENDICES:**

Peters, M., Minton, A., Phanstiel, O., Gilmour, S. (2018) A novel polyamine targeted therapy for BRAF mutant melanoma tumors, Med. Sci. (Basel), 6(1), pii:E3:10.3390/medsci6010003.

medical sciences

MDPI

Article

A Novel Polyamine-Targeted Therapy for BRAF Mutant Melanoma Tumors

Molly C. Peters [1], Allyson Minton [1], Otto Phanstiel IV [2] and Susan K. Gilmour [1,*]

[1] Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood, PA 19096, USA; PetersM@mlhs.org (M.C.P.); Mintonar@msn.com (A.M.)

[2] Biomolecular Research Annex, University of Central Florida, 12722 Research Parkway, Orlando, FL 32826-3227, USA; Otto.Phanstiel@ucf.edu

* Correspondence: GilmourS@mlhs.org; Tel.: +1-484-476-8429

Received: 29 November 2017; Accepted: 28 December 2017; Published: 5 January 2018

Abstract: Mutant serine/threonine protein kinase B-Raf (BRAF) protein is expressed in over half of all melanoma tumors. Although BRAF inhibitors (BRAFi) elicit rapid anti-tumor responses in the majority of patients with mutant BRAF melanoma, the tumors inevitably relapse after a short time. We hypothesized that polyamines are essential for tumor survival in mutant BRAF melanomas. These tumors rely on both polyamine biosynthesis and an upregulated polyamine transport system (PTS) to maintain their high intracellular polyamine levels. We evaluated the effect of a novel arylpolyamine (AP) compound that is cytotoxic upon cellular entry via the increased PTS activity of melanoma cells with different *BRAF* mutational status. Mutant BRAF melanoma cells demonstrated greater PTS activity and increased sensitivity to AP compared to wild type BRAF ($BRAF^{WT}$) melanoma cells. Treatment with an inhibitor of polyamine biosynthesis, α-difluoromethylornithine (DFMO), further upregulated PTS activity in mutant BRAF cells and increased their sensitivity to AP. Furthermore, viability assays of 3D spheroid cultures of mutant BRAF melanoma cells demonstrated greater resistance to the BRAFi, PLX4720, compared to 2D monolayer cultures. However, co-treatment with AP restored the sensitivity of melanoma spheroids to PLX4720. These data indicate that mutant BRAF melanoma cells are more dependent on the PTS compared to $BRAF^{WT}$ melanoma cells, resulting in greater sensitivity to the PTS-targeted cytotoxic AP compound.

Keywords: polyamines; α-difluoromethylornithine; polyamine transport system; melanoma; mutant BRAF

1. Introduction

Melanoma is a highly aggressive tumor with poor prognosis in the metastatic stage. Multiple oncogenic mutations (including genes encoding serine/threonine protein kinase B-Raf (BRAF), the neuroblastoma RAS homolog (*NRAS*), and the proto-oncogene receptor tyrosine protein kinase KIT) drive this highly heterogeneous disease, with mutations in the *BRAF* gene detected in half of all melanoma tumors [1]. The treatment of metastatic melanoma has been revolutionized over the last decade with the discovery of highly prevalent *BRAF* mutations, which drive constitutive activation of the RAS-RAF-MEK-ERK pathway and promote uncontrolled proliferation [1]. Ninety percent of reported *BRAF* mutations result in substitution of glutamic acid for valine at amino acid 600 (the V600E mutation) [2,3]. The subsequent rapid development of selective inhibitors of mutant $BRAF^{V600E}$ proteins (vemurafenib and dabrafenib) demonstrated a major advance in the treatment of melanoma patients harboring the $BRAF^{V600E}$ mutation. However, nearly 100% of the patients exhibit disease progression within seven months after treatment with BRAF inhibitors [4–6]. Thus, new ways

to overcome the acquired resistance to these inhibitors are urgently needed to increase survival in melanoma patients.

An alternative approach is to target a downstream pathway that is essential for survival of oncogene-addicted tumor cells. While oncogenes indeed drive proliferation, they do so via downstream effector molecules. For example, downstream of extracellular regulated kinase (ERK) signaling is c-MYC (myelocytomatosis viral proto-oncogene homolog), a known regulator of ornithine decarboxylase (ODC) transcription and polyamine biosynthesis [7]. The native polyamines (putrescine, spermidine and spermine) are amino acid-derived polycations that have been implicated in a wide array of biological processes, including cellular proliferation, differentiation, chromatin remodeling, hypusination of the eukaryotic initiation factor-5A (eIF-5A) and apoptosis [8]. Multiple oncogene-encoded proteins, including c-MYC and RAS, are known to upregulate key polyamine biosynthetic enzymes [7,9,10] as well as the cellular uptake of polyamines by activating the polyamine transport system (PTS) [11–14]. Compared to normal cells, tumor cells have been shown to contain elevated levels of polyamines [15–18]. These intracellular polyamine levels are maintained via tightly-regulated biosynthetic, catabolic, and uptake and export pathways [19]. Polyamine uptake is upregulated in many tumor types, especially in melanoma tumor cells when compared to normal cells [11,20]. Thus, melanoma tumor cells notoriously replete with multiple oncogenic mutations have a greatly increased need for polyamines compared to normal cells to meet their increased metabolic needs [20].

Our objective was to exploit the oncogene-induced polyamine transport activity in melanoma cells by selectively targeting the PTS with a novel arylmethyl-polyamine (AP) compound (Figure 1, [21]). The two-armed design of AP predicated upon a naphthyl core provides PTS hyperselectivity and high potency [21]. Key to our drug design is that both exogenous polyamines and polyamine-based drugs are imported into tumors via a specific uptake system [8,21,22]. Here, we show that polyamine uptake is increased in mutant $BRAF^{V600E}$ melanoma cells, and that AP treatment significantly increases cell death in $BRAF^{V600E}$ melanoma cells compared to $BRAF^{WT}$ melanoma cells. Furthermore, we show that BRAF inhibitor-resistance in melanoma tumor spheroid cultures can be overcome by treatment with AP. These studies provide valuable insights into developing more effective treatment strategies to restore sensitivity of melanoma tumor cells to BRAF inhibitors. In short, the mutant BRAF-driven polyamine addiction can be targeted by cytotoxic polyamine compounds, which selectively target melanoma cells with high polyamine import activity.

