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Yao Yan for the Master of Science

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Title: The Anti-melanoma Effects of Heat Shock Protein Inhibitors

Thesis Chair: _____

Abstract approved: _____

(Thesis Advisor Signature)

Abstract

BRAF inhibitor can be used as a treatment regimen for malignant melanoma cancer, but this treatment is limited by the development of drug resistance. Heat shock proteins (Hsp) have been found to be over-expressed in several cancers, suggesting the critical role of Hsps in tumor initiation and growth. This study revealed that Hsp inhibitors worked as novel anti-cancer drugs in both Vemurafenib sensitive A375 and resistant A375VR melanoma cells. Hsp inhibitors displayed potent cytotoxicity against the human malignant melanoma cells and achieved a better therapeutic effect when combined with PLX4032. In addition, Hsp90 inhibitors CAY10607 significantly inhibited cell migration in the resistant cell line A375VR, and Hsp70 inhibitor

VER155008 showed a synergistic effect with PLX4032 in inhibiting cell migration in the sensitive cell line A375. Furthermore, Hsp70 inhibitor VER155008 promoted cell apoptosis significantly in both Vemurafenib sensitive and resistant melanoma cells, and the combination of Hsp70 inhibitor and Hsp90 inhibitor synergistically increased their cytotoxic effects on melanoma cells. Finally, Hsp90 inhibitor CAY10607 suppressed the activation of Akt and Erk in both Vemurafenib sensitive melanoma cell line A375 and resistant cell line A375VR, but only inhibited the activation of Akt and Erk in the sensitive cell line A375. Therefore, Hsp inhibitors can serve as novel anti-cancer drugs by inhibiting cell migration, inducing cell apoptosis, and inactivating the PI3K/Akt and Ras-Raf-Mek-Erk pathways.

Key words: melanoma cell, $BRAF^{V600E}$ mutation, drug resistance, Vemurafenib, heat shock protein (Hsp) inhibitors, CAY10607, VER155008.

The Anti-melanoma Effects of Heat Shock Protein Inhibitors

A Thesis Presented to

The Department of Biological Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirement for the Degree

Master of Science

By

Yao Yan

July 2017

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Approved by the Department Chair

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Committee Member

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Committee Member

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Dean of the Graduate School and Distance Education

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PREFACE

The organization of this thesis references the publication style of *Cancer Letters*.

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1. Introduction

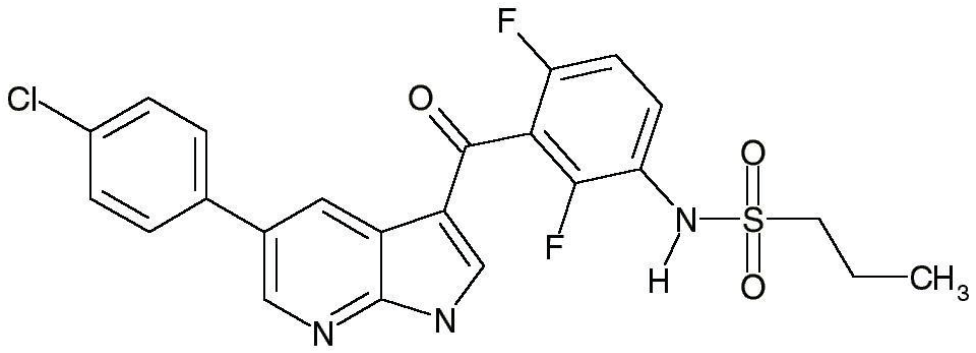
Melanoma is an invasive type of skin cancer that is classified into four stages. Melanoma at the first three stages occurs in the top layer of the skin, while stage IV melanoma is invasive and penetrates the deeper layers of skin [1]. Metastatic malignant melanoma is derived from melanocytes which are found in the basal layer of the epithelium [2]. It was reported that one in 36 American males and one out of 55 American females will develop melanoma cancer in their lifetime [3]. The increasing incidence of melanoma worldwide indicates that melanoma has become a public health threat in the international community. Exposure to solar UV radiation, possession of fair skin, symptoms of dysplastic nevi syndrome, and a family history of melanoma are all major risk factors for melanoma development [3]. Some researchers found that in 66% of cases, BRAF somatic missense mutations can be found in malignant melanoma, while this mutation occurs at a lower frequency in a large number of other types of human cancers [4].

BRAF belongs to the RAF serine/threonine kinase family which can promote cell proliferation through the Ras–Raf–Mek–Erk cascade [5]. Thus, once BRAF is activated, it can then activate its downstream genes and promote cell growth. In melanoma cells, almost all BRAF mutations occur in the kinase domain, and 80% of them have a single substitution called V600E [4]. Under this situation, BRAF can keep activating without being stimulated by growth factors such as epidermal growth factor [6], and the activated BRAF mutation leads to constitutive activation of the mitogen-activated protein kinase (MAPK) signaling pathway and then uncontrolled cell growth [7, 8]. BRAF inhibitors

can inhibit the phosphorylation of BRAF and thus abrogate the activation of Erk and the Ras–Raf–Mek–Erk signaling pathway. BRAF inhibitors have been used in clinical trials to control the proliferation of malignant melanoma cells [9].

Vemurafenib (Figure 1), a potent BRAF inhibitor, was approved in 2011 by the U.S. Food and Drug Administration (FDA) for clinical treatment of metastatic melanoma. Vemurafenib treatment initially results in significant inhibition of melanoma growth and shrinkage of tumors [10, 11]. Compared with other anti-cancer drugs, Vemurafenib causes fewer detrimental effects in normal cells since Vemurafenib cannot block BRAF in normal cells [5]. However, BRAF inhibitors can cause some side effects in patients treated with Vemurafenib alone, Dabrafenib alone, or with the combination of Vemurafenib and Dabrafenib.[12, 13]. Data show that 13% of patients developed panniculitis when treated with BRAF inhibitors Vemurafenib or Dabrafenib [13, 14]. Regardless of modest side effects, the BRAF inhibitors Vemurafenib and Dabrafenib were found to show significant anti-tumor effects in a clinical trial [15]. However, success using Vemurafenib to treat melanoma is limited by the development of drug resistance [16, 17]. Melanoma disappeared in patients 3-4 months after taking Vemurafenib, but then relapses almost inevitably occur in 6-9 months. In addition, some patients develop squamous cell carcinomas (around 20~30%) several months after treatment with Vemurafenib [16, 18, 19]. Therefore, researchers have been working on developing new treatment strategies that can synergistically improve the therapeutic effects of existing anti-melanoma drugs and alleviate the side effects that existing chemotherapy causes.

Figure 1: Vemurafenib (PLX4032) structure.


F
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N

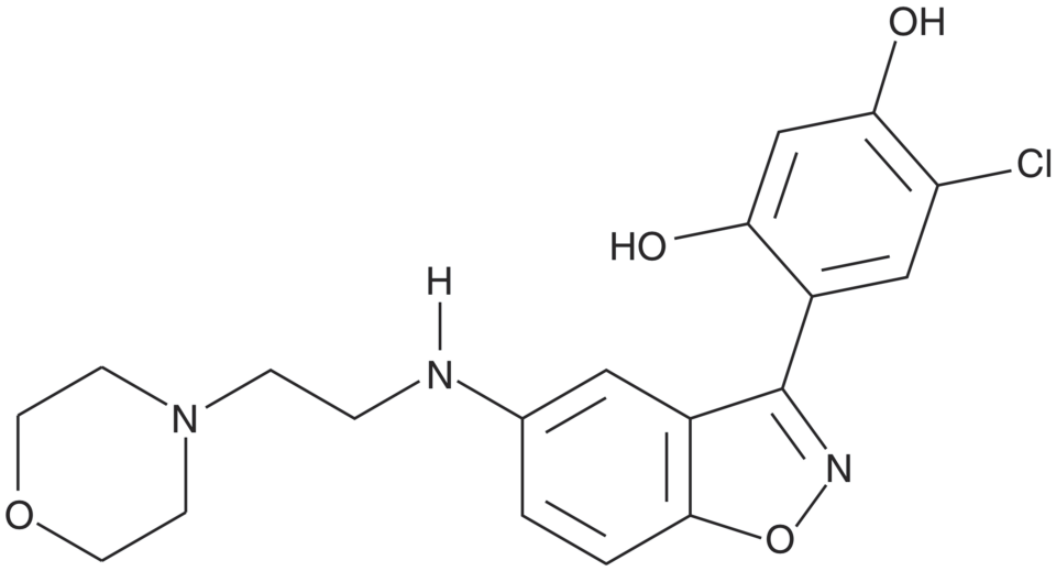
Heat shock proteins (Hsp) are a group of important cytoplasmic chaperone complexes [20] that assist proteins folding into functional conformations. Some chaperones can work individually, while other chaperones need to bind with each other and work as co-chaperones [21]. Expression of heat shock protein can be induced by different types of cell stress [22]. Hsp100, Hsp90, Hsp70, Hsp60, and Hsp40 are named according to their functions, their molecular mass (KD), and their sequence homology [21, 23, 24]. Hsp family proteins have been found to be over-expressed in several cancers, suggesting a critical role for heat shock proteins in tumor initiation and growth [25, 26]. These heat shock proteins are found to be related to apoptotic, inflammatory, and immune processes [23, 27-29] which are important in tumor initiation, development, and prognosis. For example, Hsp27 was found to be involved in cancer development, progression, and metastasis [30]. It can increase cell invasion in human cancer by increasing the transforming growth factor b (TGF-b)-stimulated MMP2 activity [30, 31]. Hsp60 can be secreted by cancer cells and is critical in transformation and cancer cell metastasis [32, 33]. By binding to the intracellular protein clusterin in neuroblastoma cells, Hsp60 can inhibit these intracellular proteins and promote cell survival [34]. Hsp70 is a highly diverse family [35]. Some papers, reported that Hsp70 inhibits cell apoptosis through binding with BAX, inhibiting the transportation of APAF-1 to the apoptosome and inhibiting the activation of AIF [30, 36]. In cancer cells, researchers found that Hsp70 can protect cancer cells from the cytotoxicity induced by TNF [37] . Some studies show that Hsp70 and Hsp90 play significant roles in folding and assembling other cellular proteins [23, 38]. It has been reported that the over-expression of Hsp90 can significantly decrease cancer survival time in many types of tumors, including melanoma

and breast cancer [25]. Hsp90 helps cancer cells survive by accelerating cell growth, invasion, metastasis, and inhibiting cell apoptosis [30]. In addition, Hsp90-β is associated with many important cellular processes, such as cell proliferation, cell differentiation, and cell apoptosis [25]. Recently, Hsp90 inhibitors have been found to have anti-cancer properties. For example, Hsp90 inhibitors stimulate the degradation of focal adhesion kinase and integrin-linked kinase (ILK) which play critical roles in promoting cell adhesion for a variety of cancer cells [39, 40]. Based on these scientific findings, we hypothesized that Hsp inhibitors can be used as novel anti-melanoma drugs to effectively treat melanoma cells.

