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Sorafenib induces synergistic effect on inhibition of vemurafenib resistant melanoma growth

Ankita Srivastava[1], Anbalagan Moorthy[1]

[1]School of Biosciences and Technology, Vellore Institute of Technology, Vellore, Tamil Nadu, India 632014

TABLE OF CONTENTS

1. ABSTRACT

Vemurafenib is a B-raf inhibitor which is widely used in treatment of melanoma patients with B-RAF V600E mutation. Majority of patients treated with vemurafenib develop resistance against the drug. Here, we asssessed the effectiveness of a combination drug therapy in vemurafenib resistant melanoma cells. Vemurafenib resistant A375 melanoma cells (A375Res cells) were developed by growing parental cells in increasing concentrations of the drug. The A375Res cells were 50 times more resistant (higher IC_{50} value), had reduced cell doubling time, were less responsive to the antiproliferative activity of Vemurafenib and showed increased tumor forming potential as compared to the parental cells. Vemurafenib inhibited phosphorylation of MEK 1/2 and ERK 1/2 at the concentrations far less than those that were effective in parental cells. Compared to the other drugs sorafenib in combination with vemurafenib significantly inhibited proliferation of A375Res cells. These findings show that Sorafenib, in combination with Vemurafenib, is a more effective method for treatment of melanoma with B-Raf 600E mutation.

2. INTRODUCTION

Transformation of melanocytes to cancerous cells leads to melanoma. Melanoma is the fifth in males and the sixth most common malignancy in women (1). Enormous advances in the treatment of melanoma have occurred in recent years. For example, the identification of molecular players which are involved in the cause of disease and the development molecules specifically targeting the signalling molecule/pathway involved in the disease. These therapeutic advances have provided foundations for further improvements in treatment protocols and management of the disease with reduced side effect and increase in survival rate

of cancer patients. One of the major drawbacks in treating cancer patients with a drug is patient's short duration of response and later developing resistance to that drug or toxicity related to the treatment of drugs. Efforts are currently being made to understand the molecular basis of resistance and also to improve treatment protocol with newer drugs.

BRAF is one of the important signalling molecules involved in cellular proliferation and deregulation of BRAF signalling has been associated with several forms of cancer. BRAF is also shown to have V600E activation somatic mutation in a wide range of cancers including 40% to 70% of malignant melanomas depending upon the geographic location (2–4). BRAF-mutated melanoma tumours have been correlated with poor response to traditional chemotherapy treatment and poor prognosis as well. (5) Targeted therapies are of great interest for these types of cancers (6) and intense research on BRAF kinase with V600E mutation has led to development of vemurafenib. (7) Vemurafenib specifically inhibits BRAF kinase with V600E mutation. This approach of using targeted drugs against oncogenic kinases with somatic activating mutations has been successful in the treatment of various cancers. Thus vemurafenib is the choice of drug for treatment of melanoma patients with BRAF V600E mutation (8). Like any other cancer, the most prevailing limitations in treatment of melanoma is the resistance developed by cancer cells to available treatment methods and acquiring resistance to the drug, thus melanoma patients with BRAF V600E mutation develop resistance for vemurafenib (9), hence improved treatment methods are needed in treating melanoma patients who develop resistance against vemurafenib.

A newer approach, which has been investigated recently, is to determine whether inhibition of two signal transduction pathways is a more effective means to treat cancer (10). Combining more than one active therapeutic compound to overcome resistances is the key to make further advances in the treatment of drug resistance cancer cells. Synergism and antagonism are two terms used in combinatorial therapy. Synergism is defined as greater effects of more than one combination of drugs instead of simple additive effect of each drug and antagonism is defined as lesser effects for the same (11). As per Chou TC (2006) (12) more than 13 different methods exists for determining synergism and for various reasons they claim that combination index (CI) is the simplest possible scale for determining synergism and antagonism, Chou TC (2010). (13)

In order to identify novel drug combinations to treat vemurafenib resistant melanoma, we have developed vemurafenib resistant A75 cell line and determined synergistic effect of various drugs in combination with vemurafenib in inhibiting A375 resistance cell proliferation.

