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Stromal cell effects on melanoma cell drug response

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**STROMAL CELL EFFECTS ON MELANOMA CELL DRUG
RESPONSE**

by

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B.S., Boston College, 2011

Submitted in partial fulfillment of the
requirements for the degree of
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DEDICATION

For Brendan Greve

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ABSTRACT

Objective: Melanoma is currently one of the deadliest forms of skin disease in the United States. However in the past decade there have been significant advances in treatment. Among the most promising recent developments, inhibitors of the serine/threonine-protein kinase B-Raf (BRAF inhibitors) such as vemurafenib show great promise and have been shown to increase the median survival of patients with melanoma cells that harbor a mutation of the BRAF gene. While BRAF inhibitors and other treatment therapies have much potential, more needs to be done to improve treatment.

As with other cancers, a major hurdle in the treatment of melanoma is the eventual tumor resistance to drug therapy. Accessory cells are thought to play a large role in mediating tumor resistance to drug treatment. Stromal cells have been known to release cytokines and growth factors that aid in cancer proliferation. They can also expression adhesion molecules that further help to aid cell growth and tumor development. It has also been demonstrated that these accessory cells can significantly alter cancer cell drug response as a result

of the factors they release or express on their surface. In this study we hypothesize that certain anti-cancer drugs will behave differently against melanoma cell line A375 in the presence versus the absence of stromal cells.

Methods: Melanoma cell line A375 was grown on 384 well plates in the presence or absence of different stromal cell lines. A number of different drugs were screened using Compartment-Specific Bioluminescence Imaging to determine if there was a difference in A375 proliferation after drug treatment in the presence versus absence of accessory cells. After an initial screen, a few drugs were chosen to generate dose-response curves to determine if different drugs had different effects at various doses in the presence or absence of stromal cells.

Results: An initial screen involving 81 FDA approved oncology drugs showed that a number of drugs had different effects on A375 cells in the presence versus absence of stromal cells. A follow-up screen of 24 compounds identified six different drugs that were effective at killing A375 cells. Four of these drugs, as well as vemurafenib, crizotinib, and bortezomib were chosen to be used in the generation of drug dose-response curves to look for differences in drug action in the presence of stromal cells. Of these drugs, cytarabine, bortezomib, and vemurafenib showed the most significant changes in drug response in the presence versus absence of stromal cells.

Conclusion: In this study, we observed that some drugs behave in a significantly different manner on A375 melanoma cells in the presence versus absence of

stromal cells. By using Compartment-Specific Bioluminescence Imaging (CS-BLI) we were able to determine exactly how much cellular proliferation levels were altered. We believe that these results indicate that further study on intercellular interactions on melanoma cell drug response is needed.

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ABBREVIATIONS

ACT	Adoptive Cell Therapy
ALM	Acral Lentiginous Melanoma
CS-BLI	Compartment-Specific Bioluminescence Imaging
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
HCL	Hydrogen Chloride
HDAC	Histone Deacetylase
HDIL2	High Dose Interleukin 2
HGF	Hepatocyte Growth Factor
MAP	Mitogen Activated Protein
OHT	Hydroxytamoxifen
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor

RNA	Ribonucleic Acid
SSM	Superficial Spreading Melanoma
TGF	Transforming Growth Factor
TIL	Tumor Infiltrating Lymphocytes
TNF	Tumor Necrosis Factor
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

INTRODUCTION

Each year over 122,000 people worldwide are diagnosed with some form of melanoma and about 65,000 people per year die from the disease. While there have been many advancements in therapy, melanoma remains the most dangerous form of skin disease.[1]

Melanoma is the malignant proliferation of melanocytes. Melanocytes are cells that produce the pigment known as melanin. Melanin gives rise to skin as well as hair color. Melanoma can originate anywhere melanocytes can be found. It can sometimes be located in the small intestine, large intestine, lungs and other internal organs.[2] However, by far most cases of melanoma present in the skin, and less frequently, the eyes. When found on the skin, melanoma typically presents as an asymmetrical dark mole or growth.

While there are a few different types of melanoma, the four main types of melanoma include superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma. [2] Superficial spreading melanoma (SSM) is the most common form of melanoma found on the skin. SSM is frequently found on sun-exposed skin. Commonly originating from pre-neoplastic lesions, this type of melanoma typically presents in two stages: a) first, a radial growth phase, and b) second, a vertical growth phase, where the population of neoplastic cells begins to develop malignant features. Like SSM, lentigo maligna melanoma tends to be located on chronically sun-exposed skin.

Originating from non-invasive lentigo maligna (skin growths), lentigo maligna melanoma generally presents as dark raised nodules on the skin.[2] Nodular melanoma is more aggressive than other types of melanoma. Nodular melanoma often has a rapid vertical growth phase and can present in locations showing no previous abnormal growths. In some cases, the growth does not exhibit dark pigment, thus delaying detection, which can be especially deadly. The last major form of melanoma is acral lentiginous melanoma (ALM). ALM can be one of the most difficult types of melanoma to identify. It can present on areas of the body that are not exposed to high levels of sunlight such as beneath nail beds, in the mouth, and on the soles of feet. This type of melanoma is the most common melanoma to affect people of Asian and African descent.[1]

Melanoma can be caused by a variety of factors. One of the most significant causes of melanoma is overexposure to UV radiation.[3] Individuals that have chronic sun damage from ultraviolet light demonstrate a significantly higher prevalence of melanoma. This can be from both UV light from the sun as well as tanning beds. In addition to overexposure to UV radiation, other significant risk factors include prolonged presence at high altitudes, fair skin, light hair, moles, weakened immune system, familial history of the disease as well as prior xeroderma pigmentosa.[2] Other genetic predispositions to the development of melanoma include mutations in MC1R or CDKNA2, which are associated with significantly higher rates of developing melanoma.[4]

There are a variety of ways in which an individual can improve his or her chances of preventing the disease. Perhaps the best way of preventing melanoma is to minimize exposure to UV radiation. Avoiding tanning beds as well as using sunscreen that blocks both UVA and UVB radiation significantly reduces one's chance of developing the disease. Wearing clothes that block sunlight can also help to prevent the disease.[2] It is important that individuals constantly monitor themselves for abnormal growths. Professional screens can also help in reducing the chances of malignancy.

Treatment

Despite methods of prevention, many individuals will develop malignant melanoma over the course of their lifetime. Treatment for the disease can be administered via multiple approaches. At present, the most effective treatment for the disease remains to be surgery. Surgical excision of tumors is the best way for completely curing the disease, as long as it has not disseminated away from the primary site.[5] In some cases, sentinel lymph nodes can also be excised in order to reduce chances of further spreading.[6] However, when melanoma has spread to other organs, surgical resection becomes less and less effective.

When surgery no longer remains feasible for curing the disease, other treatment options do exist. For instance, as with other cancers, radiation therapy can be administered, but it is generally limited to melanomas that have spread

locally. For those cases that require a more aggressive treatment, there are a variety of different chemotherapeutic strategies as well as immunotherapy options that are used to combat the disease. In the past, the two most common drugs that were used for melanoma treatment were dacarbazine and high-dose interleukin 2.[7] However both of these treatment strategies have their limitations. Dacarbazine has a very low response rate (10-15%) as well as a low overall mean survival. High dose interleukin-2 (HDIL2) works on an even smaller percentage of patients (5-10%). HDIL2 also has major side effects.[7]

Over the past decade, there has been a significant improvement in immunotherapy. One reason why immunotherapy seems to be so promising in melanoma is the highly immunogenic nature of melanoma tumors. Melanoma tumors exhibit very high levels of tumor-infiltrating lymphocytes and this characteristic has encouraged extensive research in immunotherapy. One such drug that shows promise is ipilimumab. Ipilimumab is a fully human IgG₁ monoclonal antibody that acts by blocking CTLA-4, a negative T cell regulator.[7] By blocking the negative regulator, T cells become increasingly active and can diminish size and number of tumors more effectively. With ipilimumab, median overall survival was increased by 3-4 months. However one drawback of ipilimumab is its low overall response rate (between 10-15%).[7]

There has been much research as of late to try and improve the effectiveness of ipilimumab. Some researchers suggest that a higher dose than the FDA approved 3 mg/kg could help lead to increased rate of response.[7]

Other researchers believe that a drug combination therapy including ipilimumab may be the best way to increase efficacy and rate of response. A study conducted in June 2011, showed that ipilimumab administered in combination with standard dacarbazine showed an added improvement in overall survival time.[8] Other compounds that appear to increase the average response rate when combined with ipilimumab include bevacizumab and temozolamide. Both of these drugs demonstrated higher response rates in phase 2 studies when combined with ipilimumab versus using ipilimumab alone.[8]