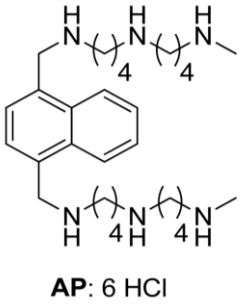


Figure 1. Structure of the arylpolyamine (AP).

2. Materials and Methods

2.1. Cell Lines and Reagents

All human melanoma cell lines including WM983B, WM3734, WM3743, WM989, WM88, WM3451, WM3211, and 1205Lu were obtained as kind gifts from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA, USA). These cells were maintained in MCDB153 (Sigma-Aldrich, St. Louis, MO, USA) and Leibovitz's L-15 (Mediatech Inc, Manassas, VA, USA) medium (4:1 ratio) supplemented with 2% fetal calf serum and 2 mmol/L $CaCl_2$. B16F10 cells were obtained from the American

Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Waltham, MA, USA) supplemented with 10% fetal bovine serum and 100 U/mL Penicillin/Streptomycin. The YUMM1.7 cell line (kindly provided by Marcus Bosenburg, Yale University, New Haven, CT, USA) that harbors a $BRAF^{V600E}$ mutation and inactivation of the Phosphatase and tensin homolog (*PTEN*) gene was maintained in DMEM/F12 (Invitrogen, Waltham, MA, USA) medium supplemented with 10% fetal bovine serum and 100 U/mL Penicillin/Streptomycin.

PLX4720, (S1152; Selleckchem, Houston, TX, USA) a derivative related to PLX4032/Vemurafenib (Plexxikon, Berkeley, CA, USA) was prepared as a 50 mM stock solution in dimethyl sulfoxide and stored at −20 °C. The synthesis of the AP compound has been described previously [21]. The compound was dissolved in phosphate buffered saline (PBS) to provide an initial stock (10 mM), which was filtered through a 0.2 um filter to ensure sterility. Subsequent dilutions were made in PBS to generate the desired stock solutions.

2.2. 3D Spheroid Culture

The nanoscale scaffolding NanoCulture plates (NCP) were purchased from (Organogenix Inc, Woburn, MA, USA). The base of each NCP is constructed with a transparent cyclo-olefin resinous sheet with a nanoscale indented pattern. To form spheroids, 1205Lu human melanoma cells were seeded in a 96-well NCP at 1×10^4 cells/well in MCDB153 (Sigma-Aldrich, St. Louis, MO, USA) and Leibovitz's L-15 (Mediatech Inc, Manassas, VA, USA) medium (4:1 ratio) supplemented with 2% heat-inactivated fetal calf serum and 2 mM $CaCl_2$ and incubated in a conventional cell incubator at 37 °C in an atmosphere of 5% CO_2 and normal O_2 levels. When visible spheroids began to form on day 3 after the cells were seeded on the NCPs, treatment with PLX4720 and/or AP was initiated. After drug treatment for 48 h, the spheroid cultures were assayed for cell viability.

2.3. Cell Viability Assay

Cell proliferation assays were conducted in 96-well plates at 25–30% starting confluence to determine the effect of exposure to increasing concentrations of PLX4720 or AP with or without 1 mM α-difluoromethylornithine (DFMO) for 72 h. Cell viability was assessed using the EZQuant Cell Quantifying Kit (Alstem, Richmond, CA, USA) in which the tetrazolium salt WST-8 is reduced by the metabolic activity of live cells to formazan dye. For spheroids treated with PLX4720 and/or AP, viability of the spheroid cells was estimated by quantification of the adenosine triphosphate present using a CellTiter-Glo Luminescent Cell Viability Assay (Promega Co., Madison, WI, USA). The 72 h half maximal inhibitory concentration (IC50) values for AP were calculated using nonlinear regression (sigmoidal dose response) of the plot of percentage inhibition versus the log of inhibitor concentration in GraphPad Prism (v5; GraphPad Software, Inc., La Jolla, CA, USA). The IC50 value is defined as the concentration of the compound required to inhibit 50% cell viability compared to an untreated control.

2.4. Radiolabeled Spermidine Transport Assays

Polyamine transport in tumor cells was evaluated essentially as described previously [23,24]. Radioactive spermidine (Net-522, Spermidine Trihydrochloride, [Terminal Methylenes-^{3}H(N)], specific activity 16.6 Ci/mmol; Perkin Elmer, Boston, MA, USA) was used. Cells were plated in 96 well plates and grown to approximately 80% confluence. Half of the cells were treated with 1 mM DFMO for 40 h. After repeated washing with PBS, ^{3}H-spermidine was added at 0.5 μM and incubated for 60 min at 37 °C. Cells were then washed with cold PBS containing 50 μM spermidine and lysed in 0.1% sodium dodecyl sulfate solution at 37 °C for 30 min with mixing. Cell lysates were then aliquoted for scintillation counting and for protein assay using a microplate Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Results were expressed as counts per minute (CPM)/μg protein.

2.5. Statistical Analysis

All in vitro experiments were performed at least in triplicate, and data were compiled from two to three separate experiments. Analyses were done using a one-way analysis of variance with a Tukey test for statistical significance or a Students *t*-test. In all cases, values of $p \leq 0.05$ were regarded as being statistically significant.

3. Results

3.1. Human Mutant $BRAF^{V600E}$ Melanoma Cells Are More Sensitive to Cytotoxic Effects of AP Than $BRAF^{WT}$ Melanoma Cells

A panel of human melanoma cell lines with different BRAF mutational status was screened for their sensitivity to the BRAF inhibitor PLX4720. We confirmed previous findings that mutant $BRAF^{V600E}$ melanoma cells, including WM983B, WM3734, 1205Lu, WM989, and WM88, demonstrated marked sensitivity to PLX4720 (IC50 values $\leq$ 3.0 μM), whereas $BRAF^{WT}$ melanoma cells, including WM3451, WM3743, and WM3211, demonstrated relative resistance to treatment with PLX4720 (IC50 > 3.0 μM) [25]. This approach allowed us to rank the relative sensitivity of each cell line to the BRAF inhibitor, PLX4720. Thus, the sensitivity of these $BRAF^{V600E}$ melanoma cells to BRAF inhibition with PLX4720 reflected their functional dependence on mutant BRAF signaling to sustain their proliferation and viability.