3. MATERIALS AND METHODS

3.1. Development of Vemurafenib-resistant A375 cells

A375 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% antibiotic solution. The cells were grown in 175-cm^2 culture flasks by incubating in a humidified incubator with 5% CO_2 at 37^0 C and sub cultured when the cells reach 80% confluency.

Vemurafenib resistant cells were developed by initially culturing the cells in Vemurafenib with a starting concentration of 10 nM. Once the cells started growing normally to the drug, concentration of the drug was increased gradually until 20µm. The cells were cultured in vemurafenib free medium for two months and tested for resistance to drug before performing other experiments.

3.2. Cell proliferation assay

For calculating IC_{50} values, $5x10^3$ cells were seeded per well in a 96 well tissue culture plate and incubated overnight. After incubation period, the cells were challenged with varying concentrations of vemurafenib. An equal concentration of vehicle (DMSO-never exceeding 0.01%) was used as a control. After 48 hrs of treatment, 10 µl of CCK-8 reagent were added to each well and incubated for 3 hrs at 37°C. After 3 hrs, the absorbance was measured on Tecan Sapphire multi-fluorescence micro-plate reader (Tecan, Germany, GmbH) at a wavelength of 450 nm corrected to 650 nm and normalized to controls. Absorbance values of DMSO treated control cells were considered to have a cell viability of 100%. The IC_{50} values were calculated from the vemurafenib logarithmic cytotoxicity curves of the parental cells and its resistant cells. The four parametric nonlinear hill slope logistic curve was determined by plotting the percentage of cell proliferation against vemurafenib concentration (log values) by using Graph Pad Prism software.

3.3. Wound healing assay for cell motility

A375 or A375Res cells were seeded (5 X 10^3 cells) per well in a 96-well plate and allowed to attach to plate for 16 hrs. By using a 200µl tip and scale a scratch was made on the monolayer of cells and floating cells were removed from the wells by rinsing twice with medium and incubated at 37°C. Monochrome images were taken at 0 hrs and after 6 hrs of incubation and analysis was performed using Image J software. The data obtained was plotted in graph pad prism software.

3.4. Cell cycle analysis

A375 and A375Res cells were serum starved for 24 hrs and incubated with 3 and 1 µM of vemurafenib for 24 hrs in complete medium, after the incubation period, cells were harvested and washed twice with ice cold PBS and fixed in ice cold 70% ethanol at 4^0C for overnight. The fixed cells were centrifuged, washed and re suspended in PBS containing RNase (1 mg/ml) and Propidium Iodide (25 ug/ml in PBS) and incubated at RT for 45 min before analysing by FACS Calibur (Becton Dickinson). The data was analysed using Flow Jo 7.6.5. software (Ashland, OR).

3.5. 3D cell culture

Spheroids were developed as described previously (14). A375 and A375Res cells ($5x10^3$ cells) in 100 µl medium per well were seeded in a 96 well ultra-low attachment U-bottom tissue culture grade plate and incubated at 37°C for overnight. After formation of spheroids (Approx. 24 hrs.), spheroid imaging was done on day 1, 4, 8, 11 and 14 by using Carl Zeiss Axio Lab A1 microscope.

3.6. Western blot analysis

A375 and A375Res cells were pre-treated with different concentrations of Vemurafenib or 0.01% DMSO for 6 Hrs. After the incubation time, cells were washed harvested and total protein extraction was done using RIPA lysis buffer. Protein quantification was done by Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Equal quantity of protein (50 µg) was loaded on pre-cast 4–20% SDS polyacrylamide gels and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Blots were blocked in 5% non-fat dry milk for 1 hrs after blocking, the blots were incubated with required dilution of the primary antibody prepared in SuperBlock Blocking Buffer at 4°C overnight with gentle rocking. The antibodies used were: rabbit anti-pERK1/2 (Cell Signaling), rabbit anti-CyclinD1 (Cell Signaling), rabbit anti-pMEK1/2 (Cell Signaling) and housekeeping protein, rabbit anti-β-actin (Sigma Aldrich) as a loading control. Following the incubation time, membranes were washed and then probed with horseradish peroxidase-conjugated polyclonal (secondary) antibody to rabbit IgG for one hr followed by washing (Calbiochem; Darmstadt, Germany). The blots were visualized and imaged with chemiluminescent peroxidase substrate (Sigma Aldrich) and the Kodax Image Station (Kodak Molecular Imaging Systems; CT, USA).