In this study, we examined the anti-melanoma effects of Hsp90 inhibitor CAY10607 (Figure 2A) and Hsp70 inhibitor VER155008 (Figure 2B). Pacey found that Hsp90 inhibitor 17AAG exhibited anti-cancer activity in melanoma cells[41], while no research has been done to study the effect of CAY10607 on melanoma cells. VER155008 has been studied as a Hsp70 inhibitor to replace temozolmide in treating U87-MG glioma [42, 43]. In order to identify the anti-melanoma effects of Hsp inhibitors, we examined whether Hsp inhibitors alone or adding Hsp inhibitors to BRAF inhibitor will produce stronger cytotoxicity against melanoma cells and overcome the drug resistance in melanoma cells with BRAFV600E mutation to the BRAF inhibitor. This work will lead to a heat-shock-protein-based novel anti-melanoma therapy.

Figure 2: The structure of Hsp90 inhibitor CAY10607 and Hsp70 inhibitor VER155008.

A.



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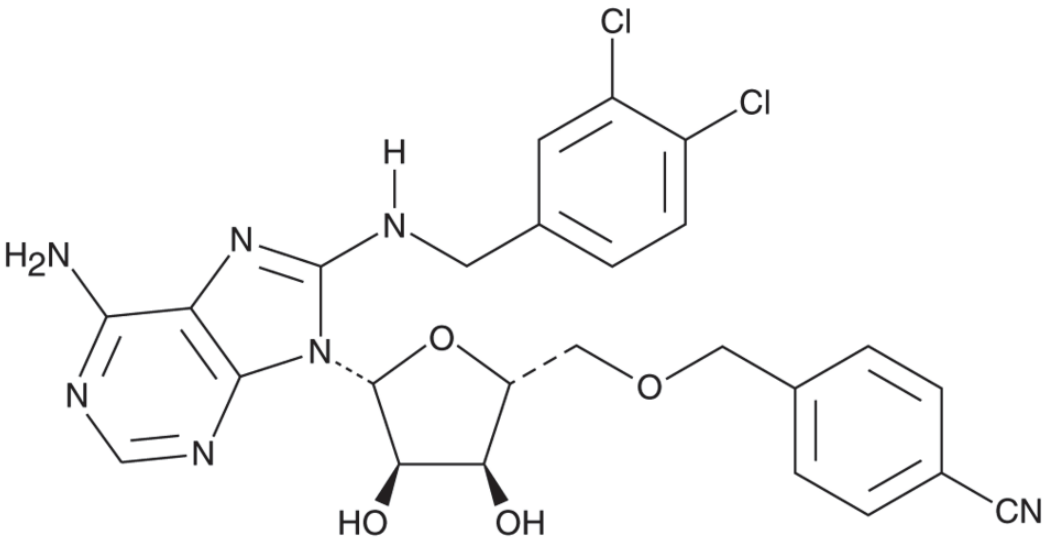


2. Materials and Methods

2.1 Chemicals

Vemurafenib (PLX4032) was purchased from Selleck Chemicals (Houston, Texas). Hsp90 inhibitors (CAY10607, Radicicol) and Hsp70 inhibitor (VER-155008) were purchased from Cayman Chemical (Ann Arbor, Michigan).

2.2 Cell Type and Culture Conditions

A375 human melanoma cell lines donated by Dr. Jaime Acquaviva and Dr. David Proia (Synta Pharmaceuticals Corp, Lexington, Massachusetts) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%) and penicillin/streptomycin (0.1%). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Vemurafenib-resistant A375 cells (A375-VR) donated by Dr. Jaime Acquaviva and Dr. David Proia were generated in continuous selective culturing by serially increasing concentrations of Vemurafenib over 8 weeks. After that, we still cultured the A375-VR cells in 5uM Vemurafenib to maintain a selective pressure for resistance.

2.3 Viability Assay

To test the cytotoxic effects of the Hsp inhibitors on the melanoma cells, A375 cells and A375VR ($3x10^4$/ml) were seeded in 96-well plates and treated with increased

concentrations of Hsp90 inhibitors alone, HSP70 inhibitors alone, PLX4032 alone, the combination of HSP90 inhibitors/PLX4032, HSP70 inhibitors/PLX4032, and the combination of PLX4032, HSP70 inhibitor and HSP90 inhibitor for 48 hrs.

There are two HSP 90 inhibitors (CAY, Radicicol) and one HSP70 inhibitor VER15508. The final concentrations of CAY were 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, and 10 μM; the final concentrations of Radicicol were 500 nM, 1 μM, 5 μM, and 10 μM. Melanoma cells were treated with VER155088 at the final concentrations of 500 nM, 1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 30 μM, and 50 μM. The cells in control group were treated with 1% of the drug vehicle DMSO.

Since we used both Vemurafenib sensitive (A375) and resistant (A375VR) cell lines, we needed to determine the working concentrations of Vemurafenib for each cell line. We treated melanoma cells A375 with PLX4032 alone at the concentration of 50 nM, 100 nM, 500 nM, 1 μM, 5 μM and 10 μM. Vemurafenib resistant A375VR cells were treated with PLX4032 at the concentrations of 10 nM, 50 nM, 100 nM, 500 nM, 1 μM, 5 μM, 10 μM, 20 μM, and 50 μM for 48 hrs.

To test if the combination of Vemurafenib and HSP90/70 inhibitors synergistically decreased the cell viability, Vemurafenib sensitive A375 melanoma cells were treated with the combinations of 1 μM PLX4032/500 nM Cay10607, 1 μM PLX4032/1 μM Cay10607, 1 μM PLX4032/1 μM VER155008, 1 μM PLX4032/5 μM

VER155008, and 1 μM PLX4032/500 nM Cay10607/1 μM VER155008 for 48 hrs. Vemurafenib resistant melanoma cells (A375VR) were treated with the combinations of 50 nM PLX4032/500 nM Cay10607, 50 nM PLX4032/1 μM Cay10607, 50 nM PLX4032/1 μM VER155008, 50 nM PLX4032/5 μM VER155008, and 50 nM PLX4032/500 nM Cay10607/1 μM VER155008 for 48 hrs. Without changing the concentration of HSP inhibitors, we replaced the 50 nM PLX4032 with 1 μM PLX4032 and 20 μM PLX4032, testing if these combinations with higher concentrations of PLX4032 could achieve a better therapeutic effect.

The viability of cells was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega Corp., Gaithersburg, MD) reading the absorbance at 490 nm by a micro-plate reader (Fisher Scientific, Hampton, NH). All experiments were performed six times and reported as mean ± SE.

2.4 Wound Healing Assay

Wound healing assay was used to test if the HSP inhibitors can inhibit the cell migration. 1×10^5 cells were seeded into 12-well plates and incubated at 37 ℃ until the cell confluence was 100%. Then we created a wound by using a 200 μl micropipette tip. Plates were washed once with Hank's solution and replaced with fresh medium. We treated the melanoma cells with increasing concentrations of PLX4032, HSP inhibitors, and the combination of PLX4032 and HSP inhibitors for 48 hrs. The images of the

wounds were captured at 40-fold magnification under an inverted microscope and the average wound distances were calculated using Image J software. We treated the melanoma cells A375 with 5 μM PLX4032, and the combinations of 5 μM PLX4032/500 nM CAY, 5 μM PLX4032/1 μM CAY, 5 μM PLX4032/5 μM VER155008, 5 μM PLX4032/10 μM VER155008, and 5 μM PLX4032/500 nM CAY/5 μM VER155008 for 48 hrs. Similarly, Vemurafenib resistant cells A375VR were treated with the combination of 1 μM PLX4032 and CAY10607 at the concentrations of 200 nM, 1 μM, and 2 μM for 48hrs.

2.5 Apoptosis Detection by Flow-cytometry

Apoptosis was used to examine whether HSP inhibitors alone or the combination of Vemurafenib/HSP inhibitors can decrease cell viability by inducing apoptosis (programed cell death). By following the manufacturer's instructions, we examined apoptosis by using an annexin V-FITC-propidium iodide (PI) dual staining kit (Biolegend). A375 melanoma cells were treated with 1 μM PLX4032, 500 nM CAY10607, 5 μM CAY10607, 10 μM VER155008, 20 μM VER155008, and the combinations of 1 μM PLX4032/500 nM Cay10607, 1 μM PLX4032/20 μM VER155008, 500 nM Cay10607/10 μM VER155008, and 1 μM PLX4032/500 nM Cay10607/10 μM VER155008 for 48 hrs. A375VR melanoma cells were treated with 5 μM PLX4032, 20 μM PLX4032, 30 μM VER155008, 5 μM CAY10607, and the combinations of 20 μM PLX4032/5 μM Cay10607, 20 μM PLX4032/30 μM VER155008 for 48 hrs. Cells were trypsinized, washed with ice-cold PBS, and re-suspended in binding buffer at a density of $1x10^6$ cells/ml. Cell suspension was stained

with Annexin V and PI and analyzed by a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA).