Likewise, we tested whether mutant $BRAF^{V600E}$ cells were more sensitive to increasing concentrations of the cytotoxic polyamine transport ligand, AP (Figure 1), compared to $BRAF^{WT}$ melanoma cells. Table 1 shows that mutant $BRAF^{V600E}$ melanoma cells demonstrated greater sensitivity to AP (IC50 < 2.5 μM) than $BRAF^{WT}$ melanoma cells (IC50 > 4.0 μM). This observation was reflected by the greater polyamine transport activity in $BRAF^{V600E}$ melanoma cells compared to $BRAF^{WT}$ melanoma cells (Figure 2A and Table 1). Since AP accumulated at a faster rate in $BRAF^{V600E}$ human melanoma cells with higher polyamine transport rates compared to $BRAF^{WT}$ human melanoma cells (Figure 2A), $BRAF^{V600E}$ melanoma cells were significantly ($p < 0.01$) more sensitive to AP exposure than $BRAF^{WT}$ melanoma cells (Figure 2B). In summary, cell lines with high polyamine import activity were more sensitive to the cytotoxic polyamine compound.

Table 1. Polyamine transport activity and sensitivity to AP in human melanoma cells with different BRAF mutational status cultured ± DFMO [a].

		− DFMO		+ DFMO	
Cell Line	**BRAF Mutational Status**	**IC50 (μM AP)**	**PTS Activity [b] (cpm ^{3}H Spd/μg Protein)**	**IC50 (μM AP)**	**PTS Activity [b] (cpm ^{3}H Spd/μg Protein)**
WM983B	V600E	2.6	487 ± 64	0.9	706 ± 78 *
WM3734	V600E	2.2	444 ± 46	1.2	654 ± 59 *
1205Lu	V600E	0.7	230 ± 22	0.6	299 ± 54
WM989	V600E	1.2	304 ± 28	1.0	348 ± 48
WM88	V600E	0.8	302 ± 18	0.2	343 ± 59
WM3451	WT	9.0	130 ± 21	5.1	157 ± 34
WM3743	WT	8.9	117 ± 23	10.9	110 ± 25
WM3211	WT	4.5	278 ± 58	5.2	251 ± 46

[a] The mean values for half maximal inhibitory concentration (IC50) for AP and polyamine transport system (PTS) activity for human melanoma cells expressing mutant $BRAF^{V600E}$ are compared with that for melanoma cells expressing wild type (WT) BRAF under conditions where cells were cultured without added DFMO or with 1 mM α-difluoromethylornithine (DFMO). [b] PTS activity expressed as counts per minute (CPM) ^{3}H Spermidine (Spd)/μg protein ± standard deviation. PTS assays and cell viability assays were performed at least three times with each cell line. * $p \leq 0.001$ when compared to the PTS activity in the absence of DFMO.

It is well known that lowering intracellular levels of polyamines with inhibitors of polyamine biosynthesis can increase uptake of extracellular polyamines as well as exogenous polyamine analogues [20,26]. WM983B and WM3743 melanoma cells were pretreated for 40 h with 1 mM

DFMO, an inhibitor of ODC, the first and rate-limiting enzyme in polyamine biosynthesis, before measuring their polyamine transport activity. DFMO treatment dramatically increased polyamine transport activity in $BRAF^{V600E}$ WM983B melanoma cells, but not in the $BRAF^{WT}$ WM3743 melanoma cells (Supplementary Materials Figure S1A). In general, polyamine depletion with DFMO treatment enhanced polyamine uptake more in the screened human $BRAF^{V600E}$ melanoma cells compared to that seen in $BRAF^{WT}$ melanoma cells (Table 1, Figure 2A). Since DFMO treatment increases polyamine transport activity, we tested whether co-treatment with AP and DFMO will increase the sensitivity of melanoma cells to AP. For instance, $BRAF^{V600E}$ WM983B melanoma cells are significantly ($p \leq 0.0001$) more sensitive to AP treatment when co-treated with DFMO (IC50 = 0.7 μM) compared to that with AP alone (IC50 = 2.3 μM) (Supplementary Materials Figure S1C). In contrast, sensitivity to AP was not increased in DFMO-co-treated $BRAF^{WT}$ melanoma cells (Table 1, Figure 2B). These data indicate that human $BRAF^{V600E}$ melanoma cells demonstrate greater polyamine transport activity and increased sensitivity to AP compared to $BRAF^{WT}$ melanoma cells, and their sensitivity can be increased by inhibition of polyamine biosynthesis with DFMO.

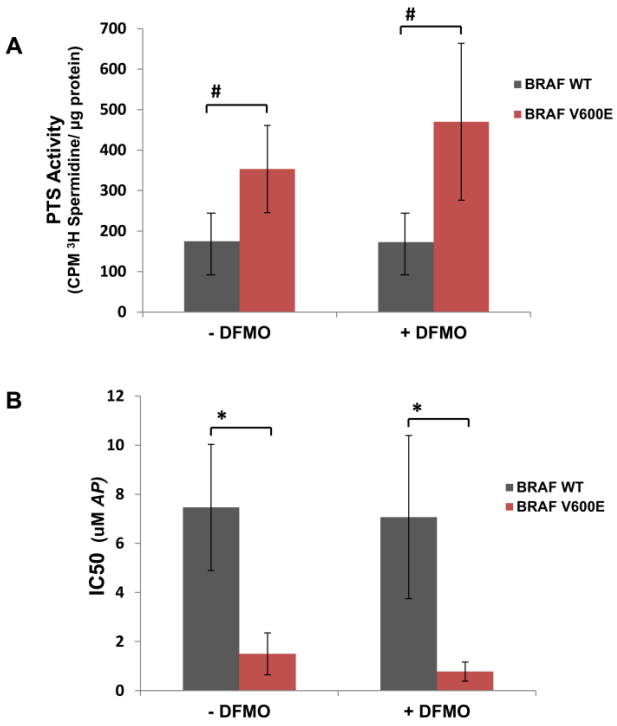


Figure 2. Greater PTS activity and increased sensitivity to AP in mutant $BRAF^{V600E}$ human melanoma cells compared to wild type (WT) $BRAF^{WT}$ cells. (**A**) $BRAF^{V600E}$ human melanoma cells (WM983B, WM3734, 1205Lu, WM989, and WM88) and $BRAF^{WT}$ human melanoma cells (WM3451, WM3743, and WM3211) were cultured with and without 1 mM DFMO for 40 h and then pulsed with 0.5 μM ^{3}H-spermidine for 60 min at 37 °C. Cell lysates were assayed for CPM ^{3}H-spermidine per mg protein by scintillation counting. The mean PTS activity ± SD for $BRAF^{WT}$ melanoma cells is compared with that of $BRAF^{V600E}$ melanoma cells under conditions where cells were cultured without added DFMO or with 1 mM DFMO. (**B**) $BRAF^{V600E}$ human melanoma cells (WM983B, WM3734, 1205Lu, WM989, and WM88) and $BRAF^{WT}$ human melanoma cells (WM3451, WM3743, and WM3211) were treated with increasing doses of AP with or without 1 mM DFMO, using 5–6 samples per dose of AP. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay (Alstem, Richmond, CA, USA). AP IC50 values were calculated by GraphPad Prism 6. The mean AP IC50 values ± SD for $BRAF^{WT}$ melanoma cells is compared with that of $BRAF^{V600E}$ melanoma cells under conditions where cells were cultured without added DFMO or with 1 mM DFMO; # $p \leq 0.05$; * $p < 0.01$.