3.7. Combination studies

For calculating synergy between drugs, $1.5x10^3$ cells were seeded per well in a 384 well tissue culture plate and incubated overnight. After incubation period, the cells were challenged with varying concentrations of individual drugs as well as combination of drugs in various ratios. An equal concentration of vehicle (DMSO-never exceeding 0.01%) was used as a control. After 72 hrs of treatment, 3 µl of CCK-8 reagent were added to each well and incubated for 3 hrs at 37°C. After 3 hrs, the absorbance was measured on Tecan Sapphire multi-fluorescence micro-plate reader (Tecan, Germany, GmbH) at a wavelength of 450 nm corrected to 650 nm and normalized to controls. Raw data were normalized to DMSO-treated controls and IC_{50} values were calculated via nonlinear regression curve fit, with error bars representing 95% confidence intervals (GraphPad Prism 6, La Jolla, CA). Calcusyn software (Biosoft.) was used to calculate synergy based on the methodology and algorithms of Chou. (12). The synergy values obtained were plotted in microsoft excel in a heatmap.

4. RESULTS

4.1. Development of vemurafenib resistant A375 cells (A375Res cells)

A375 cells are human melanoma cell lines with BRAF V600E mutation. We wanted to develop vemurafenib resistant A375 cells which could be used to identify synergy between vemurafenib and other cancer treating drugs so that a good combination of drugs could be arrived to treat cancer patients with Raf V600E mutation, who develop resistance to vemurafenib treatment. Towards this objective, A375 Res cells were developed by culturing A375 cells with gradual increase in vemurafenib concentration as mentioned in material and methods. These resistant cells developed had an IC_{50} of 5 ± 0.1 µM for vemurafenib compared to the non- resistant A375 cells which had an IC_{50} value of 0.05 ± 0.01 µM (Figure 1a).

4.2. Rate of proliferation of A375 Res is higher compared to the A375 cells

Proliferation rate of A375 and A375Res cells were measured in terms of a) doubling time and b) ability to form tumours in *in vitro* 3D model system. Equal number of A375 and A375Res cells were cultured for indicated time points and doubling time for the cells were calculated. As seen in Figure 1b, the doubling time for A375 cells were 12.99 hrs and A375Res cells were reduced to 10.94 hrs. In 3D growth assay, As seen in Figure 1c, volume of the tumor gradually increased from day 1 till day 11 beyond which much growth was not observed. However, compared to A375 cells, the rate of increase in the volume of tumour formed by A375Res cells was high. On day 11, the radius of spheroids formed by A375 cells were smaller (5.11 ± 0.11 µm) compared to A375Res cells (9.5 ± 0.2 µm).