2.6 Cell Cycle Analysis

This assay was applied to test if the cocktail treatment can arrest cell cycle. Cells were seeded at 0.5×10^6 cells per T-25 cell culture flask (Corning Costar, Cambridge, MA). Vemurafenib sensitive cell line A375 cells were treated with 1 μM PLX4032/500 nM Cay10607, 1 μM PLX4032/1 μM Cay10607, 1 μM PLX4032/1 μM VER155008, 1 μM PLX4032/5 μM VER155008, and 1 μM PLX4032/500 nM Cay10607/1 μM VER155008 for 48 hrs. Vemurafenib resistant melanoma cell line A375VR was treated with 20 μM PLX4032, 1 μM Cay10607, 10 μM Cay10607, 10 μM VER155008, 30 μM VER155008 for 48 hrs. 48 hrs later, cells were harvested by trypsinization, washed with PBS twice, and fixed in cold 90% ethanol for 1 h at room temperature. The fixed cells were centrifuged at 200 × g for 5 min and re-suspended in 100 μl PBS. The cells were incubated in the dark with PI (50 μg/ml) for 1 hr. The cells were then incubated with RNase (100μg/ml final concentration) at 37 ℃ for 30 min. The DNA content was measured by the Accuri C6 Flow Cytometer® System (Accuri Cytometers Inc., Ann Arbor, MI). The cell fractions in G0/G1, S, and G2/M phases were expressed as the percentage of total gated cells.

2.7 Western Blot

Western blot was applied to analyze the effect of Hsp inhibitors and the combination of BRAF inhibitor and Hsp inhibitors on the RAS-RAF-MEK-ERK signaling pathway in melanoma cells. A375 melanoma cells were treated with the 1 μM PLX, 1 μM CAY10607, 15 μM VER155008, and the combinations of 1 μM PLX4032/500 nM Cay10607, 1 μM PLX4032/1 μM Cay10607, 1 μM PLX4032/1 μM VER155008, 1 μM PLX4032/5 μM VER155008, 1 μM PLX4032/15 μM VER155008, 1 μM CAY10607/15 μM VER155008, and 1 μM PLX4032/500 nM Cay10607/1 μM VER155008, 1 μM PLX4032/1 μM Cay10607/15 μM VER155008 for 48 hrs. A375VR melanoma cells were treated with 20 μM PLX4032, 5 μM CAY10607, 30 μM VER155008, and the combinations of 20 μM PLX4032/500 nM Cay10607, 20 μM PLX4032/5 μM Cay10607, 20 μM PLX4032/30 μM VER155008, 5 μM CAY10607/30 μM VER155008, and 20 μM PLX4032/5 μM Cay10607/30 μM VER155008 for 48 hrs. Cells were trypsinized and washed two times with PBS and then lysed in lysis buffer (100 μL/1x10^6 cells) for 30 min on ice. Supernatant was obtained after centrifugation at 12,000 rpm for 20 min at 4℃. The protein concentration was determined by Bradford Protein Assay. The protein samples were mixed with the loading buffer, which was made from 95% of Laemmli sample buffer (Bio-Rad, Hercules, CA) and 5% of 2-Mercapto-ethanol (β-ME) (Sigma, St. Louis, MO) at a ratio of 1:1. Total protein was then separated by electrophoresis and blotted onto a nitrocellulose membrane at 300 mA for 1.5 hrs in transfer buffer. The membrane was then blocked in TBST for 2 hrs at room temperature with 5% non-fat milk. The membrane was washed for three times and the membrane was immunoblotted with primary antibody for rabbit anti-GAPDH, anti-P44/42 MAPK, anti-Akt, anti-phospho-P44/42 MAPK, anti-phospho-Akt, anti-LC3B (Cell Signaling

Technology, Danvers, MA) with dilutions of 1: 10000, 1: 1000, 1: 1000, 1: 1000, 1: 1000 and 1: 1000 respectively, at 4ºC overnight (less than 18 hrs). The membrane was washed three times (10 min per time) and then treated with the HRP-linked secondary antibody (1:2500) at RT for 2 hrs. The image was developed by Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA) after washing with TBST buffer for three times. The band intensity was determined by the FluorChem™ E system (Protein Simple, Santa Clara, CA).

2.8 Statistics

All values were represented as mean ± standard deviation (SD) from at least three independent experiments. To compare the statistical difference among multiple groups, one-way analysis of variance (ANOVA) followed by Tukey post-test was applied. For comparisons of mean values between two groups, the unpaired t test was used. P value in all experiments is considered significant at less than or equal to 0.05.

3. Results

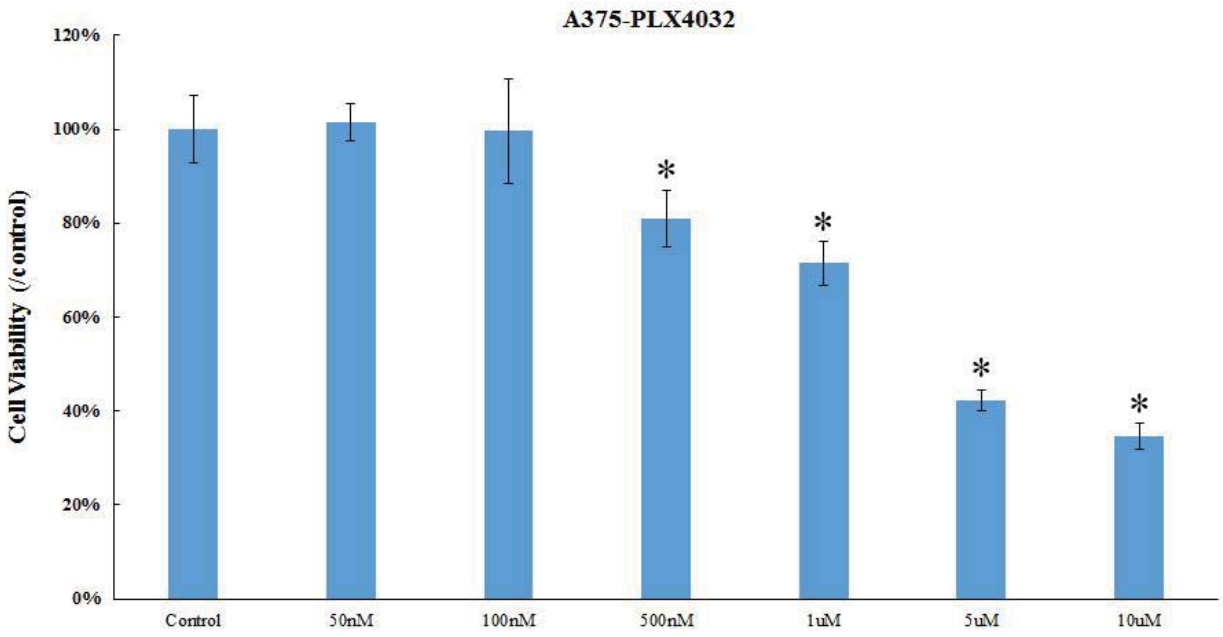
3.1 Heat shock protein inhibitors exhibited potent cytotoxicity against human malignant melanoma cells and achieved a better therapeutic effect when combined with PLX4032.

The cytotoxicity of Hsp inhibitors was determined in the Vemurafenib sensitive melanoma cell line A375 and the Vemurafenib resistant melanoma cell line A375VR by using the MTS assay. Braf inhibitor PLX4032 (PLX), Hsp90 inhibitor radicicol (RAD) and CAY10607 (CAY) significantly decreased cell viability in A375 cells, as shown in Figures 3A, 3B and 3C, respectively, indicating the high cytotoxicity of Hsp90 inhibitors in the Vemurafenib sensitive cell line A375. Compared with Hsp90 inhibitors, Hsp70 inhibitor VER155008 (VER) inhibited cell viability at a much higher working concentration, which is around 20 μM (Figure 3D). However, 1μM VER exhibited a synergistic effect with 1 μM PLX4032 in reducing the viability of the A375 melanoma cells (Figure 3E).

A similar cytotoxic effect of Hsp90 inhibitor and Hsp70 inhibitor was also observed in the Vemurafenib resistant cell line A375VR (Figures 3G, 3H). It is worthwhile to mentation that exposure of A375VR cells to PLX4032 at concentrations ranging from 50 nM to 50 μM caused a substantial increase of cell viability in a dose-dependent manner (Figure 3F). However, the combination of 500 nM CAY and 20 μM PLX synergistically reduced cell viability even though 20 μM PLX alone significantly increased cell viability. Compared to the Hsp inhibitors alone, the cocktail treatment of PLX, CAY, and VER achieved a better therapeutic effect (Figure 3I).

Figure 3: The effect of Hsp inhibitors on cell viability in both Vemurafenib sensitive and resistance melanoma cell lines. The cytotoxicity of the Heat Shock Protein inhibitors on melanoma cells was determined by MTS assay after A375 and A375 VR melanoma cells were treated with Hsp inhibitors for 48 hours. The concentration for each drug is shown in the figure below. Each experiment was repeated three times with six replicated reactions in every independent MTS assay. One asterisk means there were significant differences between the experimental group and control group ($p<0.05$). Two asterisks indicate a significant difference exists when we compared the group PLX4032 alone with other cocktail groups ($p<0.05$).

A.