3.2. AP Is More Cytotoxic to BRAF^{V600E} Murine Melanoma Cells Than BRAF^{WT} Melanoma Cells

Since human melanoma cells possess multiple oncogenic mutations in addition to BRAF^{V600E}, we compared AP cytotoxicity and PTS activity in the murine B16F10 melanoma cell line that is BRAF^{WT} with BRAF^{V600E} YUMM1.7 cell line that was derived from a melanoma tumor that spontaneously developed in a BRAF^{V600E}/PTEN^{null} transgenic mouse [27]. As expected, YUMM1.7 cells were very sensitive to PLX4720 with a lower IC50 compared to B16F10 cells (Figure 3A). In addition, BRAF^{V600E} YUMM1.7 cells were significantly ($p < 0.0001$) more sensitive to AP and had a much lower IC50 value for AP compared to BRAF^{WT} B16F10 cells (Figure 3B). In particular, DFMO co-treatment increased the sensitivity of YUMM1.7 cells to AP (IC50 = 0.8 μM AP without DFMO and IC50 = 0.2 μM AP with DFMO co-treatment), and this correlated with a marked DFMO-induction of PTS activity in BRAF^{V600E} YUMM1.7 cells (Figure 3D). In contrast, BRAF^{WT} B16F10 cells demonstrated no significant induction in polyamine uptake following DFMO treatment (Figure 3D). However, B16F10 cells retrovirally infected to express the mutant BRAF^{V600E} protein exhibited a similar PTS activity profile as that seen with YUMM1.7 cells. DFMO treatment was shown to enhance polyamine uptake in the B16F10-BRAF^{V600E} cells as was seen with YUMM1.7 cells (Figure 3D). AP was also more cytotoxic in B16F10-BRAF^{V600E} cells (IC50 = 24.4 μM) compared to control-infected B16F10-pBABE cells that were infected with retrovirus expressing the empty plasmid (IC50 = 36.4 μM). These data suggest that melanoma cells with a mutant BRAF^{V600E} protein are more dependent on the polyamine uptake system compared to cells with a BRAF^{WT} protein, resulting in greater sensitivity to the PTS-targeted cytotoxic AP compound that enters and kills melanoma cells via the polyamine transport system.

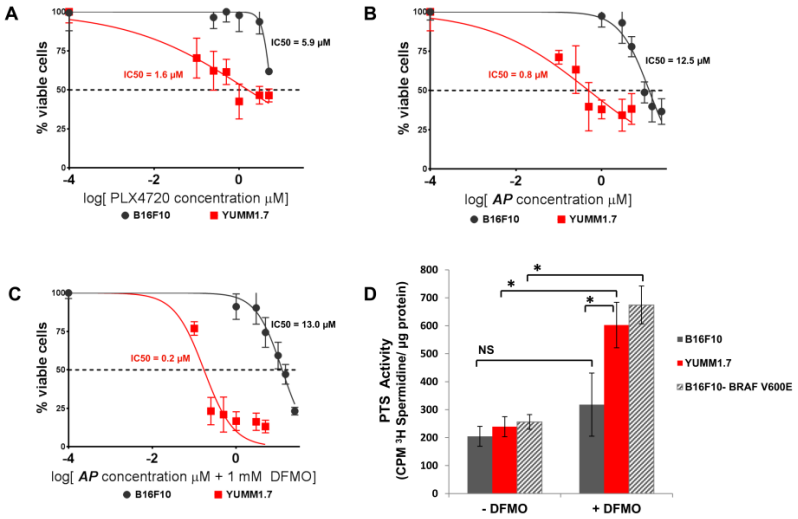


Figure 3. BRAF^{V600E} murine melanoma cells are more sensitive to AP than BRAF^{WT} melanoma cells. (**A**) Murine BRAF^{V600E} YUMM1.7 melanoma cells and BRAF^{WT} B16F10 melanoma cells were treated with increasing doses of PLX4720. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p = 0.0013$. (**B**) Murine BRAF^{V600E} YUMM1.7 melanoma cells and BRAF^{WT} B16F10 melanoma cells were treated with increasing doses of AP. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p < 0.0001$. (**C**) Murine BRAF^{V600E} YUMM1.7 melanoma cells and BRAF^{WT} B16F10 melanoma cells were treated with increasing doses of AP ± 1 mM DFMO. After 72 h of culture, cell survival was determined via EZQuant Cell Quantifying assay. IC50 values were calculated by GraphPad Prism 6; $p < 0.0001$. (**D**) YUMM1.7 and B16F10 melanoma cells and B16F10 cells retrovirally infected to express the mutant BRAF^{V600E} protein were cultured with and withoutS 1 mM DFMO for 40 h and then pulsed with 0.5 μM ^{3}H-spermidine for 60 min at 37 °C. Cells were washed with cold PBS containing 50 μM spermidine, and cell lysates were assayed for CPM ^{3}H-spermidine per mg protein by scintillation counting; * $p < 0.0001$; NS: not significant.

3.3. Increased Resistance of Spheroid Melanoma Cells to PLX4720 Is Overcome with AP Co-Treatment

Because growth of cells in a 3D culture system has been found to be more representative of the in vivo microenvironment, we cultured 1205Lu human melanoma cells using a nanoscale, scaffold-based NCP in which tumor cells easily form 3D spheroids [28]. Although these tumor spheroid cultures are grown in ambient air, the spheroid microenvironment closely resembles that in tumors with a hypoxic core and is more relevant for drug sensitivity compared to that seen with monolayer cultures [28–30]. Similar to previous reports [31], $BRAF^{V600E}$ mutant 1205Lu melanoma cells grown as spheroids on NCPs were more resistant to 48 h treatment with PLX4720 (25 μM) compared to the same cells grown in 2D monolayer cultures in ambient air (Figure 4). Both spheroid and monolayer cultures were similarly sensitive to 48 h treatment with a high concentration of AP (25 μM) alone. We then tested the effect of AP treatment on the PLX4720-resistant phenotype of the 1205Lu spheroid and monolayer cultures. Co-treatment with both PLX4720 (25 μM) and AP (25 μM) led to a dramatic reduction in cell viability in the spheroid cultures unlike monolayer cultures that showed no further reduction in cell viability when compared to PLX4720 treatment alone (Figure 4). Thus, the increased resistance of the melanoma spheroid cultures to PLX4720 was eliminated with AP co-treatment.