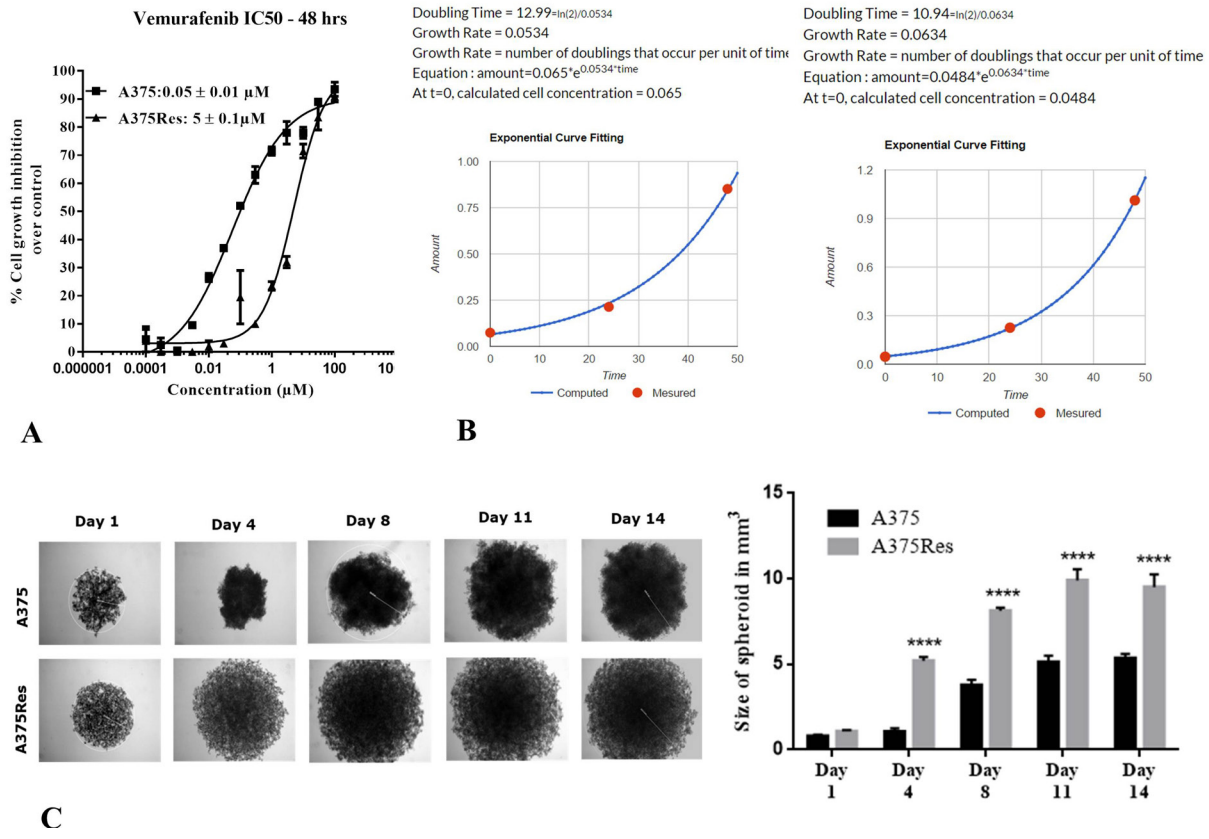

Figure 1. a) Vemurafenib IC_{50} values for A375 and A375Res cells. Vemurafenib strongly reduces proliferation of human melanoma cell lines while the effect of vemurafenib is greatly reduced in A375Res cells as reflected in IC50 values (0.01 µM A375 Vs 0.1 µM A375Res). b) Doubling time for A375 and A375Res cells. A375Res cells divide faster compared to A375 cells as reflected in doubling time, The doubling time of A375 cells is almost 13 hrs while the doubling time of A375Res cells was 10.94 hrs. c). Rate of *in vitro* tumour formation in A375 and A375 Res cells. Rate of *in vitro* tumour formation is faster in A375Res cells compared to A375 cells, The tumor radius A375 tumor at the end of day 14 was 5.33 ± 0.08µm while its was 9.51 ± 0.22 µm in the case of A375Res tumor.

4.3. Resistant cells had higher metastatic potential and resistant to vemurafenib mediated cell cycle arrest

Since cancer patients who developed resistance to Vemurafenib are also reported to have higher incidence of metastasis and recurrence (15), we have compared the metastatic potential of A375 and A375Res cell lines. Scratch assay, a well-known assay to evaluate metastatic potential of a cell. By considering wound percentage of wound closure in A375Res cells as 100 %, the percentage of wound closure was 21.3% in A375 cells (Figure 2a). Vemurafenib, being inhibitor of BRAF with V600E mutation, it blocks cells at G1 stage of cell cycle (16), in this regard we wanted to compare the cell cycle pattern of A375 and A375Res cells in presence of vemurafenib. It can be observed that Vemurafenib arrests the A375 cells in G1 phase, while in case of A375Res cells close to 20% of cells were in S phase of cell cycle even in the presence of vemurafenib (Figure 2b).

4.4. Vemurafenib inhibits ERK signalling only in A375 cells and not in A375Res cells

Vemurafenib acts on Raf signalling molecule with V600E mutation and inhibiting further downstream signalling pathway, we have tested the effect of vemurafenib in A375 and A375Res cell on levels of active signalling molecules such as phospho-MEK1/2 level, phospho - ERK 1/2 and cyclin D1; the downstream signalling molecules of Raf signalling leading to activation of cell cycle. Parental and resistant cell lines were treated with either DMSO (vehicle control) or vemurafenib in various concentrations for 6 hrs and Western blot analysis was carried out using antibodies for the above mentioned proteins. Vemurafenib dramatically and serially inhibited levels of pMEK 1/2 and pERK 1/2 in A375 cells but in the case of A375Res cells the inhibition was not observed at 0.1 µM vemurafenib and a significant inhibition of phosphorylation of the signalling molecules was observed only at 10 µM concentration. As observed