A375-PLX4032
Cell Viability (/control)
120%
100%
80%
60%
40%
20%
0%
Control
50nM
100nM
500nM
1uM
5uM
10uM
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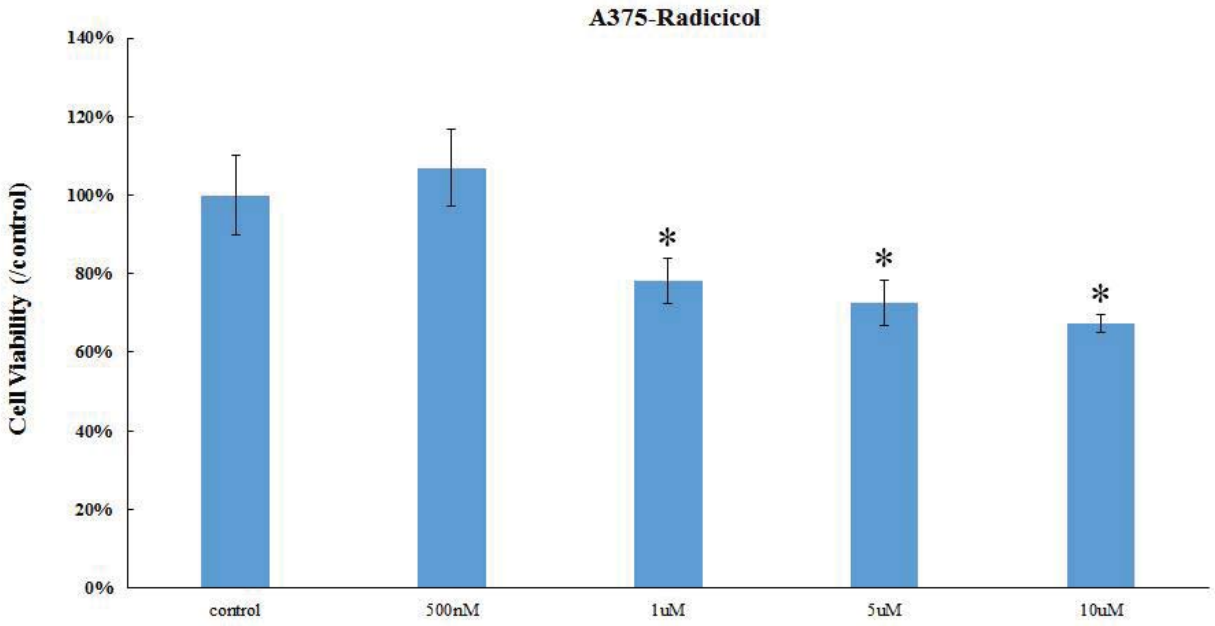
B.


A375-Radicicol
Cell Viability (/control)
140%
120%
100%
80%
60%
40%
20%
0%
control
500nM
1uM
5uM
10uM
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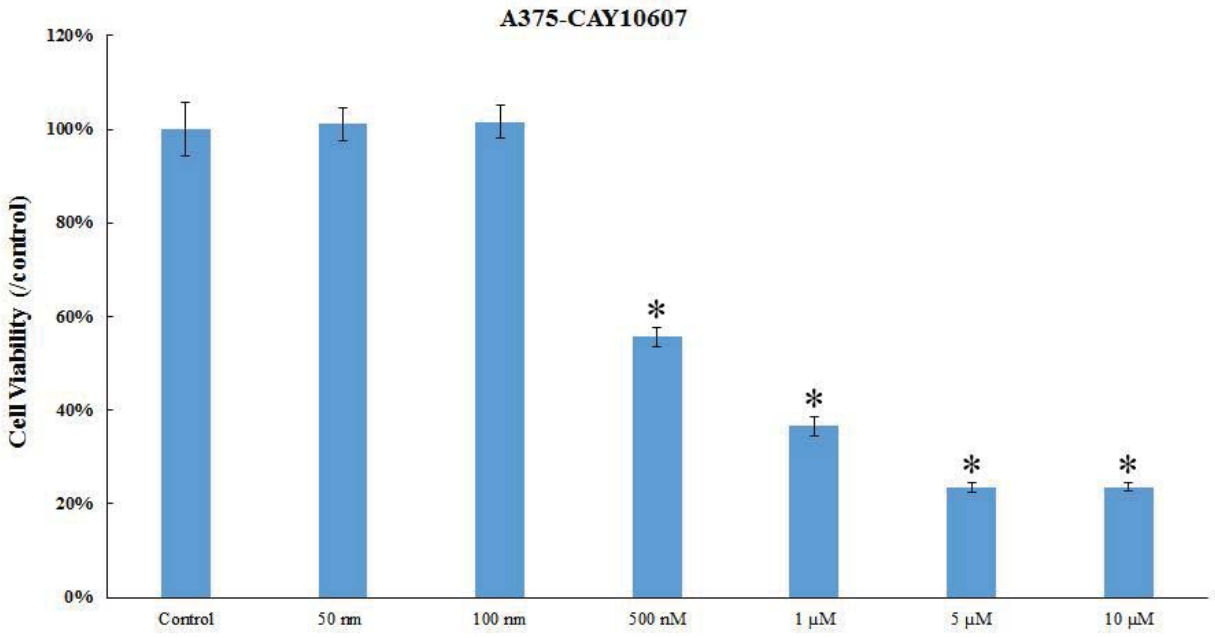
*
*

C.


A375-CAY10607
Cell Viability (/control)
120%
100%
80%
60%
40%
20%
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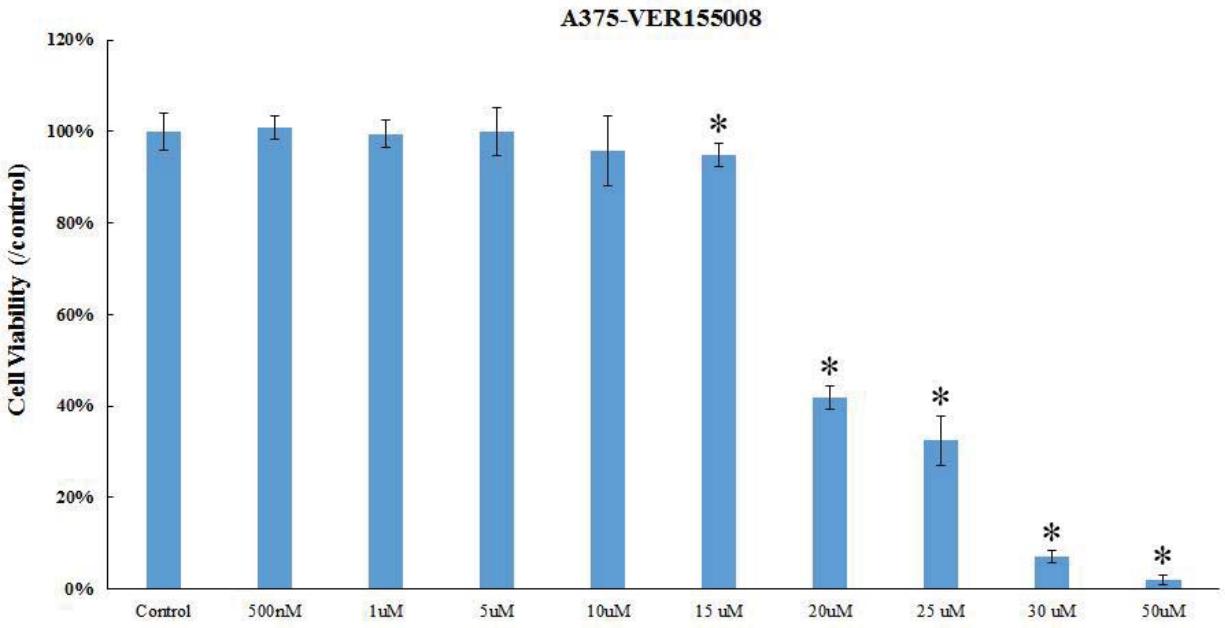
Control
50 nm
100 nm
500 nM
1 μM
5 μM
10 μM

D.


A375-VER155008
Cell Viability (/control)
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80%
60%
40%
20%
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Control
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25 uM
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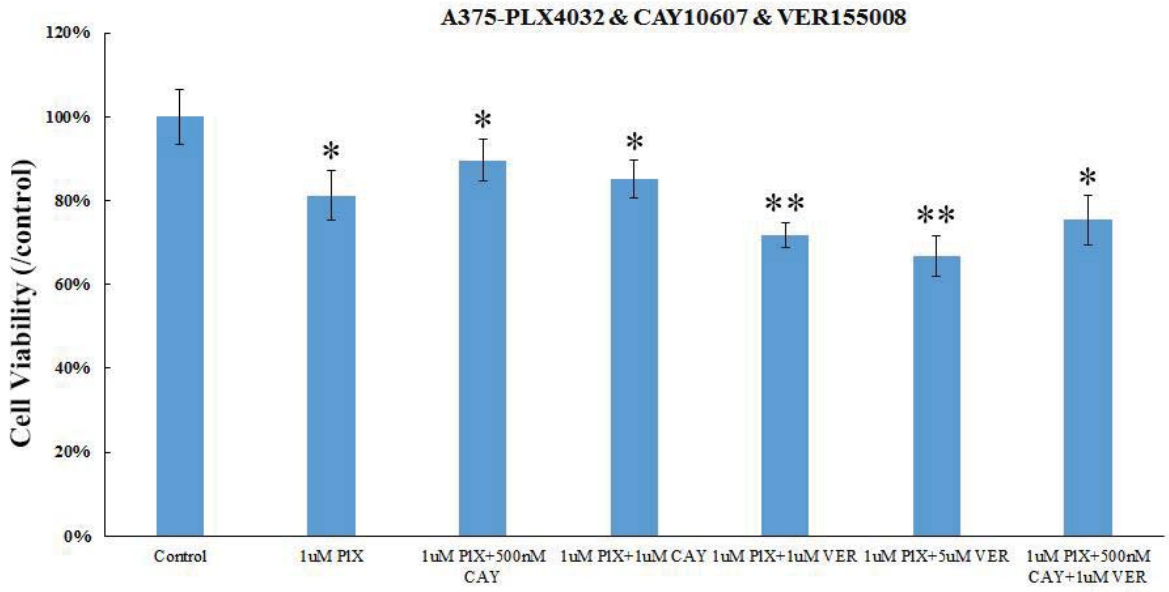
E.