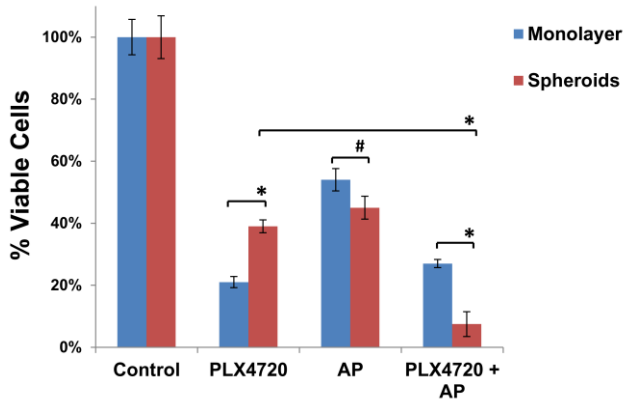


Figure 4. Increased resistance of spheroid melanoma cells to PLX4720 is overcome with AP co-treatment. $BRAF^{V600E}$ mutant 1205Lu melanoma cells were seeded at 1×10^4 cells in each well of 24-well NanoCulture plates (NCPs). When spheroids were formed on day 3, the 3D cultures of spheroids were treated with PLX4720 (25 μM) and/or AP (25 μM). 2D monolayer cultures of 1205Lu melanoma cells were also treated with PLX4720 (25 μM) and/or AP (25 μM). After drug treatment for 48 h, the viability of spheroids and monolayer cultures was assayed using the CellTiter-Glo Luminescent Cell Viability Assay. The percent cell survival in each treatment group was calculated relative to cells treated with medium only under the same conditions. As controls, the growth of cells without drug treatment under each condition was normalized as 100% separately. The means are presented ± SD; * $p < 0.0001$; # $p = 0.0028$.

4. Discussion

Melanoma is challenging to treat due to its genetic heterogeneity, and successful therapy requires targeting multiple molecular vulnerabilities. Our data show that melanoma tumor cells expressing mutant $BRAF^{V600E}$ exhibit a high demand for polyamine growth factors and a greatly upregulated PTS. Utilizing the PTS for drug delivery, the AP compound attacks the melanoma cells via one of its key modes of survival. Indeed, we propose that polyamines are essential for the survival of melanomas. Polyamine levels are dramatically elevated in tumor cells compared to normal cells, often the result of oncogenic induction [11,20]. Previous studies have shown that the c-MYC and RAS can upregulate polyamine biosynthesis [9,10] and increase cellular uptake of polyamines by inducing PTS activity [12–14]. Although melanoma cells are notoriously replete with multiple oncogenic mutations, more than half of all melanoma tumors express a mutant BRAF protein [20]. Our data suggest that

$BRAF^{V600E}$ melanoma tumors have a greatly increased metabolic need for polyamines compared to normal cells. We have exploited the $BRAF^{V600E}$-induced PTS activity in metastatic melanoma cells by targeting the PTS with AP.

Putrescine, spermidine, and spermine play key roles in cellular proliferation, signal transduction, gene expression, and autophagic states that contribute to tumor survival [32–35]. These endogenous polyamines and the polyamine-based AP compete to be imported into tumors via the PTS [21]. However, studies suggest that arylmethyl-polyamines similar to AP have enhanced cytotoxic potency via their multiple electrostatic interactions with DNA [36] and topoisomerase II [37]. Since AP selectively targets tumor cells with high polyamine transport rates, normal cells are significantly less sensitive to AP since they have low PTS activity [21]. Studies have shown that polyamine biosynthesis and cellular uptake are induced in hypoxic regions of tumors and in tumor spheroids [38]. Moreover, depletion of polyamines during hypoxia resulted in increased apoptosis [38], indicating that polyamines play an essential role in the ability of tumor cells to adapt to hypoxic stress and reactive oxygen species. Indeed, polyamines are known to exert anti-oxidant functions [39]. Although the melanoma spheroid cultures in this study were cultured with ambient air, it is well documented that the cells at the center of spheroids are hypoxic, thus modeling the heterogeneous 3D structure of in vivo melanoma tumors that often contain hypoxic regions [28,31]. Solid tumors contain poorly vascularized, hypoxic regions that contribute to tumor progression by activating a hypoxia stress response via hypoxia inducible factor-1α that promotes cell survival, tumor angiogenesis, and metastasis [40,41]. Studies have shown that hypoxic tumor cells and spheroid cultures are more resistant to chemotherapy including BRAF inhibitors [31,42,43]. Likewise, we have found that 3D cultures of 1205Lu melanoma cells grown as spheroids on NCPs are more resistant to PLX4720 treatment compared to 1205Lu cells grown in 2D monolayer culture in ambient air. Knowing that polyamine uptake is induced in hypoxic regions of tumor spheroids, we hypothesized that treatment with the PTS ligand AP would increase the sensitivity of 1205Lu spheroid cells to PLX4720. Indeed, the increased resistance of melanoma spheroids to PLX4720 was overcome with AP co-treatment. In contrast, AP co-treatment had no significant effect on the sensitivity of 2D monolayer cultures of 1205Lu cells to PLX4720.