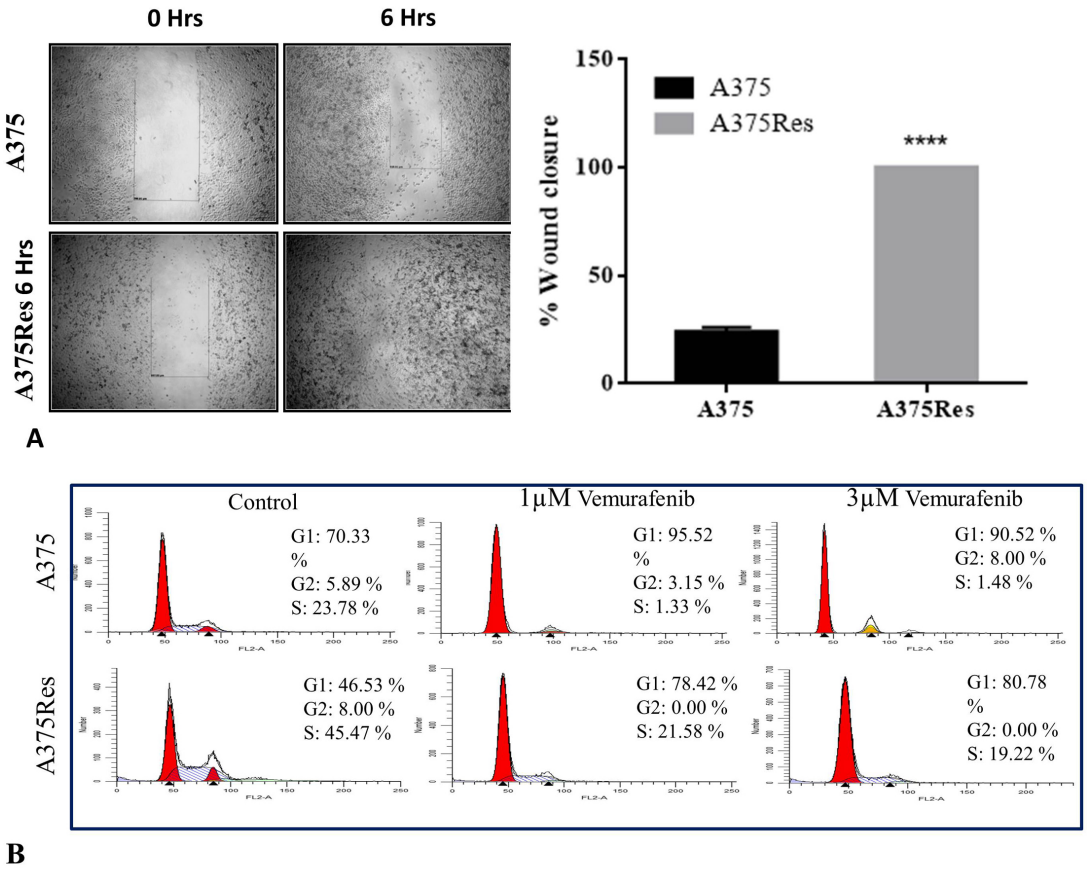

Figure 2. a) Cell motility assay. Images of culture plates taken after creating scratch and after 6 hrs of incubation. The wound was completely closed within 6 hrs is case of A375Res cells while hardly any closure was observed in the case of A375 cells. b) Effect of Vemurafenib on proliferation of A375 and A375 Res cells. FACS analysis shows number of A375Res cells in S phase in presence of vemurafenib is more compared to A375 cells. (21.58 % vs 1.33% at 1uM and 19.22 % compared to 1.48 % at 5 uM.

in p-MEK1/2 and p-ERK 1/2, levels the effect of vemurafenib was reduced on cyclin D1 levels.