A375-PLX4032 & CAY10607 & VER155008
Cell Viability (/control)
120%
100%
80%
60%
40%
20%
0%
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Control
1uM PlX
1uM PlX+500nM CAY
1uM PlX+1uM CAY
1uM PlX+1uM VER
1uM PlX+5uM VER
1uM PlX+500nM CAY+1uM VER

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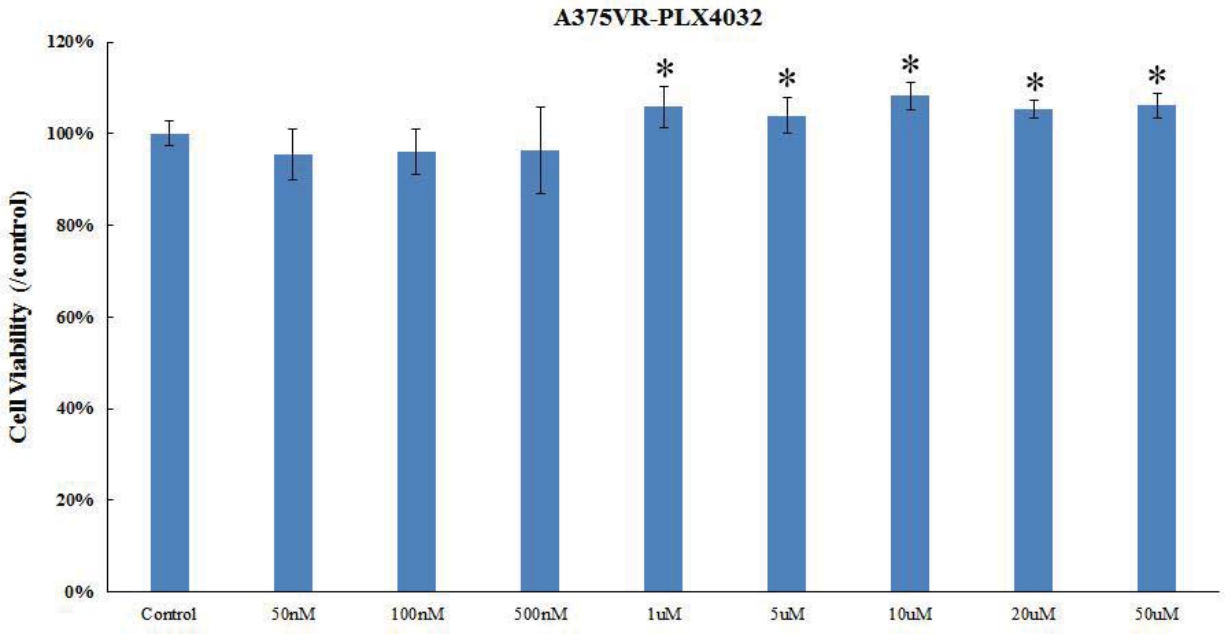
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60%
40%
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Control
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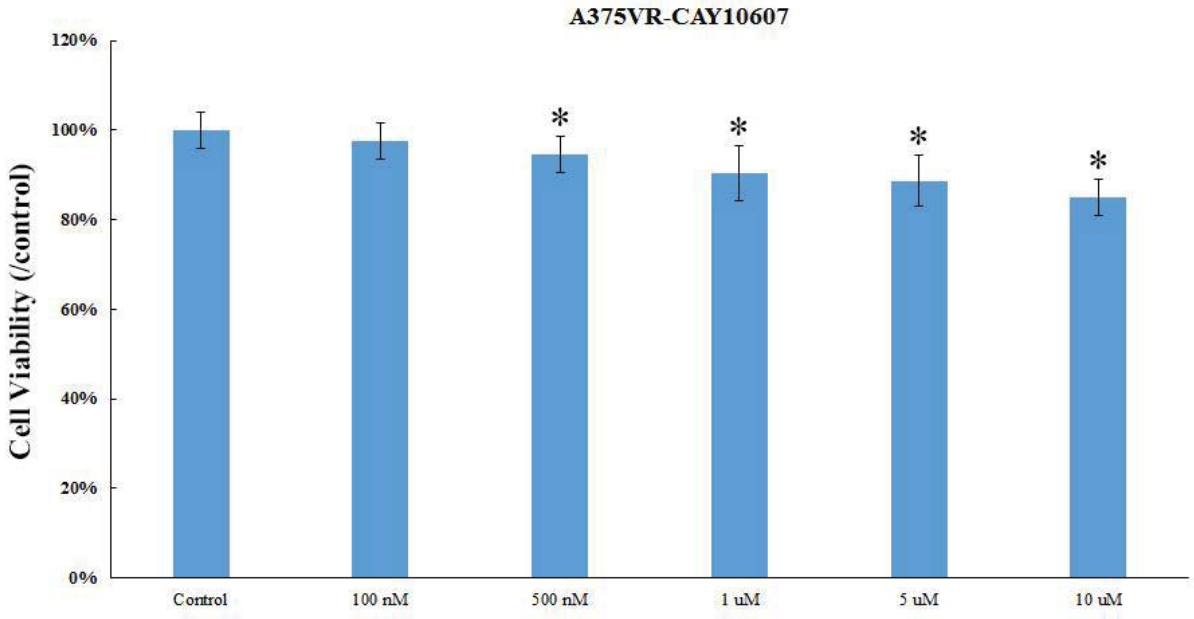
G.


A375VR-CAY10607
Cell Viability (/control)
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100%
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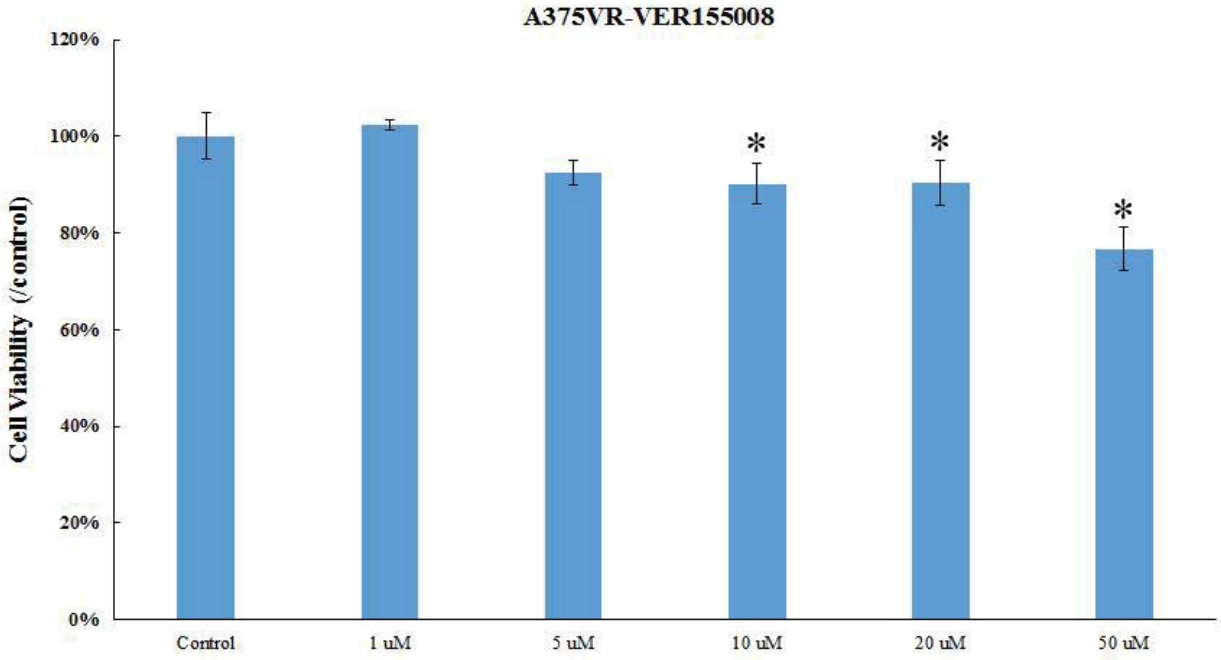
H.


A375VR-VER155008
Cell Viability (/control)
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20%
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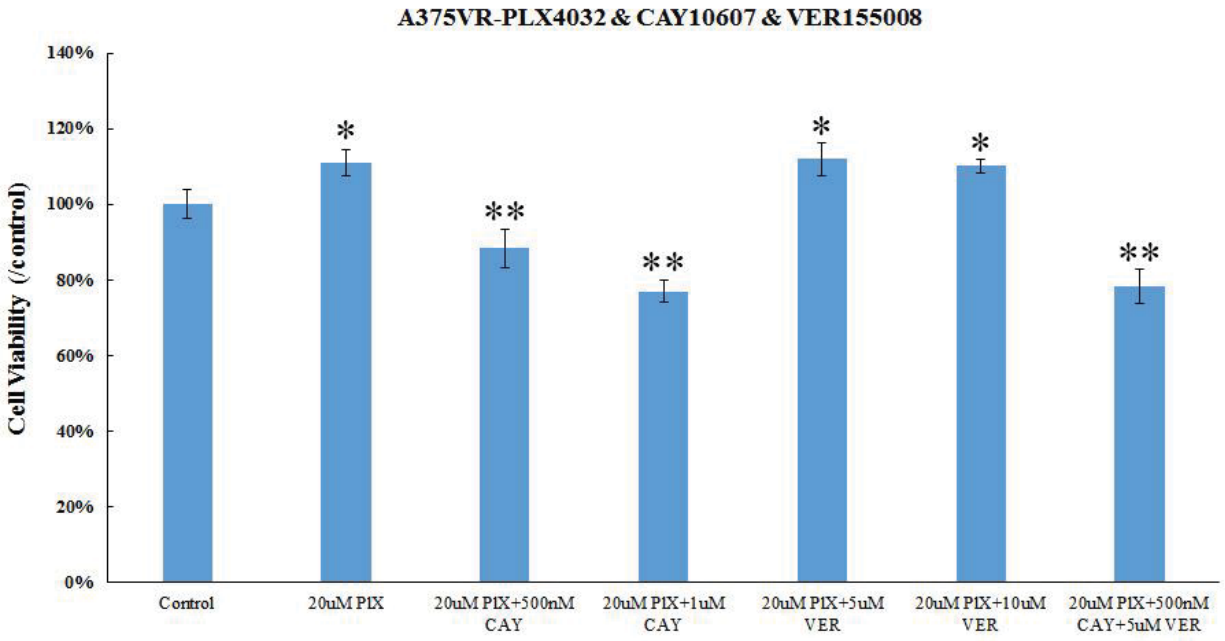
1 uM
5 uM
10 uM
20 uM
50 uM

I.