In addition to ipilimumab, other immunotherapies are currently being developed. One of the most promising immunotherapies is adoptive cell therapy. Taking advantage of the high levels of tumor-infiltrating lymphocytes found in melanoma tumors, adoptive cell therapy involves extraction of these TIL's. These cells are then expanded and injected back into the patient. ACT has been shown to have a response rate of around 30%. However when combined with lymphodepletion of regulatory T cells, the response rates have increased to over 70%.[9] Aside from lymphodepletion, other adjunct therapies are also being evaluated. Some investigators are trying to genetically modify autologous T cells not found in the tumor to express antigens that can recognize and target melanoma cell specific antigens. Other studies have reported that by pretreating individuals with interferon gamma, increased tumor immunogenicity develops which could result in a higher overall response rate.[7]

BRAF

One of the most significant advances in the past few years for the treatment of melanoma is the development of BRAF inhibitors. The BRAF gene is an important part of the MAP kinase signaling pathway that can lead to cell growth and is an important pathway for tumorigenesis in melanoma as well as a few other types of cancer types. BRAF is responsible for producing a protein called B-Raf. B-Raf is a serine/threonine protein kinase that helps activate cell growth, division and secretion.[10] In cutaneous melanoma, BRAF mutations have been detected in over 40% of patients. The vast majority of BRAF mutations are due to a substitution of glutamate for valine at amino acid 600 (V600E).[11] The substitution at this site leads to chronic activation of the MAP kinase pathway and therefore provides a means for increased growth of many melanoma tumors.

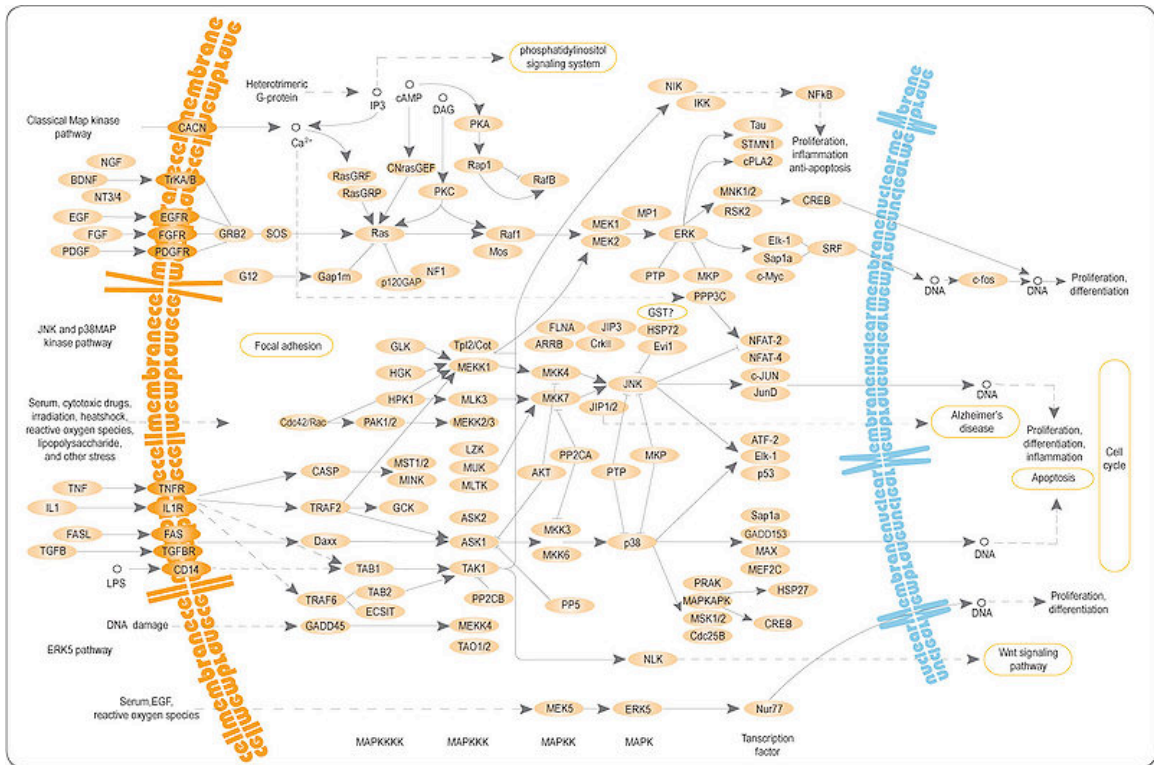


Figure 1: Overview of the MAPK pathway

The MAPK pathway is an important growth pathway for cell survival and proliferation. As seen above, BRAF plays an important part in the pathway.

Early attempts at mutant BRAF inhibition used a BRAF inhibitor known as sorafenib. Sorafenib had significant side effects however due to its poor selectivity against BRAF mutants and its activity against many other targets.[7] Recently, BRAF inhibitors that have demonstrated a much greater specificity for BRAF mutants have been developed. In 2011, the FDA approved for use in patients with metastatic melanoma a BRAF inhibitor, developed by Plexxikon called vemurafenib (also known as PLX4032). In a phase 1 trial, vemurafenib proved to be efficacious in over 80% of patients who presented with a V600E

mutation.[12] While not yet approved by the FDA, vemurafenib also appears to have some effect in patients with a V600K mutation (the valine is replaced by lysine).[7] For patients with the V600E mutation that respond to the drug, vemurafenib has shown to increase life span for nearly 6 months on average.[7] While other selective BRAF inhibitors such as GSK2118436 have been developed, vemurafenib remains to be the most common selective BRAF inhibitor prescribed to patients with metastatic melanoma.

While the drug has been a major breakthrough for the treatment of melanoma, resistance to vemurafenib has been the major drawback of treatment. After about 6 months, most patients begin to develop major resistance to the drug. One suggested means of developing BRAF resistance is that tumor cells begin to produce elevated levels of platelet-derived growth factor receptor-beta (PDGFRB).[13] PDGFRB is a tyrosine kinase receptor present on the surface of cells that can provide an alternate pathway for cell proliferation in patients treated with BRAF inhibitors. An alternative mechanism proposed for resistance is activation of the NRAS pathway.[10] Activation of NRAS leads to a reactivation of the MAP kinase pathway and ultimately resistance to vemurafenib.

The Role of Stromal Cells

Stromal cells have long been known to aid in tissue growth and regulation. In normal tissues, neighboring cells found in the ECM as well as other local sites help aid in cell growth or destruction based on the needs of the cells or tissues

involved. This is done through the release of a plethora of cytokines and growth factors and whose levels change depending on the levels of stress mediated on the tissue. Lately the role of the tumor microenvironment as well as the role of stromal cells is becoming an increasingly popular topic in cancer research. As tumors develop and change from normal cells, so do stromal cells. For many cancers, the compounds released by these stromal cells can help aid in growth and malignancy. By releasing compounds such as TNF alpha, EGF, FGF, HGF, TGF, PDGF, VEGF, as well as a wide variety of others, stromal cells can have a substantial effect on tumor proliferation [14], thus functioning as “accessory cells”.

Of particular interest in this study is the role which stromal cells and the tumor microenvironment play in regards to drug resistance. Previous studies have confirmed that stromal cells can significantly affect how cancer cells respond to drug treatment. [15] Stromal cells have been shown to increase, as well as provide resistance to, drug efficacy. Stromal cell-mediated resistance to drug treatment is a major problem for diverse drug therapies. By reactivating certain growth pathways or activating previously silent growth pathways, stromal cells can lead to rapid drug resistance and ineffectiveness.

Like in other types of cancers, stromal mediated resistance to drug therapy is believed to be a major factor for drug resistance in melanoma. One such study proving this was performed in 2012 by Dr. Ravid Straussman. In this study, Straussman proposed that HGF produced by stromal cells played a

significant role in melanoma resistance to selective treatments such as BRAF inhibition.[16]

Compartment-Specific Bioluminescence Imaging (CS-BLI)

One method of determining the effect of stromal cell-mediated resistance is through the use of the CS-BLI platform, developed by Dr. Constantine Mitsiades and Dr. Douglas McMillin. CS-BLI is a system that takes advantage of luciferase expression.[17] The first step is to make cancer cells stably express luciferase. Once they stably express luciferase they can be grown in culture with stromal cells of interest that are luciferase-negative. Once the cells have been plated they can be treated with different agents and after the end of treatment the plates can be placed inside a luminometer in order to read the bioluminescence of each well. By reading the amount of light given off by each well, the luminometer can then indirectly determine the amount of cells in each well, or at least give the user a relative percentage of cells compared to a control as the bioluminescence increases linearly with cell number.