Accumulating literature shows that treatment with a BRAF inhibitor such as PLX4720 enriches a slow-cycling cancer stem cell-like (CSC) subpopulation of melanoma cells that is characterized by stem cell markers such as Lysine-Specific Demethylase 5B (JARID1B) and spheroid formation [44,45]. It is thought that cancer stem cell populations exist in a hypoxic microenvironment [46–48]. Roesch et al. [45] have found that endogenous reactive oxygen species (ROS) levels are increased in slow cycling $JARID1B^{high}$ melanoma cells as a result of increased mitochondrial respiration and oxidative phosphorylation. This high oxygen consumption contributes to hypoxic conditions that have been shown to favor the $JARID1B^{high}$ slow-cycling CSC-like phenotype [44]. Using 1205Lu melanoma cells stably transduced with a JARID1B-promoter-green fluorescent protein (GFP)-reporter construct [44], we found that a short 2-day exposure to PLX4720 led to a 4-fold enrichment of JARID1B-driven GFP expressing 1205Lu melanoma cells grown as spheroids (data not shown). Our data show that PLX4720-resistant melanoma spheroids are made more sensitive to PLX4720 with AP co-treatment, and it is likely that AP is targeting CSC subpopulations that are enriched in the PLX4720-resistant melanoma spheroids.

Polyamines may also contribute to tumor survival by inducing an autophagic state [32–35]. For instance, spermidine has been shown to induce autophagy in multiple systems including yeast cells, *Caenorhabditis elegans*, *Drosophila melanogaster*, and human tumor cells [35,49] and to increase survival of pluripotent stem cells in culture [50]. Autophagy has recently emerged as a common survival process that tumors undergo when assaulted by chemotherapy and radiation [51]. It is induced by cellular stress such as nutrient deprivation, withdrawal of growth factors, and hypoxia [52]. In established tumors, autophagy is also a resistance mechanism to many therapeutic modalities including BRAF inhibitors [53]. Increased polyamine uptake provides a mechanism for BRAFi-resistant

melanoma cells to acquire sufficient polyamines to undergo autophagy to survive treatment with BRAF inhibitors.

Previous clinical trials have tested the anti-tumor efficacy of the ODC inhibitor DFMO. However, treatment with DFMO alone demonstrated only moderate success in treating cancer patients [54]. Subsequent studies discovered that DFMO-inhibition of polyamine biosynthesis leads to upregulation of PTS activity with resulting increased uptake of polyamines from the diet and gut flora into the tumor cells [18]. Our findings show that DFMO induces PTS activity and increases AP sensitivity in melanoma cells that harbor a mutated BRAF protein. In summary, treatment with AP, with or without DFMO, offers an exciting potential as adjunct cancer therapy to overcome drug resistance in mutant $BRAF^{V600E}$ melanoma.

Supplementary Materials: The following are available online at www.mdpi.com/2076-3271/6/1/3/s1, Figure S1: Greater PTS activity and increased sensitivity to AP in $BRAF^{V600E}$ human melanoma cells compared to $BRAF^{WT}$ cells.

Acknowledgments: We thank Meenhard Herlyn (The Wistar Institute, Philadelphia, PA, USA) and Marcus Bosenburg (Yale University, New Haven, CT, USA) for kindly providing melanoma cell lines. This work was supported by the United States Department of Defense grant CA150356 (S.K.G.).

Author Contributions: S.K.G. conceived and designed the experiments; M.C.P. and A.M. performed the experiments; S.K.G., M.C.P. and A.M. analyzed the data; O.P. provided materials and edited the paper; and S.K.G. wrote the paper.

Conflicts of Interest: Both the composition of matter and use of the AP with DFMO in the treatment of cancers have been patented by the University of Central Florida (UCF). Commercialization of this intellectual property is being conducted jointly by both UCF and the Lankenau Institute for Medical Research under a collaborative research agreement. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Davies, H.; Bignell, G.R.; Cox, C.; Stephens, P.; Edkins, S.; Clegg, S.; Teague, J.; Woffendin, H.; Garnett, M.J.; Bottomley, W.; et al. Mutations of the *BRAF* gene in human cancer. *Nature* **2002**, *417*, 949–954. [CrossRef] [PubMed]
2. Solit, D.B.; Rosen, N. Resistance to *BRAF* inhibition in melanomas. *N. Engl. J. Med.* **2011**, *364*, 772–774. [CrossRef] [PubMed]
3. Haq, R.; Fisher, D.E. Targeting melanoma by small molecules: Challenges ahead. *Pigment Cell Melanoma Res.* **2013**, *26*, 464–469. [CrossRef]
4. Flaherty, K.T.; Puzanov, I.; Kim, K.B.; Ribas, A.; McArthur, G.A.; Sosman, J.A.; O'Dwyer, P.J.; Lee, R.J.; Grippo, J.F.; Nolop, K.; et al. Inhibition of mutated, activated *BRAF* in metastatic melanoma. *N. Engl. J. Med.* **2010**, *363*, 809–819. [CrossRef]
5. Sosman, J.A.; Kim, K.B.; Schuchter, L.; Gonzalez, R.; Pavlick, A.C.; Weber, J.S.; McArthur, G.A.; Hutson, T.E.; Moschos, S.J.; Flaherty, K.T.; et al. Survival in *BRAF* V600-mutant advanced melanoma treated with vemurafenib. *N. Engl. J. Med.* **2012**, *366*, 707–714. [CrossRef]
6. Hauschild, A.; Grob, J.J.; Demidov, L.V.; Jouary, T.; Gutzmer, R.; Millward, M.; Rutkowski, P.; Blank, C.U.; Miller, W.H., Jr.; Kaempgen, E.; et al. Dabrafenib in *BRAF*-mutated metastatic melanoma: A multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **2012**, *380*, 358–365. [CrossRef]
7. Bello-Fernandez, C.; Packham, G.; Cleveland, J.L. The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7804–7808. [CrossRef]
8. Casero, R.A., Jr.; Marton, L.J. Targeting polyamine metabolism and function in cancer and other hyperproliferative diseases. *Nat. Rev. Drug Discov.* **2007**, *6*, 373–390. [CrossRef]
9. Forshell, T.P.; Rimpi, S.; Nilsson, J.A. Chemoprevention of B-cell lymphomas by inhibition of the Myc target spermidine synthase. *Cancer Prev. Res.* **2010**, *3*, 140–147. [CrossRef]
10. Origanti, S.; Shantz, L.M. Ras transformation of RIE-1 cells activates cap-independent translation of ornithine decarboxylase: Regulation by the Raf/MEK/ERK and phosphatidylinositol 3-kinase pathways. *Cancer Res.* **2007**, *67*, 4834–4842. [CrossRef]