A375VR-PLX4032 & CAY10607 & VER155008
Cell Viability (/control)
140%
120%
100%
80%
60%
40%
20%
0%
*
**
**
*
*
**
Control
20uM PIX
20uM PIX+500nM CAY
20uM PIX+1uM CAY
20uM PIX+5uM VER
20uM PIX+10uM VER
20uM PIX+500nM CAY+5uM VER

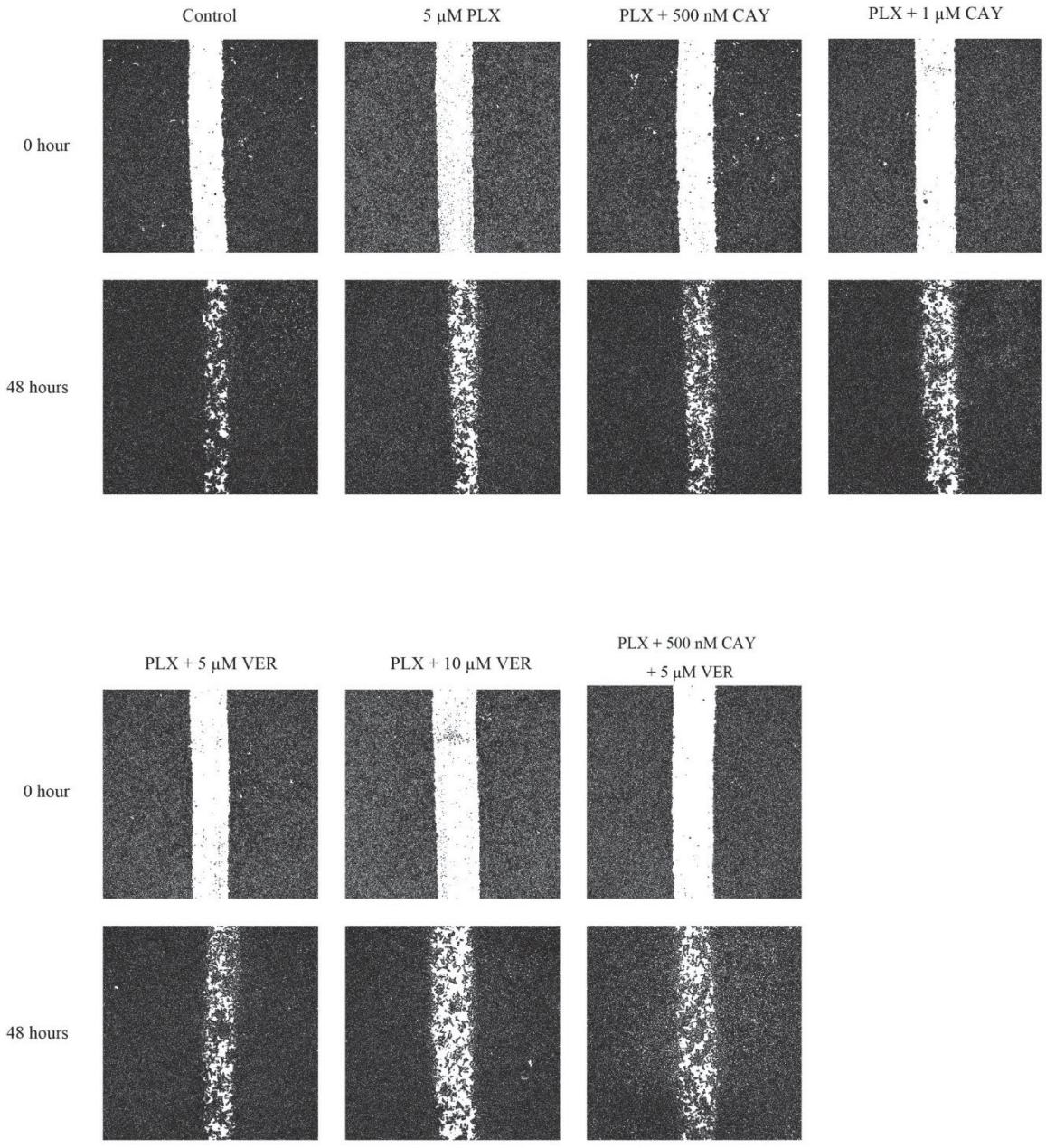
3.2 Hsp inhibitors inhibited cell migration in both the Vemurafenib sensitive melanoma cell line A375 and the resistant cell line A375VR.

The migration ability of melanoma cells was examined by a wound healing assay. Melanoma cells A375 & A375VR were treated with the Hsp inhibitors alone or the combination of Hsp inhibitors and PLX4032. In A375 cells, Hsp70 inhibitor VER at high concentrations showed a synergistic effect with PLX4032 in inhibiting cell migration, while VER at low concentrations alleviated the inhibitory effect of cell migration caused by PLX4032 (Figure 4B). The Hsp90 inhibitor CAY at a low concentration also showed similar results when it combined with the PLX4032 (Figure 4B).

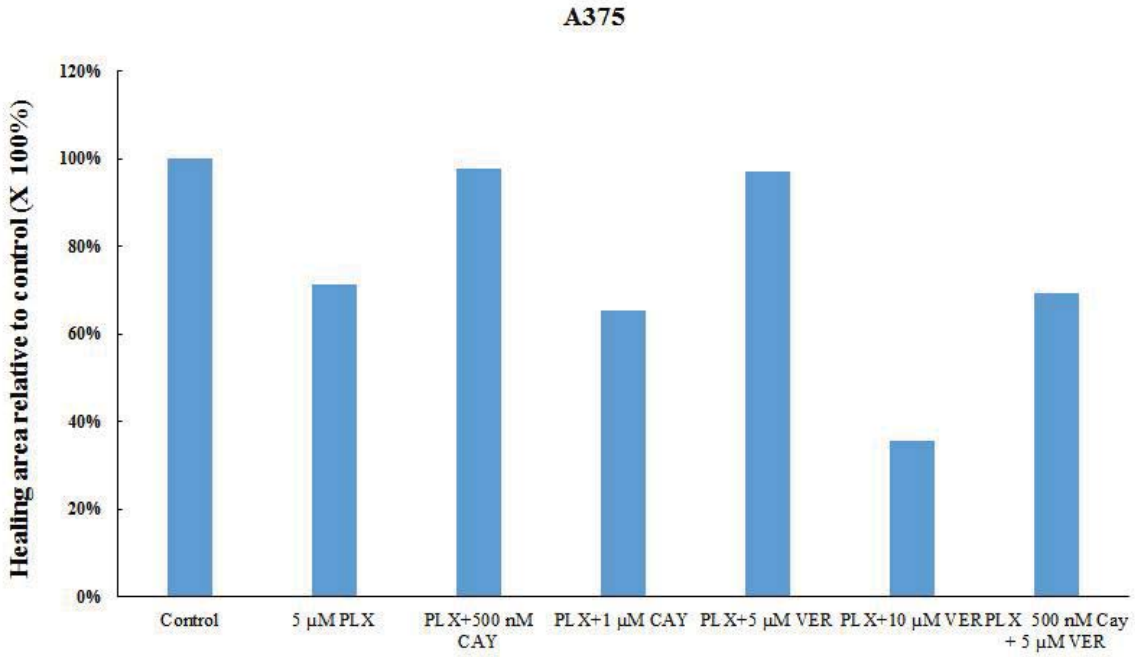
In the Vemurafenib resistant melanoma cell line A375VR, a high concentration of Hsp90 inhibitor CAY significantly inhibited the cell migration ability in a dose dependent manner (Figure 4D). However, there was no synergistic effect when cells were treated with drug cocktail groups (Figure 4D).

Figure 4: The inhibition of cell migration by Hsp inhibitors and the combination of PLX4032 and Hsp inhibitors. The wound healing assay was employed to measure the cell migration ability in A375 (Figures 2A, 2B) and A375VR (Figures 2C, 2D) cells when they were treated with Hsp inhibitors individually or in the combination of Hsp inhibitors with PLX4032. A wound was created by scraping a confluent monolayer of cells with a 200 μM pipette. The results were captured by inverted phase contrast microscope under 40X magnification after 48 hrs.