This study has identified multiple compounds that show diminished killing efficacy in the presence of stromal cells. Importantly, the platform can also be used to identify any stromal mediated improvements in killing efficacy.[17]

The Present Study

Since BRAF mutations have recently become such a popular area of study we determined that using a BRAF mutant (V600E) would be of the highest value. Therefore we decided to use melanoma cell line A375.

In an effort to find as many cases of altered drug response we used a variety of chemotherapeutics. We also used a variety of stromal cell lines as multiple lines would produce different growth factors and cytokines and would therefore be more likely to elicit drug resistance or sensitization.

At present, there are few studies that aim to determine how different stromal cells alter melanoma cell drug response. The purpose of this experiment is to demonstrate the importance of stromal cell effects in order to improve melanoma study models. We believe that the addition of stromal cells will alter how A375 responds to certain drugs.

Specific Aims

In order to determine the effect of stromal cell mediated drug resistance and or sensitization to drug treatment on melanoma cells we will:

1. Screen melanoma cell line A375 with a variety of FDA-approved oncology compounds to identify drugs that show a change in activity in the presence of stroma. At the same time we hope to determine a positive control for drug resistance.

2. Establish dose response curves and examine possible stromal cell-mediated differences in drug response for drugs identified in the FDA Oncology Set screen.

3. Identify other well-known chemotherapeutics that show significant efficacy in killing A375 melanoma cells.

4. Establish dose response curves for drugs selected previously which proved efficacious at killing A375. Determine if these compounds demonstrate any stromal mediated resistance and/or sensitization to drug treatment.

We expect to see:

1. An initial screen will show that a number of compounds exhibit altered drug activity in the presence versus absence of stroma.

2. Some drugs selected for further study will generate different dose response curves in the presence versus absence of stroma.

Methods

Cell Lines and Reagents

Stromal cell lines CCD13LU, CCD1065SK, HU88, and LL86 were all obtained from ATCC. Melanoma line A375 was also obtained from ATCC and had previously been transduced to express luciferase.

Vemurafenib stock solution was obtained from Sara Buhrlage (Nathanael Gray Lab, Dana Farber Cancer Institute). The FDA oncology set drugs were obtained from the National Cancer Institute. The remaining drugs were obtained from Sigma-Aldrich and stored at -20°C.

Cell Culture and Plating

Cell lines were maintained at 37°C. Cells were grown in Dulbecco's Modified Eagle's Medium. Heat-inactivated FBS was added to the media to a final concentration of 10%. The media was also supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin.

Cells were plated using a Thermo Scientific Multidrop Combi. The cells were plated at a ratio of 400 melanoma cells to 1000 stromal cells per well to a volume of 45 microliters. Five microliters of drug in DMSO was added after 24 hours of co-culture either by repeater pipette or by the JANUS Automated Workstation to a final volume of 50 microliters. All experiments were performed on white bottom 384 well plates.

CS-BLI Reading

To measure bioluminescence, a Biotek luminometer was used. On the day of measurement, 5 microliters of beetle luciferin was added to each well of each 384 well plate used. The plates were allowed to incubate at 37° for 30 minutes then they were placed in the luminometer to be read.

FDA Oncology Drug Screen

The FDA-approved Oncology Set III drug plates (384 wells per plate, 2 plates) were obtained by the NCI. Two concentrations for each plate (0.1 and 0.5 micromolar) were also used. A total of 81 compounds were tested. The compounds were stored in 384 well plates with each well containing 20 microliters of a 10 mM DMSO solution.

Cells were plated in co-culture on day one onto 384 white bottom plates using a Thermo Scientific Multidrop Combi. On day 2, 100 nanoliters of drug was added to a final concentration of 0.1 or 0.5 micromolar. Bioluminescence was measured using a Biotek luminometer after 72 hours of drug treatment.

In Vitro Testing of Anti-Tumor Agents Against A375 Cells

On day one, A375 cells were plated onto 384 well plates using a Thermo Scientific Multidrop Combi (45 microliters). On day two, 10 serial concentrations of drug (5 microliters per well) were added to different wells in order to generate

dose response curves. Bioluminescence was measured using a Biotek luminometer at 24, 48, 72, or 96 hours for the different drugs.

A375 Co-Culture Comparison

On day one, A375 cells and either CCD13LU, HS88LU, or LL86 were plated in co-culture onto 384 well plates using a Thermo Scientific Multidrop Combi (45 microliters). On day two, 10 different concentrations of drug were added to different wells in order to generate dose response curves (5 microliters per well). Bioluminescence was measured using a Biotek Luminometer at 24, 48, 72, or 96 hours for the different drugs.

Dose Response Curve Generation

For all generation of dose response curves, Graphpad Prism Version 6 was used. All curves were normalized to their respective no drug control and each data point was the average of at least 4 replicate values.

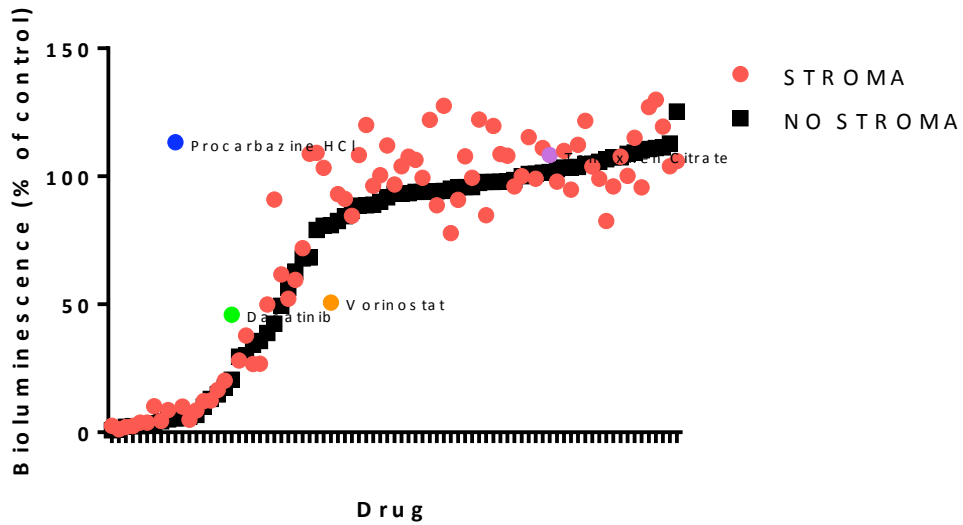
Results

FDA ONCOLOGY SCREEN

The screen involved 81 FDA approved oncology drugs. Certain drugs were subject to sensitization and others to resistance in the presence of stroma. This was the case for both A375 with CCDK1065SK, as well as A375 with CCD13LU. This was also seen at both the 0.1 micromolar dose as well as the 0.5 micromolar dose. In regards to drug mediated sensitization, it appeared that vorinostat and tamoxifen HCL showed drug sensitization over the four different conditions. For drug resistance, procarbazine HCL and dasatinib showed resistance when co-cultured with stroma (both CCD13LU cells as well as CCD1065SK cells).

There were some drugs that showed an altered activity in only one of the stromal lines. Sunitinib malate showed slight drug resistance when in the presence of CCD1065SK at 0.5 micromolar, but in the presence of CCD13LU there was slightly more A375 killing then compared to the no stroma control.

A



B

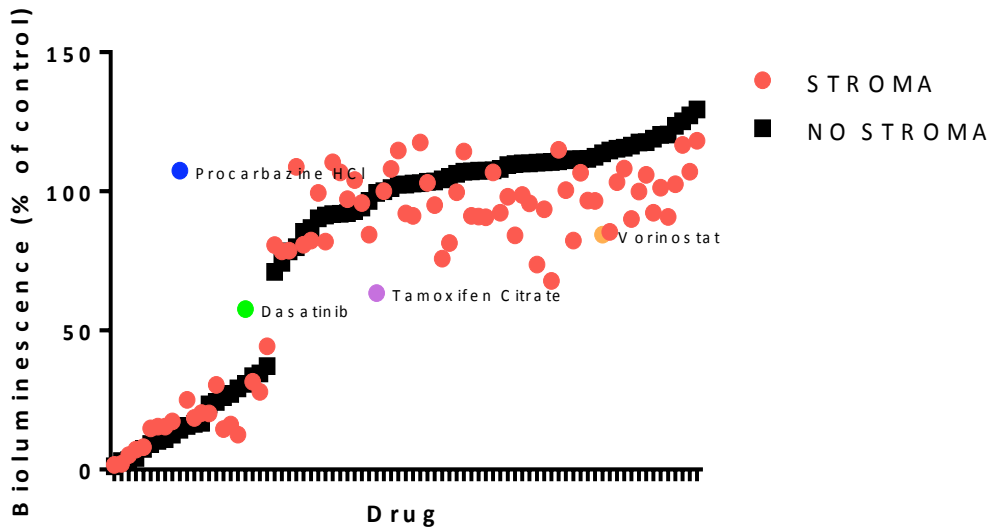


Figure 2: FDA Oncology Set Plate Screen Using A375 and CCDK1065SK Cells.