4.5. Vemurafenib has synergistic potential on resistant cells when combined with other anti-cancer small molecule inhibitors

Combination therapy is widely used in treatment of various diseases, where more than one active ingredient is used in the treatment. With the growing interest in combination therapies, we have tested ability of vemurafenib to synergise with different cancer treating drugs such as a) Etoposide (DNA damaging agent), b) Sorafenib (general kinase inhibitor) c) BEZ 235 (PI3 kinase inhibitor) d) Pacilitaxel (microtubules stabilizing agent) in inhibiting proliferation of A375 and A375Res cells. The cell lines were treated with different concentration of Vemurafenib and along with any one of the anti-cancer agents in different concentration and combinations, as well as individually for 72 hrs. The synergy was calculated using CI (Combination Index). The lower the CI, the higher the synergy is. As mentioned in materials and methods, syngery between molecules was calculated using Calcusyn software and plotted on Microsoft excel. As can be seen in the Figure 3, there was not much difference in synergetic values between A375 and A375Res cells in vemurafenib - etoposide as well as in vemurafenib - Pacilitaxel combinations. It was interesting to note that Vemurafenib – BEZ235 and Vemurafenib – Sorafenib exhibited good synergy for a wide range of concentration in A375Res cells compared to A375 cells.

5. DISCUSSION

Cancer cells ability to develop resistance against a particular drug is one of the main concerns in treating cancer patients globally irrespective of the type of cancer (17). Cancer cells that show resistance to a particular drug either produces a mutant protein (by somatic mutation) on which the drug can't act anymore or goes through a rescue signalling molecule/pathway to overcome the drug mediated inhibition (18). Vemurafenib targeting BRAF with V600E mutant is a very good drug to