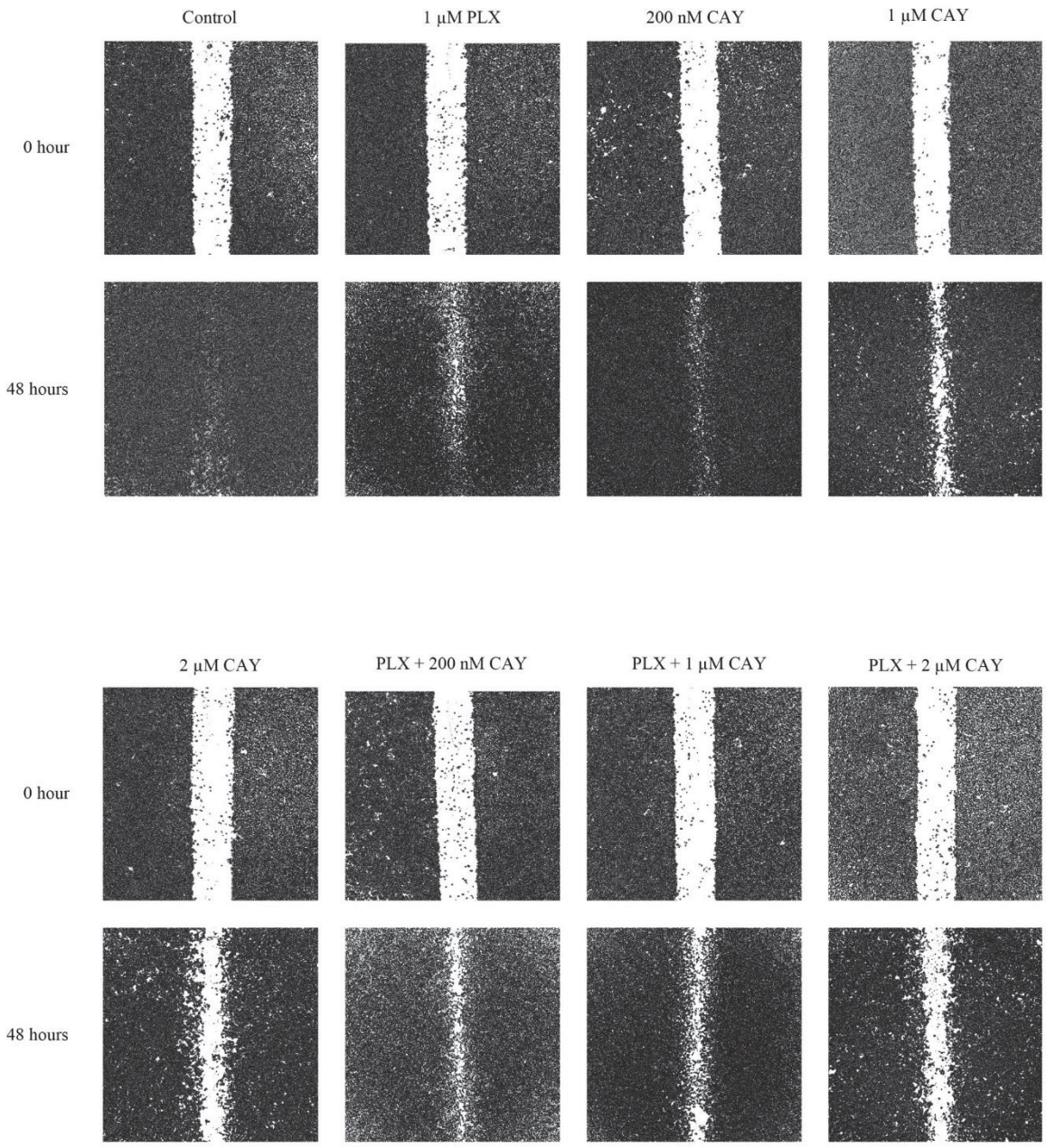
A.


Control
5 μM PLX
PLX + 500 nM CAY
PLX + 1 μM CAY
0 hour
48 hours
PLX + 5 μM VER
PLX + 10 μM VER
PLX + 500 nM CAY
+ 5 μM VER
0 hour
48 hours

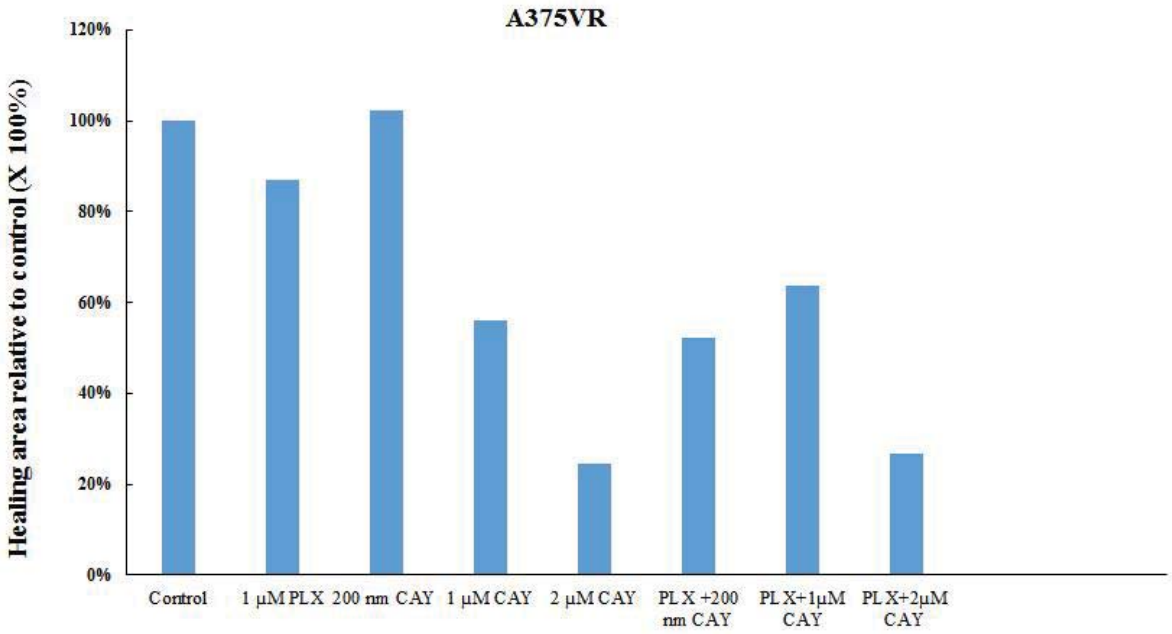
B.


A375
Healing area relative to control (X 100%)
120%
100%
80%
60%
40%
20%
0%
Control
5 µM PLX
PLX+500 nM CAY
PLX+1 µM CAY
PLX+5 µM VER
PLX+10 µM VER
PLX 500 nM Cay + 5 µM VER

C.


Control
1 μM PLX
200 nM CAY
1 μM CAY
0 hour
48 hours
2 μM CAY
PLX + 200 nM CAY
PLX + 1 μM CAY
PLX + 2 μM CAY
0 hour
48 hours

D.


A375VR
Healing area relative to control (X 100%)
120%
100%
80%
60%
40%
20%
0%
Control
1 µM PLX
200 nm CAY
1 µM CAY
2 µM CAY
PLX +200 nm CAY
PLX+1µM CAY
PLX+2µM CAY

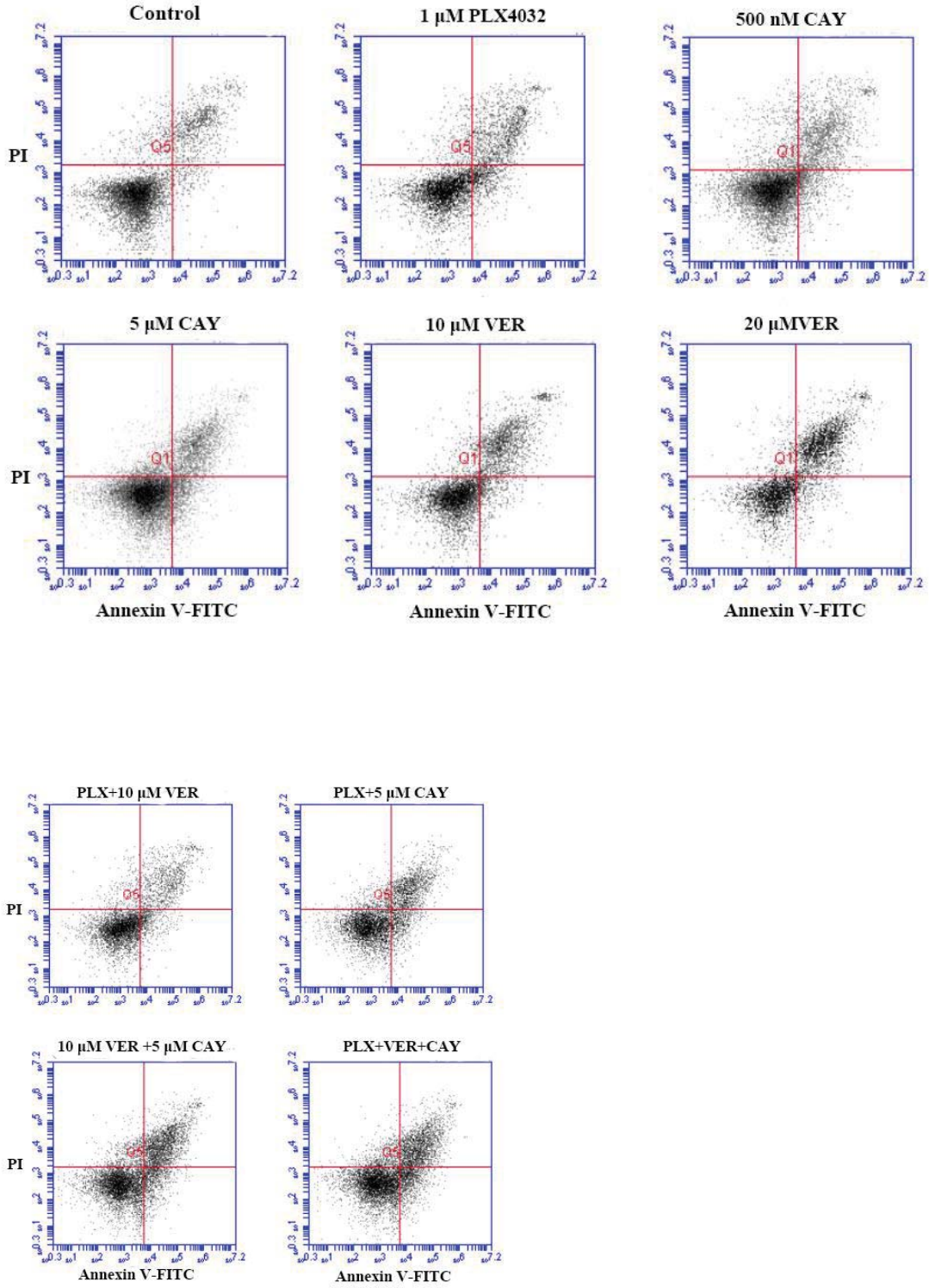
3.3 Hsp70 inhibitor VER promoted cell apoptosis significantly in both Vemurafenib sensitive and resistant melanoma cells.

To examine if the heat shock protein inhibitors induce cell apoptosis or necrosis, melanoma cells were treated with different concentrations of Hsp inhibitors for 24 hours and analyzed by flow cytometry. Exposure of A375 cells to Hsp70 inhibitor VER at concentrations of 10 μM and 20 μM caused a significant increase in the number of apoptotic cells exhibiting positive Annexin V-FITC staining (Figure 5A). Compared with Hsp90 inhibitor CAY or Hsp70 inhibitor VER alone, the combination of 10 μM VER and 5 μM CAY highly increased the cell apoptotic percentage in A375 melanoma cells (Figure 5B). Thus, Hsp70 inhibitor VER potently induced cell apoptosis either alone or combined with Hsp90 inhibitor CAY in Vemurafenib sensitive melanoma cells.