The following conditions were used to screen for potential drugs for further evaluation. (A) A375 with CCDK1065sk cells were treated with 0.1 micromolar of drug. (B) A375 with CCDK1065sk cells were treated with 0.5 micromolar of drug.

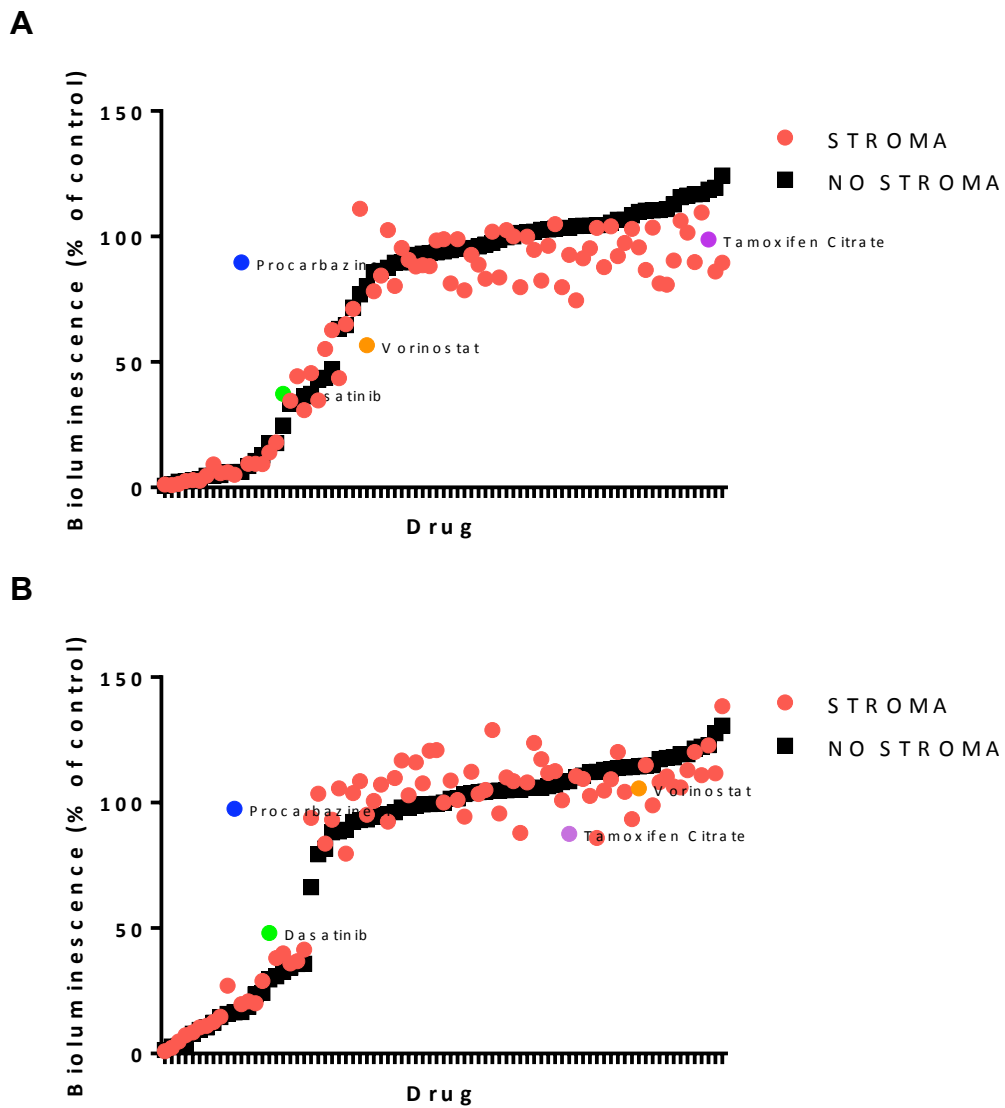
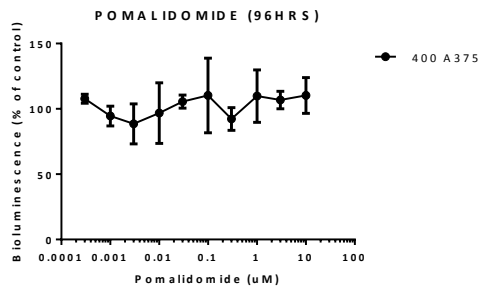
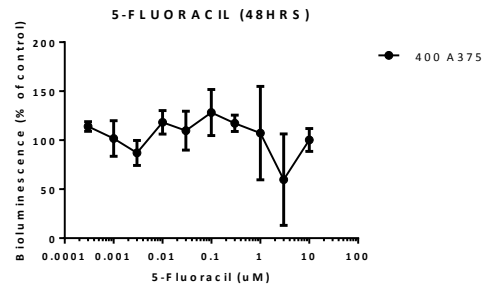
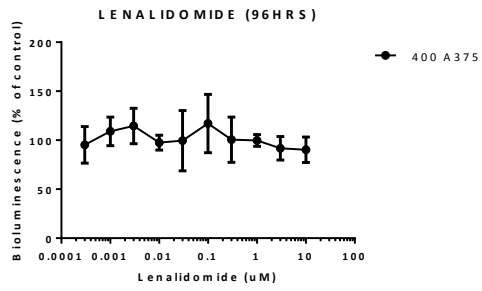
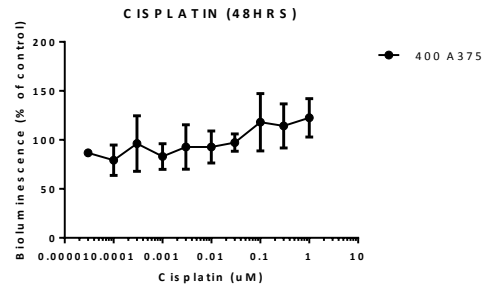
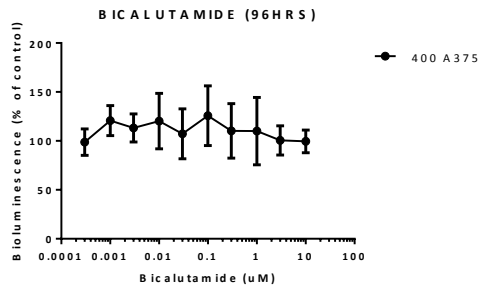
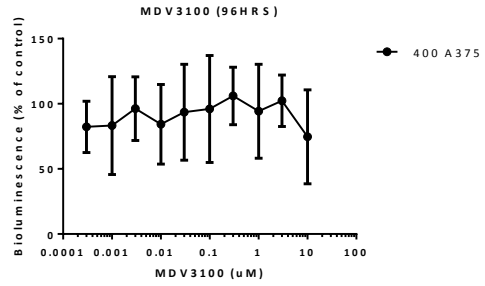
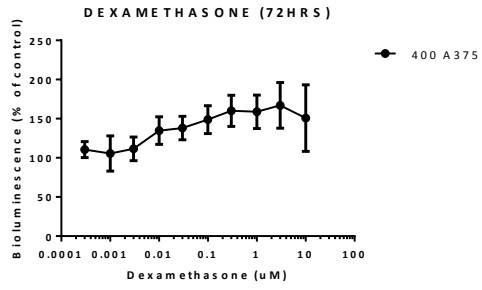


Figure 3: **FDA Oncology Set Plate Screen Using A375 and CCD13LU Cells.** The following conditions were used to screen for potential drugs for further evaluation. (A) A375 with CCD13LU cells were treated with 0.1 micromolar of drug. (B) A375 with CCD13LU cells were treated with 0.5 micromolar of drug.