11. Poulin, R.; Casero, R.A.; Soulet, D. Recent advances in the molecular biology of metazoan polyamine transport. *Amino Acids* **2012**, *42*, 711–723. [CrossRef] [PubMed]
12. Bachrach, U.; Seiler, N. Formation of acetylpolyamines and putrescine from spermidine by normal and transformed chick embryo fibroblasts. *Cancer Res.* **1981**, *41*, 1205–1208. [PubMed]
13. Chang, B.K.; Libby, P.R.; Bergeron, R.J.; Porter, C.W. Modulation of polyamine biosynthesis and transport by oncogene transfection. *Biochem. Biophys. Res. Commun.* **1988**, *157*, 264–270. [CrossRef]
14. Roy, U.K.; Rial, N.S.; Kachel, K.L.; Gerner, E.W. Activated *K*-RAS increases polyamine uptake in human colon cancer cells through modulation of caveolar endocytosis. *Mol. Carcinog.* **2008**, *47*, 538–553. [CrossRef] [PubMed]
15. Pegg, A.E. Polyamine metabolism and its importance in neoplastic growth as a target for chemotherapy. *Cancer Res.* **1988**, *48*, 759–774. [PubMed]
16. Tabor, C.W.; Tabor, H. Polyamines. *Ann. Rev. Biochem.* **1984**, *53*, 749–790. [CrossRef] [PubMed]
17. Pegg, A.E. Recent advances in the biochemistry of polyamines in eukaryotes. *Biochem. J.* **1986**, *234*, 249–262. [CrossRef] [PubMed]
18. Gerner, E.W.; Meyskens, F.L., Jr. Polyamines and cancer: Old molecules, new understanding. *Nat. Rev. Cancer* **2004**, *4*, 781–792. [CrossRef] [PubMed]
19. Wallace, H.M.; Fraser, A.V.; Hughes, A. A perspective of polyamine metabolism. *Biochem. J.* **2003**, *376*, 1–14. [CrossRef] [PubMed]
20. Seiler, N.; Delcros, J.G.; Moulinoux, J.P. Polyamine transport in mammalian cells. An update. *Int. J. Biochem. Cell Biol.* **1996**, *28*, 843–861. [CrossRef]
21. Muth, A.; Kamel, J.; Kaur, N.; Shicora, A.C.; Ayene, I.S.; Gilmour, S.K.; Phanstiel, O. Development of polyamine transport ligands with improved metabolic stability and selectivity against specific human cancers. *J. Med. Chem.* **2013**, *56*, 5819–5828. [CrossRef] [PubMed]
22. Phanstiel, O.; Kaur, N.; Delcros, J.G. Structure-activity investigations of polyamine-anthracene conjugates and their uptake via the polyamine transporter. *Amino Acids* **2007**, *33*, 305–313. [CrossRef] [PubMed]
23. Kramer, D.L.; Miller, J.T.; Bergeron, R.J.; Khomutov, R.; Khomutov, A.; Porter, C.W. Regulation of polyamine transport by polyamines and polyamine analogs. *J. Cell. Physiol.* **1993**, *155*, 399–407. [CrossRef] [PubMed]
24. Nilsson, J.A.; Keller, U.B.; Baudino, T.A.; Yang, C.; Norton, S.; Old, J.A.; Nilsson, L.M.; Neale, G.; Kramer, D.L.; Porter, C.W.; et al. Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* **2005**, *7*, 433–444. [CrossRef] [PubMed]
25. Schayowitz, A.; Bertenshaw, G.; Jeffries, E.; Schatz, T.; Cotton, J.; Villanueva, J.; Herlyn, M.; Krepler, C.; Vultur, A.; Xu, W. Functional profiling of live melanoma samples using a novel automated platform. *PLoS ONE* **2012**, *7*, e52760. [CrossRef] [PubMed]
26. Alhonen-Hongisto, L.; Seppanen, P.; Janne, J. Intracellular putrescine and spermidine deprivation induces increased uptake of the natural polyamines and methylglyoxal bis(guanylhydrazone). *Biochem. J.* **1980**, *192*, 941–945. [CrossRef] [PubMed]
27. Obenauf, A.C.; Zou, Y.; Ji, A.L.; Vanharanta, S.; Shu, W.; Shi, H.; Kong, X.; Bosenberg, M.C.; Wiesner, T.; Rosen, N.; et al. Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature* **2015**, *520*, 368–372. [CrossRef] [PubMed]
28. Yoshii, Y.; Waki, A.; Yoshida, K.; Kakezuka, A.; Kobayashi, M.; Namiki, H.; Kuroda, Y.; Kiyono, Y.; Yoshii, H.; Furukawa, T. The use of nanoimprinted scaffolds as 3D culture models to facilitate spontaneous tumor cell migration and well-regulated spheroid formation. *Biomaterials* **2011**, *32*, 6052–6058. [CrossRef] [PubMed]
29. Yamada, K.M.; Cukierman, E. Modeling tissue morphogenesis and cancer in 3D. *Cell* **2007**, *130*, 601–610. [CrossRef] [PubMed]
30. Haycock, J.W. 3D cell culture: A review of current approaches and techniques. In *3D Cell Culture: Methods and Protocols*; Springer: Berlin, Germany, 2011; pp. 1–15.
31. Qin, Y.; Roszik, J.; Chattopadhyay, C.; Hashimoto, Y.; Liu, C.; Cooper, Z.A.; Wargo, J.A.; Hwu, P.; Ekmekcioglu, S.; Grimm, E.A. Hypoxia-driven mechanism of vemurafenib resistance in melanoma. *Mol. Cancer Ther.* **2016**, *15*, 2442–2454. [CrossRef] [PubMed]
32. Cufi, S.; Vazquez-Martin, A.; Oliveras-Ferraros, C.; Martin-Castillo, B.; Vellon, L.; Menendez, J.A. Autophagy positively regulates the $CD44^{+}$ $CD24^{-/low}$ breast cancer stem-like phenotype. *Cell Cycle* **2011**, *10*, 3871–3885. [CrossRef] [PubMed]