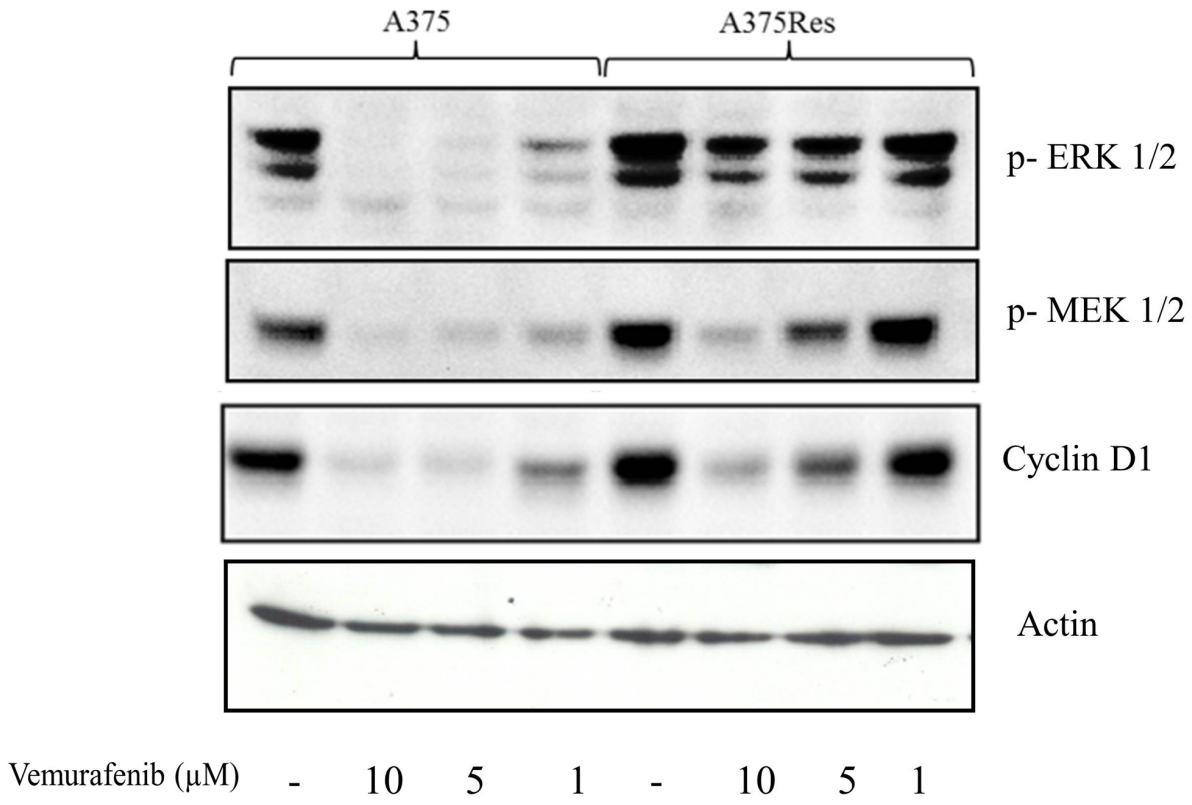

Figure 3. Comparison of synergy between vemurafenib and other small molecules in inhibiting proliferation of A375 and A375Res cells. Based on Chou-Talalay's Combination Index Theorem, combination index and synergy was calculated and the values are plotted as heat map graph. Comparison of synergy between vemurafenib and other small molecule inhibitors in inhibiting proliferation of A375 and A375Res cells shows better synergy in case of A375Res cells, especially with Sorafenib and BEZ235.

treat melanoma patients with little side effect (19), however some patients developed resistance against the drug (20). In order to address this issue we have developed A375 melanoma cell line which has 50 fold more resistance to vemurafenib compared to the parent cell line (Figure 1a). The A375 Res cells were dividing faster compared to the parental cell lines as evidenced by decreased doubling time and increased rate of *in vitro* tumour formation (Figure 1b). Increased rate of proliferation was also associated with increased metastatic potential of the resistant cells as evidenced by increased rate of cell motility by resistant cells compared to the parental cells as assessed by wound healing assay (Figure 2a).

Drug resistance in cancer cell lines is associated with many molecular changes in the cell involving several somatic mutations; all leading to survival and proliferation of the resistance cells (21). Vemurafenib was not able to block the cell division and ERK 1/2 phosphorylation in resistant cells compared to parental cells (Figure 4). All these observations confirm the molecular changes that had taken place in the parental cell lines during transformation of vemurafenib resistant cells. Considering these cells as equivalent to the melanoma cells that acquire resistance to vemurafenib in patients; we have tested combination of drugs that could synergize with vemurafenib in inhibiting growth of vemurafenib resistant cell lines. Combining mode of action of drugs and molecular basis of drug resistance in cancer cells will lead to development of combinatorial therapy with combination of novel drugs to address the problem of drug resistance in patients.

Cellular signalling involves interaction between multiple proteins which are seen deregulated at more than single point in a diseased condition; therefore using compounds that act in multiple signalling pathways for treatment are sought for various diseases (22). Already combination therapy are sought and found successful in treating diseases such as tuberculosis, leprosy, malaria, HIV/AIDS, including Cancer (23–26). It is generally accepted that cancer cells in a tumour is not a homogenous population and therefore it is wise to treat the patients with combination of drugs, where they act at multiple points in the cell. Thus combination therapy is one of the effective strategies to treat cancer(22).

Based on basic biochemical and biophysical equations such as Michaelis-Menten, Hill, Henderson-