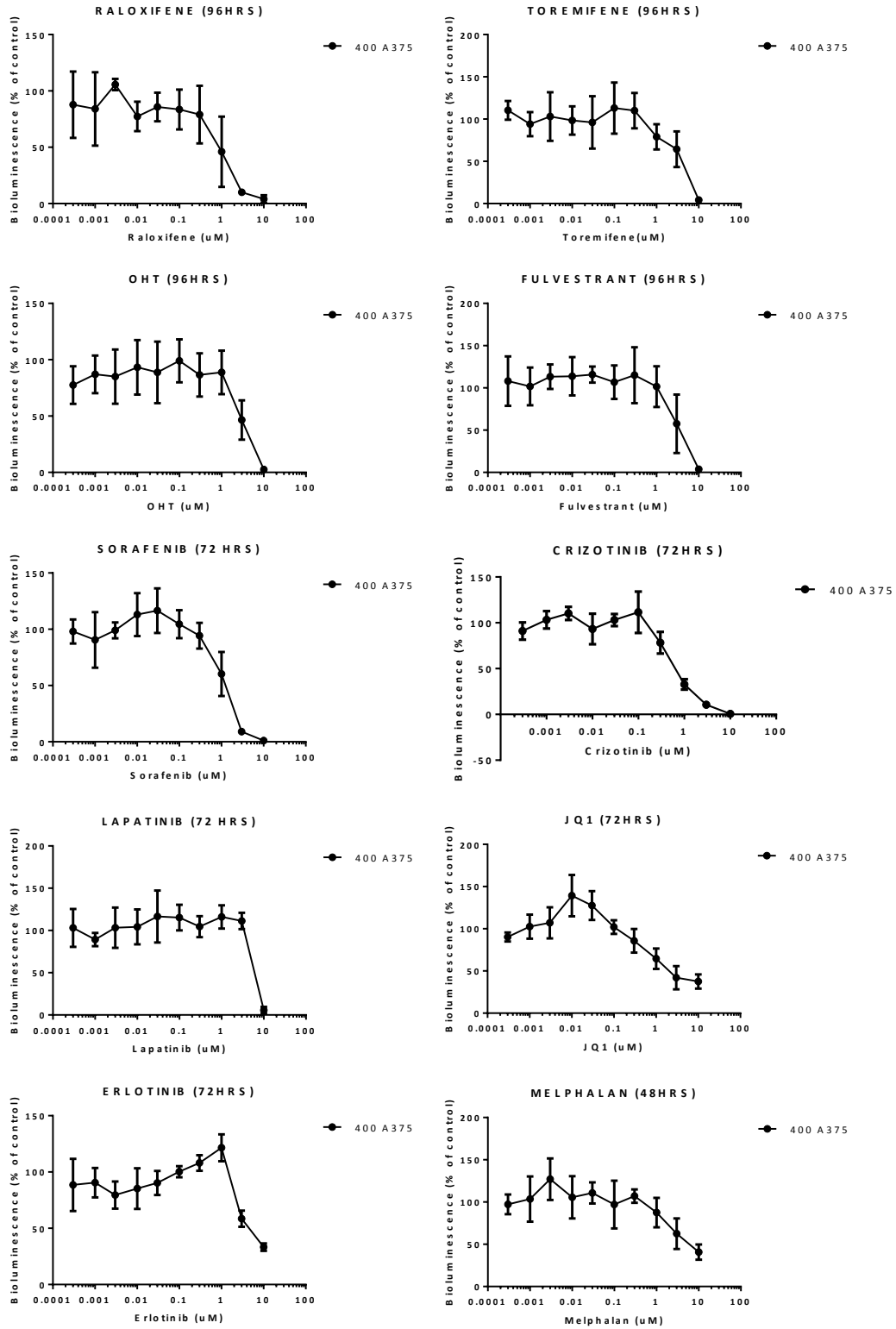
IN VITRO TESTING OF ANTI-TUMOR AGENTS AGAINST A375 CELLS

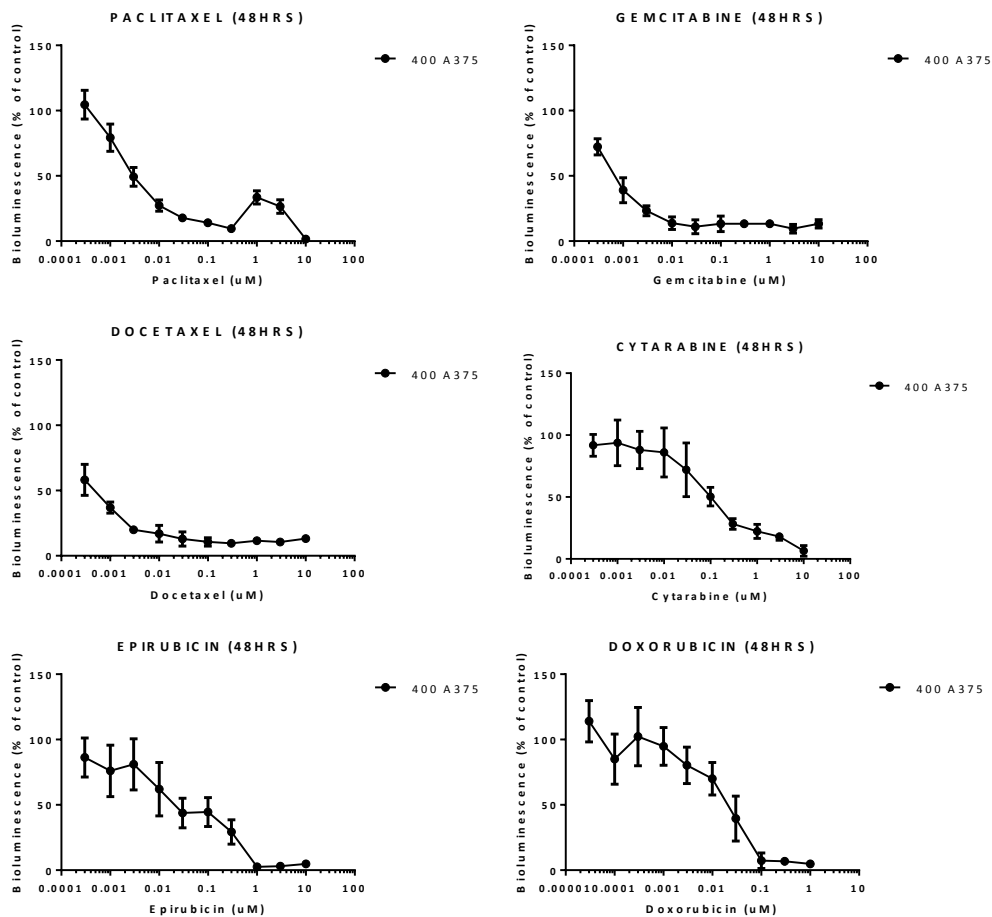
Twenty-four anti-cancer drugs were tested on A375. Each drug was individually dosed and then read at 24, 48, 72, or 96 hours to generate dose response curves. The viability of A375 cells in wells receiving drugs were compared to the viability of A375 cells in control wells that were not treated with any drug. The compounds then fell into three categories depending on how effectively they killed A375. (A) The first category included drugs that had little effect on A375 at the given dosages. These included MDV3100, bicalutamide, dexamethasone, cisplatin, 5-fluoracil, pomalidomide, and lenalidomide. Dexamethasone, pomalidomide, and cisplatin even showed a very slight increase in viability at higher doses. (B) The next category included those that only showed killing at very high doses. Those included toremifene, raloxifene, OHT, fulvestrant, sorafenib, lapatinib, JQ1, erlotinib, melphalan, and doxorubicin. Erlotinib even showed an increase in viability at 1 μM followed by a sharp drop off in proliferation. (C) The last category included drugs that showed significant killing of A375 even at lower doses. These drugs included crizotinib, bortezomib, cytarabine, epirubicin, gemcitabine, paclitaxel, and vemurafenib. Of these drugs, docetaxel, gemcitabine, and vemurafenib proved to be the most effective at killing A375. Docetaxel and gemcitabine treated A375 showed less than 50% proliferation compared to the untreated control at doses as little as 0.001 μM .

A



B.



C**Figure 4: Drug Selection**

Twenty-four known anti-cancer drugs were tested on A375 cells. Various concentrations were used in order to generate a dose-response curve to look for A375 cell killing compared against a no drug control. Graphpad Prism 6 was used to generate the dose-response curves. (A) Drugs that showed no or little killing of A375 cells. (B) Drugs that showed A375 cell killing at high doses. (C) Drugs that showed killing even at lower doses.