33. Mirzoeva, O.K.; Hann, B.; Hom, Y.K.; Debnath, J.; Aftab, D.; Shokat, K.; Korn, W.M. Autophagy suppression promotes apoptotic cell death in response to inhibition of the PI_3K—mTOR pathway in pancreatic adenocarcinoma. *J. Mol. Med.* **2011**, *89*, 877–889. [CrossRef] [PubMed]
34. Morselli, E.; Galluzzi, L.; Kepp, O.; Marino, G.; Michaud, M.; Vitale, I.; Maiuri, M.C.; Kroemer, G. Oncosuppressive functions of autophagy. *Antioxid. Redox Signal.* **2011**, *14*, 2251–2269. [CrossRef] [PubMed]
35. Morselli, E.; Marino, G.; Bennetzen, M.V.; Eisenberg, T.; Megalou, E.; Schroeder, S.; Cabrera, S.; Benit, P.; Rustin, P.; Criollo, A.; et al. Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. *J. Cell Biol.* **2011**, *192*, 615–629. [CrossRef] [PubMed]
36. Dallavalle, S.; Giannini, G.; Alloatti, D.; Casati, A.; Marastoni, E.; Musso, L.; Merlini, L.; Morini, G.; Penco, S.; Pisano, C.; et al. Synthesis and cytotoxic activity of polyamine analogues of camptothecin. *J. Med. Chem.* **2006**, *49*, 5177–5186. [CrossRef] [PubMed]
37. Wang, H.; Davis, A.; Yu, S.; Ahmed, K. Response of cancer cells to molecular interruption of the CK2 signal. *Mol. Cell Biochem.* **2001**, *227*, 167–174. [CrossRef] [PubMed]
38. Svensson, K.J.; Welch, J.E.; Kucharzewska, P.; Bengtson, P.; Bjurberg, M.; Pahlman, S.; Ten Dam, G.B.; Persson, L.; Belting, M. Hypoxia-mediated induction of the polyamine system provides opportunities for tumor growth inhibition by combined targeting of vascular endothelial growth factor and ornithine decarboxylase. *Cancer Res.* **2008**, *68*, 9291–9301. [CrossRef] [PubMed]
39. Mozdzan, M.; Szemraj, J.; Rysz, J.; Stolarek, R.A.; Nowak, D. Anti-oxidant activity of spermine and spermidine re-evaluated with oxidizing systems involving iron and copper ions. *Int. J. Biochem. Cell Biol.* **2006**, *38*, 69–81. [CrossRef] [PubMed]
40. Pouyssegur, J.; Dayan, F.; Mazure, N.M. Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* **2006**, *441*, 437–443. [CrossRef] [PubMed]
41. Keith, B.; Simon, M.C. Hypoxia-inducible factors, stem cells, and cancer. *Cell* **2007**, *129*, 465–472. [CrossRef] [PubMed]
42. O'Connell, M.P.; Marchbank, K.; Webster, M.R.; Valiga, A.A.; Kaur, A.; Vultur, A.; Li, L.; Herlyn, M.; Villanueva, J.; Liu, Q. Hypoxia induces phenotypic plasticity and therapy resistance in melanoma via the tyrosine kinase receptors ROR1 and ROR2. *Cancer Discov.* **2013**, *3*, 1378–1393. [CrossRef] [PubMed]
43. Pucciarelli, D.; Lengger, N.; Takáčová, M.; Csaderova, L.; Bartosova, M.; Breiteneder, H.; Pastorekova, S.; Hafner, C. Hypoxia increases the heterogeneity of melanoma cell populations and affects the response to vemurafenib. *Mol. Med. Rep.* **2016**, *13*, 3281–3288. [CrossRef] [PubMed]
44. Roesch, A.; Fukunaga-Kalabis, M.; Schmidt, E.C.; Zabierowski, S.E.; Brafford, P.A.; Vultur, A.; Basu, D.; Gimotty, P.; Vogt, T.; Herlyn, M. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **2010**, *141*, 583–594. [CrossRef] [PubMed]
45. Roesch, A.; Vultur, A.; Bogeski, I.; Wang, H.; Zimmermann, K.M.; Speicher, D.; Korbel, C.; Laschke, M.W.; Gimotty, P.A.; Philipp, S.E.; et al. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B^{high} cells. *Cancer Cell* **2013**, *23*, 811–825. [CrossRef] [PubMed]
46. Schwab, L.P.; Peacock, D.L.; Majumdar, D.; Ingels, J.F.; Jensen, L.C.; Smith, K.D.; Cushing, R.C.; Seagroves, T.N. Hypoxia inducible factor-1α promotes primary tumor growth and tumor-initiating cell activity in breast cancer. *Breast Cancer Res.* **2012**, *14*, R6. [CrossRef] [PubMed]
47. Mathieu, J.; Zhang, Z.; Zhou, W.; Wang, A.J.; Heddleston, J.M.; Pinna, C.M.; Hubaud, A.; Stadler, B.; Choi, M.; Bar, M. HIF induces human embryonic stem cell markers in cancer cells. *Cancer Res.* **2011**, *71*, 4640–4652. [CrossRef] [PubMed]
48. Mohyeldin, A.; Garzon-Muvdi, T.; Quinones-Hinojosa, A. Oxygen in stem cell biology: A critical component of the stem cell niche. *Cell Stem Cell* **2010**, *7*, 150–161. [CrossRef] [PubMed]
49. Eisenberg, T.; Knauer, H.; Schauer, A.; Buttner, S.; Ruckenstuhl, C.; Carmona-Gutierrez, D.; Ring, J.; Schroeder, S.; Magnes, C.; Antonacci, L.; et al. Induction of autophagy by spermidine promotes longevity. *Nat. Cell Biol.* **2009**, *11*, 1305–1314. [CrossRef] [PubMed]
50. Chen, T.; Shen, L.; Yu, J.; Wan, H.; Guo, A.; Chen, J.; Long, Y.; Zhao, J.; Pei, G. Rapamycin and other longevity-promoting compounds enhance the generation of mouse induced pluripotent stem cells. *Aging Cell* **2011**, *10*, 908–911. [CrossRef] [PubMed]
51. Strohecker, A.M.; White, E. Targeting mitochondrial metabolism by inhibiting autophagy in *BRAF*-driven cancers. *Cancer Discov.* **2014**, *4*, 766–772. [CrossRef] [PubMed]

52. Kroemer, G.; Marino, G.; Levine, B. Autophagy and the integrated stress response. *Mol. Cell* **2010**, *40*, 280–293. [CrossRef] [PubMed]
53. Ma, X.H.; Piao, S.F.; Dey, S.; McAfee, Q.; Karakousis, G.; Villanueva, J.; Hart, L.S.; Levi, S.; Hu, J.; Zhang, G.; et al. Targeting ER stress-induced autophagy overcomes *BRAF* inhibitor resistance in melanoma. *J. Clin. Investig.* **2014**, *124*, 1406–1417. [CrossRef] [PubMed]
54. Seiler, N. Thirty years of polyamine-related approaches to cancer therapy. Retrospect and prospect. Part 1. Selective enzyme inhibitors. *Curr. Drug Targets* **2003**, *4*, 537–564. [CrossRef] [PubMed]