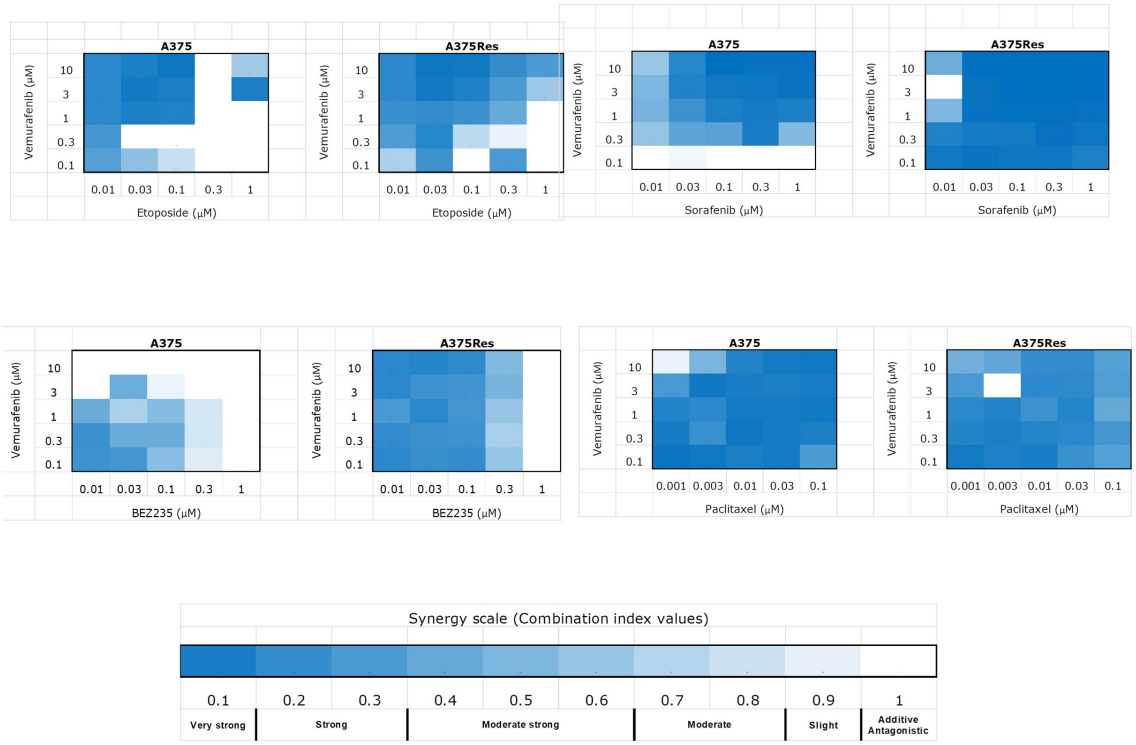

Figure 4. Effect of vemurafenib on signalling molecules involved in ERK pathway/ cellular proliferation. Cells were treated with different concentrations of Vemurafenib as indicated for 24 hrs and levels of p-ERK 1/2, p-MEK 1/2 and Cyclin D1 was analysed by Western blot analysis. The levels of Actin served as internal control. Vemurafenib mediated inhibition of phosphorylation of ERK 1/2, Mek 1/2 and Cyclin D levels at low concentrations in A375 cells was not observed in A375Res cells and actin levels were not affected.

Hasselbalch, and Scatchard equations, combination index theorem (CI) of Chou – Talalay was developed. This method offers a quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) for drug combinations. This theory also provides algorithms for automated computer simulation for synergism and/or antagonism at any effect and dose level and isobologram can be obtained for the experiments (13)**.** This method has been widely used in various models to identify(27) (28) (29) (30)**.**

Based on the synergy values/pattern obtained in this study, though it is highly speculative to discuss on molecular mechanisms; we have observed that drugs that doesn't act on signalling molecules such as DNA damaging agent, etoposide and cytoskeleton destabiliser paclitaxel, did not show any differences in the synergy between the resistant and non-resistant cells. Etoposide is an inducer of apoptosis by damaging the DNA rather than inhibiting molecules involved in cell cycle (31) thus it can be assumed that there are not many changes in the molecules involved in apoptotic signalling between the A375 and A375Res cells which could explain the similarity in synergy for Vemurafenib – etoposide combinations. For the same reason paclitaxel doesn't act on signalling molecules involved in activation of cell cycle; instead they inhibit cell division by preventing micro tubule polymerization (32) and hence did not show much difference in the synergy between A375 and A375Res cell.

Both PI3 kinase inhibitor BEZ 235 and multiple kinase inhibitor sorafenib show good synergy in inhibiting the growth of A375 res cells compared to A375 cells. It will be interesting to test whether mediators of PI3 kinase pathway that activate ERK 1/2 pathway is activated in A375Res cells; which could explain the differences in the synergy values between the two cell lines. Cancer cell population is considered to be a heterogeneous population cells with mixed genomic population of cells. Sorafenib being a multi kinase inhibitor (33) this drug is able to synergise with vemurafenib in inhibiting proliferation of A375Res cells. Future studies understanding the molecular mechanism behind the synergy of Sorafenib and vemurafenib will lead to extrapolating the observations made in this study to melanoma patients who develop resistance against vemurafenib.

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Send correspondence to: Anbalagan Moorthy, CBMR Lab # 213, Vellore Institute of Technology, Vellore, Tamil Nadu, India 632014, Tel: 91-9489 333 231, Fax: 91-4162240411, E-mail: Anbalagan.m@vit.ac.in