STROMAL CO-CULTURE FOR SELECTED DRUGS

Seven anti-cancer drugs that were previously selected for A375 killing were tested on A375 in the presence and absence of different stroma. Five were

selected from the category of drugs that showed a pronounced effect on A375 cells at lower doses. In addition to these, both crizotinib and bortezomib were also used. Each drug was individually dosed and then read at 24, 48, 72, or 96 hours to generate dose response curves. The results for each curve were normalized to their respective no drug control. (A) Dose response curves in the presence and absence of CCD13LU stromal cells were created for the seven drugs. Bortezomib, crizotinib, epirubicin, gemcitabine, and paclitaxel did not show any changes in drug response in the presence of CCD13LU cells. Cells treated with cytarabine at low doses (up to 0.0005 μ M) showed a slight stromal cell-mediated resistance to this agent in the presence of CCD13LU cells. At higher doses no resistance was observed. Vemurafenib was subject to slight resistance when cells were treated in the presence of CCD13LU cells. (B) Dose response curves were also created for A375 when co-cultured with HS888LU. Crizotinib, cytarabine, epirubicin, gemcitabine, and paclitaxel all showed no significant differences in response when co-cultured with HS888LU. Bortezomib showed very slight sensitization for A375 killing at 0.03 μ M. Vemurafenib had slightly reduced killing on A375 in the presence of HS888LU. (C) Dose response curves were also created for A375 when co-cultured with LL86. It did not appear that co-culture with LL86 led to significant drug resistance or sensitization for any of the seven compounds tested.

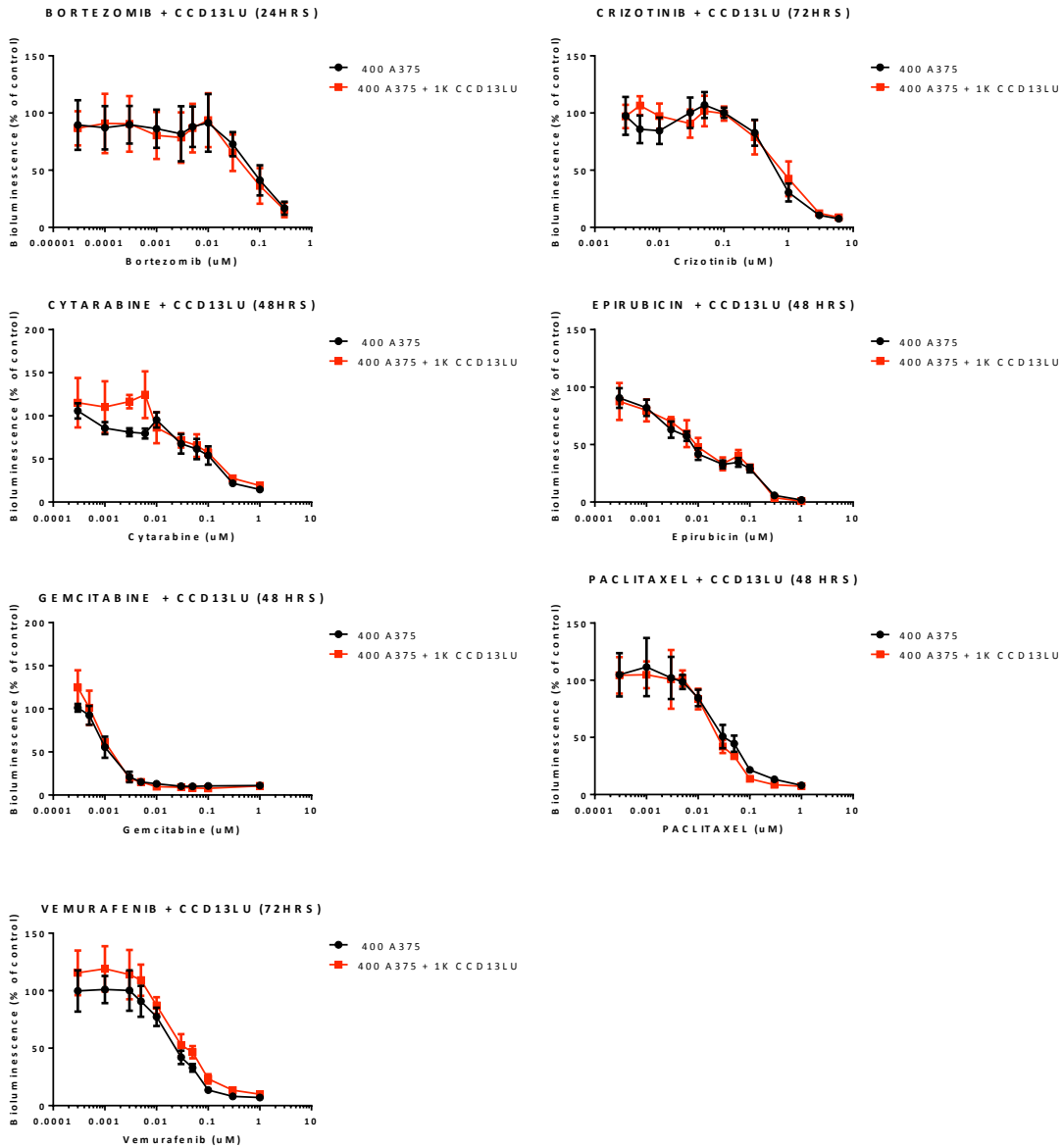


Figure 5: Dose Response Curves with CCD13LU Cells for Selected Drugs
 Seven drugs were tested on A375 cells in the presence of CCD13LU cells. Various concentrations were used in order to generate a dose-response curve to look for A375 cell killing compared to a no drug control. Dose-response curves for A375 cells in the presence and absence of stroma were superimposed on the same graph to show differences between the two. Graphpad Prism 6 was used to generate the dose response curves.

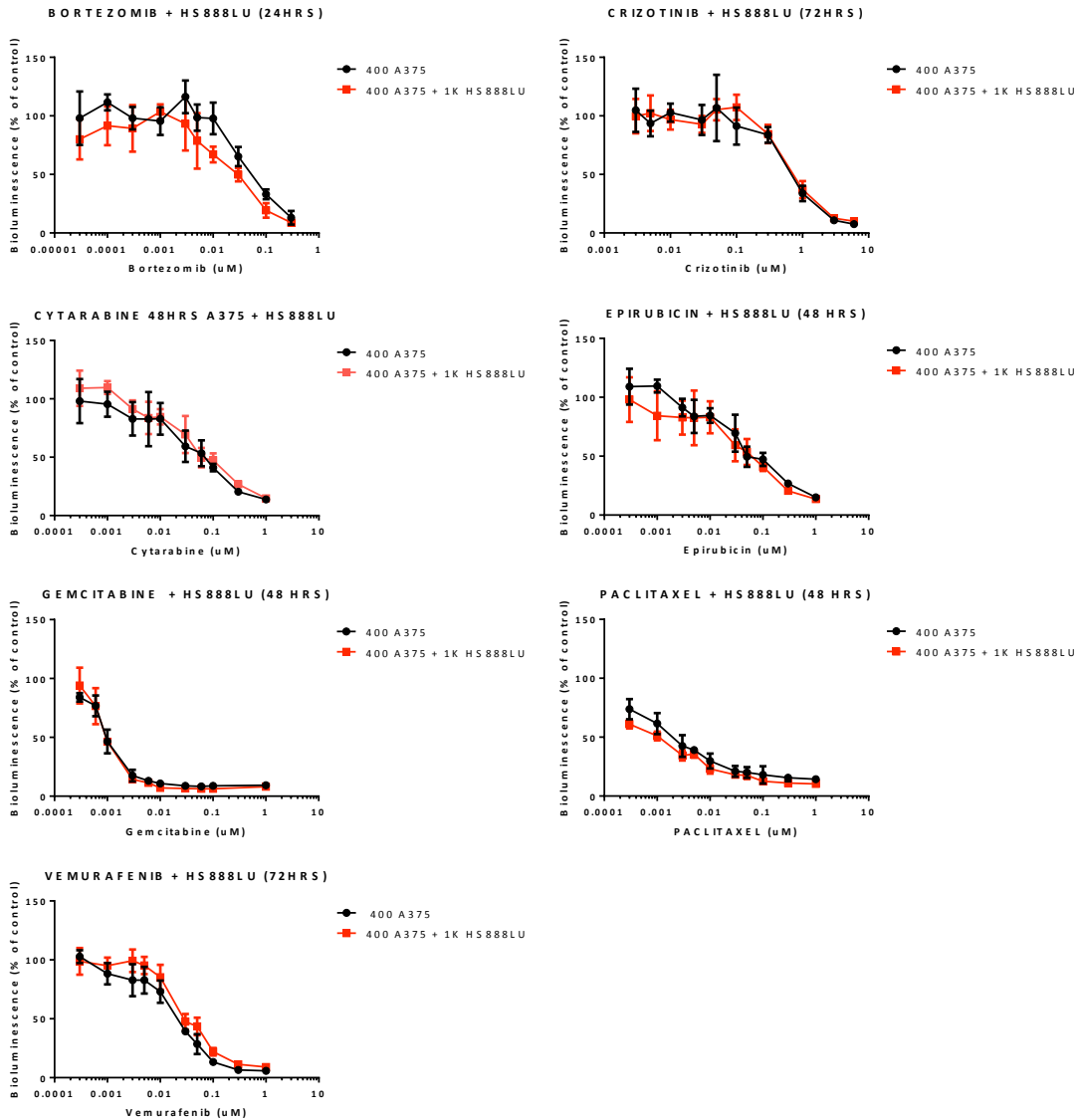


Figure 6: Dose Response Curves with HS888LU Cells for Selected Drugs
 Seven drugs were tested on A375 cells in the presence of HS888LU cells. Various concentrations were used in order to generate a dose-response curve to look for A375 cell killing compared to a no drug control. Dose-response curves for A375 cells in the presence and absence of stroma were superimposed on the same graph to show differences between the two. Graphpad Prism 6 was used to generate the dose response curves.

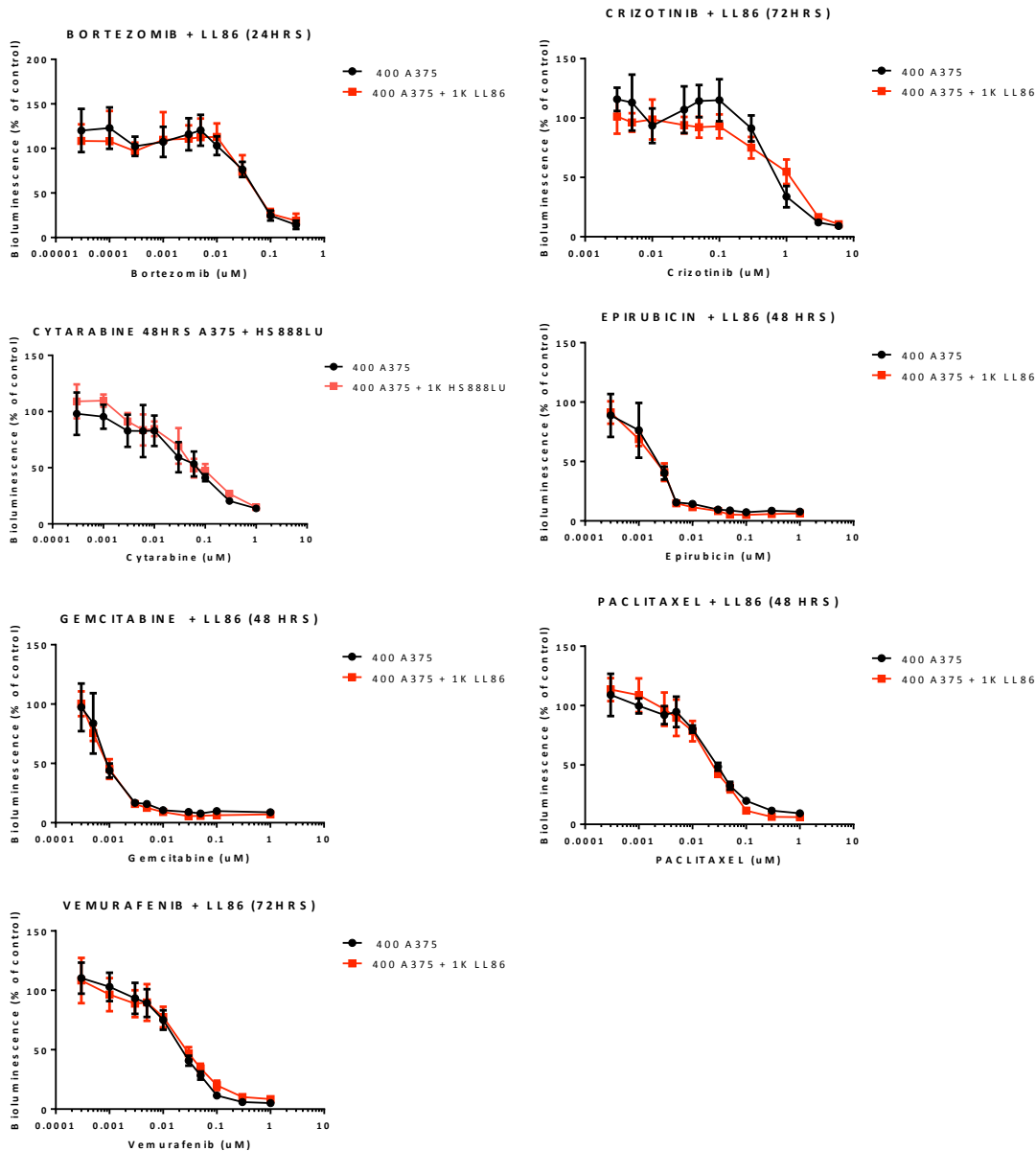


Figure 7: Dose Response Curves with LL86 Cells for Selected Drugs

Seven drugs were tested on A375 cells in the presence of LL86 cells. Various concentrations were used in order to generate a dose-response curve to look for A375 cell killing compared to a no drug control. Dose-response curves for A375 cells in the presence and absence of stroma were superimposed on the same graph to show differences between the two. Graphpad Prism 6 was used to generate the dose response curves.

DISCUSSION

This study demonstrated that certain drugs do behave differently on the melanoma cell line A375 in the presence versus absence of stromal cells. Some key findings of this study include: (i) Vorinostat appears to have an increased effect on A375 cells in the presence of stromal cells. (ii) Procarbazine HCL has significantly less killing efficiency in the presence of CCD1065SK or CCD13LU cells. (iii) Paclitaxel, gemcitabine, docetaxel, cytarabine, epirubicin, doxorubicin, and vemurafenib all showed significant killing of A375 cells even at low doses. (iv) Of these drugs, only vemurafenib and cytarabine showed some reduced efficacy on A375 in the presence of stromal cells. These findings suggest that stromal cells influence the melanoma cell drug response.

FDA Oncology Screen

In order to get a comprehensive assessment of the potential of stromal cells to influence melanoma cell drug response to diverse established anti-tumor agents, while simultaneously identifying new drugs of interest, a screen using FDA approved oncology plates was performed. While not all of the compounds included in this analysis are used clinically for melanoma treatment, they are all used (or have been approved in the past) as cancer chemotherapeutics for various neoplasias. Eighty-one different compounds at two different concentrations and with two different stromal lines a few drugs of interest were identified. Of the eighty-one compounds, four drugs were identified to be of the

most interest. A drug with notable results was procarbazine HCL. The effects of procarbazine HCL were diminished significantly in the presence of both CCD1065SK as well as CCD13LU cells. In the absence of stroma cells, A375 proliferation was on average across all four different conditions, 10.6% compared to the no drug control (100%). In the presence of stroma, the average survival percentage across the four different conditions was 102%. An average difference of over 90% survival was the largest margin of resistance seen for any drug during this study. Procarbazine HCL, more commonly known as Matulane, is a hydrazine derivative anti-neoplastic agent. As an alkylating agent, the drug impairs DNA and RNA synthesis. Specifically it is thought to impair trans-methylation of methionine and thus leads to inactive t-RNA.[18] The drug is typically used to treat Hodgkin's lymphoma however presently other therapeutic options for this agent are being explored.

Another drug that showed some resistance in the presence of stromal cells was dasatinib. A375 showed resistance to dasatinib at both concentrations and for both doses. While not as pronounced as in the case of procarbazine HCL there still seemed to be a decrease in activity of dasatinib in the presence of stromal cells. Dasatinib is FDA-approved for the treatment of Chronic Myeloid Leukemia.[19]

Some drugs showed greater killing in the presence of stroma. One such drug was vorinostat. Vorinostat is an HDAC inhibitor that is generally used to treat cutaneous T cell lymphoma.[20] This drug is of particular interest as

vorinostat has been proven to be effective against melanoma. Not only has this been effective in cutaneous melanoma but also as a treatment for uveal melanoma.[21, 22]

One last drug on interest identified from this experiment was tamoxifen citrate. Also known as Nolvadex, tamoxifen citrate is a precursor that gets metabolized to hydroxytamoxifen. Tamoxifen is an estrogen receptor antagonist and has been shown to be effective in treating breast cancer.[23] The largest increase in drug effectiveness was seen in the presence of CCD1065SK cells at 0.5 micromolar. Interestingly enough, when combined with CCD1065SK cells at 0.1 micromolar the drug was less effective then when treated on A375 alone.

Identification of compounds effective against A375

In order to determine which drugs were effective at killing A375 cells, a screen using 24 different cancer drugs were used at multiple doses against A375. The first compounds identified were those that had no effect on A375 killing. These drugs included dexamethasone, bicalutamide, MDV3100, cisplatin, lenalidomide, 5-fluoracil, and pomalidomide. None of these drugs had any effect on A375 cell death. Interestingly enough dexamethasone even increased proliferation at higher doses. This was expected as dexamethasone is a glucocorticoid that has been known to stimulate melanin production. As seen in a study by Tang et al., A375 cells are known to have a slight increase in proliferation when treated with dexamethasone.[24] Also as expected, anti-

angiogenic thalidomide derivatives such as lenalidomide and pomalidomide had no effect on A375 cell killing, as the cells have not developed into a tumor. Bicalutamide and MDV100, androgen receptor antagonists also had no effect even up to 10 micromolar suggesting that this pathway does not play a significant role in A375 cell signaling and growth pathways. Cisplatin also had little effect on A375 cells. It has been hypothesized that endogenous nitric oxide helps the cell to evade Cisplatin induced apoptosis and could perhaps explain a lack of effect. [25]

The most interesting drug in this group was 5-fluoracil. Of the drugs in this category 5-fluoracil is the drug most commonly prescribed for skin cancer. 5-fluoracil can be used topically to treat basal cell carcinomas (the most common type of cancer).[26] Acting as pyrimidine analog, the drug interferes with DNA synthesis and eventually leads to apoptosis.[26] However, it had little effect on A375 cells.

The next group of compounds were the drugs that showed killing of A375 only at higher doses. Of these 10 compounds, 4 can be categorized as anti-estrogens. Raloxifene, OHT, toremifene, and fulvestrant are estrogen receptor antagonists. It has been documented that estrogen receptors play a potential role in melanoma, including the process of metastasis. [27] Most of these compounds started killing A375 cells between 0.1 and 1 micromolar, with toremifene and raloxifene being slightly more effective in that range.

The next class of compounds to make up a majority of the high dose responders include the kinase inhibitors. These include: sorafenib, lapatinib, erlotinib, and crizotinib. Of these, crizotinib and sorafenib were the most effective at A375 killing. Sorafenib is of particular interest because like vemurafenib, it can inhibit the Raf kinases. Sorafenib however is more likely to bind and inhibit CRAF than BRAF.[7] At higher doses both are inhibited and this can explain the observation of more killing at higher doses. Lapatinib and erlotinib are both EGFR inhibitors and only started to kill A375 cells at doses above 1 micromolar. The last two drugs in this category include JQ1 and melphalan. Melphalan has had mixed reviews in regards to therapeutic uses for melanoma. Some studies have shown effectiveness while others claim the drug has little use.[28]

The last group of drugs were the drugs that significantly killed A375 cells, even at lower doses (as low as 0.0003 micromolar). These drugs included paclitaxel, docetaxel, epirubicin, gemcitabine, cytarabine, and doxorubicin. Paclitaxel and docetaxel are both potent anti-mitotics that interfere with microtubule assembly and thus cell division.[29] Docetaxel proved to be the most effective drug at killing A375 cells (along with gemcitabine). Docetaxel is known to be effective in treatment for metastatic melanoma.[30] The remaining drugs (gemcitabine, doxorubicin, epirubicin, and cytarabine) all interfere with DNA synthesis. Interestingly, none seem to be effective in patients with metastatic melanoma.[31, 32]

Stromal Co-culture with A375 Selective Drugs

After the compounds which proved to be effective at killing A375 were identified, the next step was to determine if any of these drugs (as well as bortezomib and crizotinib) showed added or reduced activity on A375 in the presence of three different stromal cell lines. The three stromal cell lines that were selected were CCD13LU, HS888LU, and LL86. CCD13LU, HS888LU, and LL86 are all human lung fibroblasts. The main reason for why these cell lines were chosen was that they had been used in a previous study by Straussman that tried to determine differences in melanoma cell drug response in the presence of various stromal lines. [16] These cell lines were able to significantly rescue some melanoma lines from vemurafenib. This was determined to be an effect of the high levels of HGF that these cell lines produced. However in regards to A375, they were unable to cause significant resistance to vemurafenib. For this study, we hoped to determine if these cell lines could have an affect on any of the other drugs that showed significant killing on A375 cells.

We tested the chosen cell lines in the presence of CCD13LU cells. Bortezomib, gemcitabine, crizotinib, epirubicin, and paclitaxel showed no significant change in killing when in the presence of CCD13LU. Interestingly, CCD13LU cells seemed to increase A375 proliferation when treated with cytarabine at lower doses (0.001-0.005 micromolar). However at levels at or higher than 0.01 micromolar, the two curves were no longer significantly different.

Also as expected CCD13LU cells had a slight rescue effect on A375 cells when the latter were treated with vemurafenib. This was expected, and had been previously seen. (Straussman et al.)

The second stromal line that was evaluated was HS888LU. HS888LU seemed to slightly increase the effectiveness of bortezomib at both lower and higher doses. One study that involved bortezomib and HGF has shown that patients suffering from multiple myeloma with higher levels of HGF respond worse to bortezomib when they had higher levels of HGF.[33] This suggests that HS888LU potentially secretes another cytokine or growth factor that increases the effectiveness of bortezomib. Vemurafenib also had a slightly reduced effect on A375 in the presence of HS888LU. This was also expected and can be attributed to the higher levels of HGF. The rest of the drugs tested did not seem to respond differently in the presence of HS888LU.

The last stromal line tested was LL86. Interestingly, LL86 did not have an effect on any of the drugs tested.

Limitations to the Study

Having compiled these data, it is understood that some additional studies could have made this study more insightful. For example, it would have been of interest to conduct a much larger screen of compounds with more stromal cell lines. For this study, only lung and skin fibroblasts were used primarily because melanoma mainly originates in the skin and the lungs are a major site of

metastasis. It could be of interest to see how liver fibroblasts, astrocytes, or fibroblasts of other organs might affect melanoma cell drug response. Another limitation of this study was that only one melanoma line was tested. For follow-up studies, it would be valuable to compare different types of melanoma cell lines and how they respond differently to drugs in the presence or absence of stroma. Ideally at least one cell line would be wild type for BRAF mutation status. This could lead to additional studies of interest especially in regards to vemurafenib and other BRAF mutant selective drugs. Additionally several follow-up mechanistic experiments could have been completed.

Ideas for Future Studies

As previously indicated, perhaps the most important future study would be to screen for candidate anti-cancer drugs in the presence of higher numbers of stromal lines. Specifically, stromal lines from organs/tissues which melanoma is known to frequently metastasize to. For instance, testing more lung fibroblasts, hepatocytes, astrocytes, and other accessory cells could yield interesting results. Another idea is to determine exactly how the stromal cells are affecting drug response. As seen in the study of Straussman et al, they were able to identify HGF as the main mediator for stromal cell-mediated resistance to vemurafenib in a variety of melanoma cell lines. However it may be possible that other growth factors or cytokines are responsible for altered drug response in other stromal cell lines. By identifying exactly what molecule(s) are conferring drug resistance

or sensitization, it would be much easier to identify targets for possible therapeutic interventions. One last idea for future studies include using a wider variety of doses. As seen in the experiments of the current study, some doses showed a difference in activity while other doses for the same experimental conditions showed no change in drug activity at all.

Conclusion

In this study we wanted to observe if stromal cell-mediated alterations in drug response played a significant role in melanoma. By first developing a screen of a large number of compounds using multiple stromal cell lines we were able to identify a few compounds which showed a significant amount of altered drug activity in the presence versus the absence of stromal cells. Procarbazine HCL, tamoxifen citrate, vorinostat, and dasatinib showed the most dramatic changes in activity when exposed to the presence of stromal cells. Then, after selecting for drugs that showed significant A375 killing, we were able to generate dose response curves in the presence versus absence of stroma in order to observe stromal mediated changes in A375 drug response. We found that vemurafenib, bortezomib, and cytarabine showed the largest changes in drug activity when in the presence of stroma.

As seen by this study, stromal cells can significantly alter melanoma cell drug response. This fact has numerous implications for studies involving acquired tumor resistance to chemotherapy, as well as overall tumorigenesis. We consider that further study needs to be done to identify the effects of human

stromal lines on cancer development. Based on our observations in this study, we also propose that current models for drug identification need to account for stromal mediated changes in drug response in order to more accurately estimate how anti-cancer drugs may perform in vivo.

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