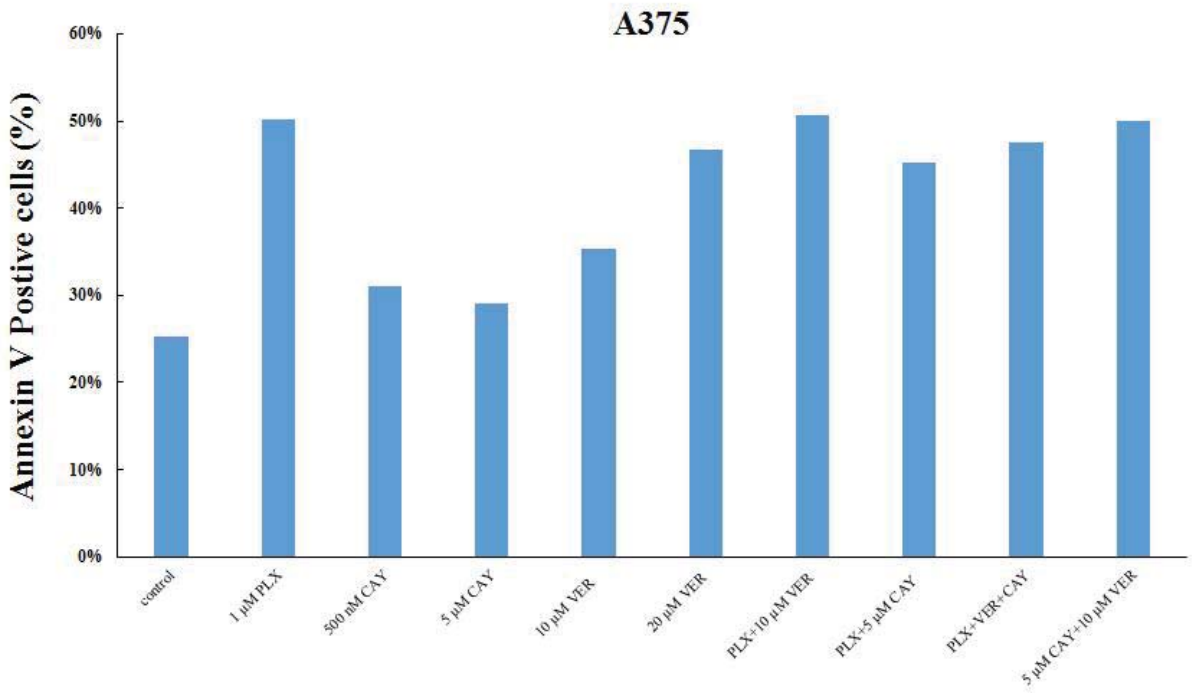
Similar results were shown in the Vemurafenib resistant melanoma cell line A375VR. Treating cells with a high concentration of Hsp70 inhibitor VER (30 μM) alone or in conjunction with PLX4032 or HSp90 inhibitor CAY led to a higher percentage of apoptotic cells exhibiting positive Annexin V-FITC staining (Figures 5C, 5D). Therefore, the Hsp70 inhibitor VER inhibited cell proliferation by inducing cell apoptosis in A375VR melanoma cells.

Figure 5: Hsp70 inhibitor VER significantly induced cell apoptosis in both Vemurafenib sensitive and resistant melanoma cells. The Vemurafenib sensitive melanoma cell line A375 was treated with 1 μM PLX4032, 500 nM CAY, 5 μM CAY, 10 μM VER, 20 μM VER, PLX/ 10 μM VER, PLX/5 μM CAY, 10 μM VER/ 5 μM CAY, and PLX/CAY/VER for 24 hrs. After that, the Vemurafenib sensitive cell line A375 (Figure 5A) and the Vemurafenib resistant cell line A375VR (Figure 5C) were stained with Annexin V-FITC and PI for 15 mins. Finally, these stained cells were analyzed by flow cytometry. The percentage of apoptotic cells is presented in bar graphs for A375 cells (5B) and A375VR (Figure 5D).

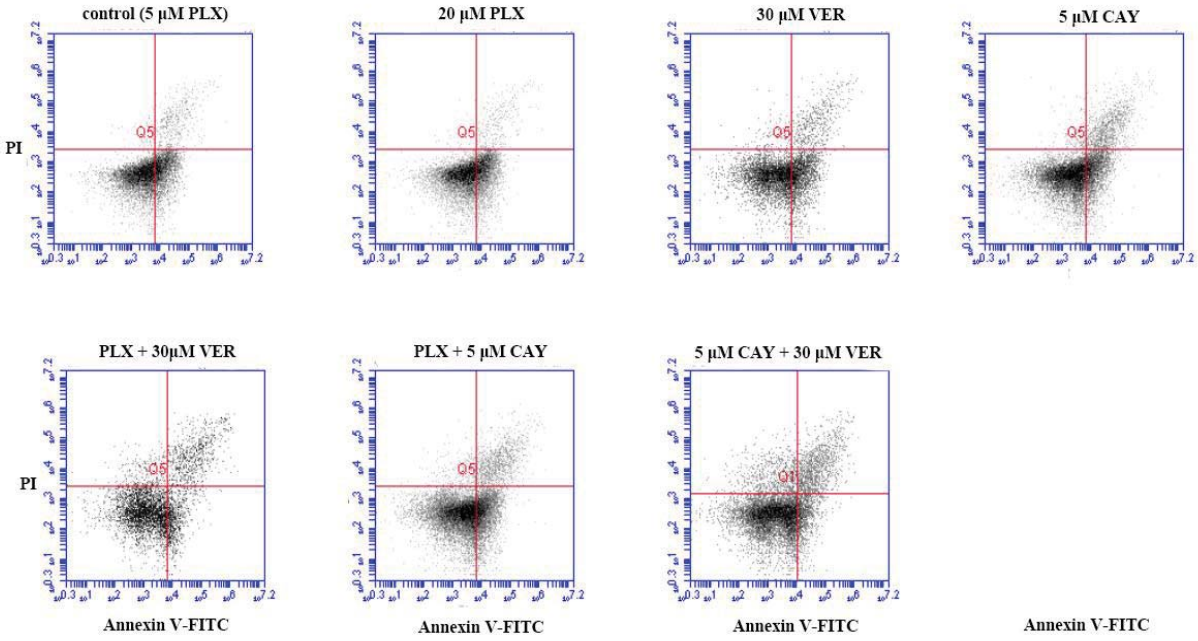
A.


Control
1 µM PLX4032
500 nM CAY
5 µM CAY
10 µM VER
20 µMVER
PLX+10 µM VER
PLX+5 µM CAY
10 µM VER +5 µM CAY
PLX+VER+CAY
PI
Annexin V-FITC
Q5
Q1

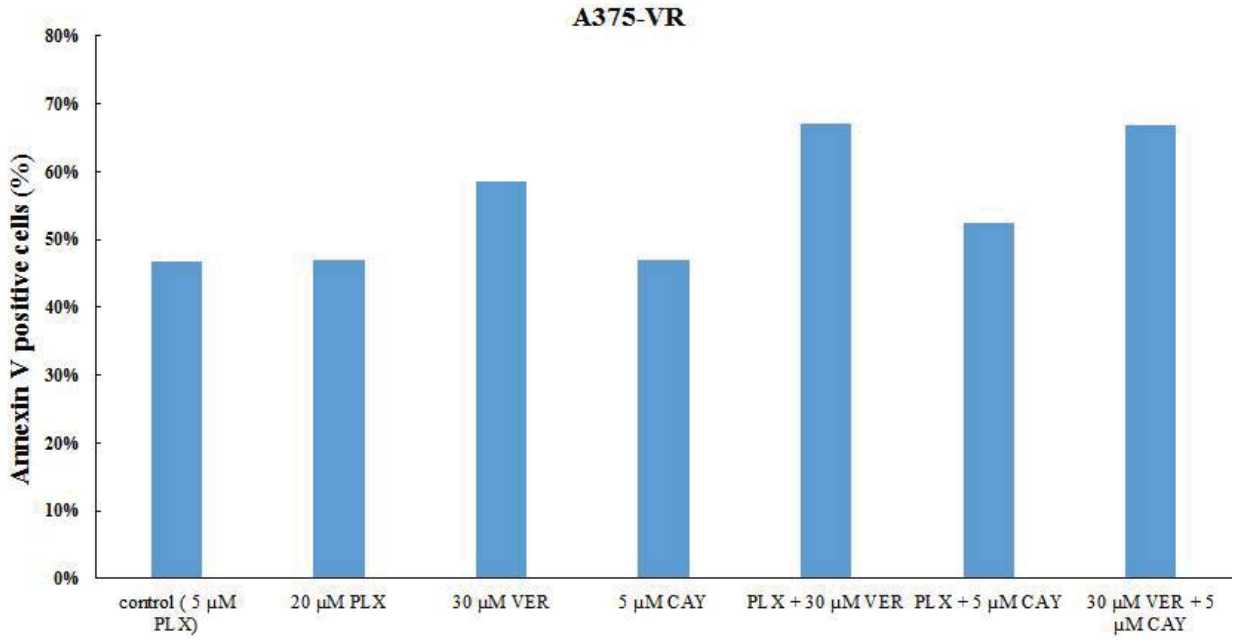
B.


A375
Annexin V Postive cells (%)
60%
50%
40%
30%
20%
10%
0%
control
1 μM PLX
500 nM CAY
5 μM CAY
10 μM VER
20 μM VER
PLX+10 μM VER
PLX+5 μM CAY
PLX+VER+CAY
5 μM CAY+10 μM VER

C.


control (5 µM PLX)
20 µM PLX
30 µM VER
5 µM CAY
PLX + 30µM VER
PLX + 5 µM CAY
5 µM CAY + 30 µM VER
PI
Annexin V-FITC
Q5
Q1

D.


A375-VR
Annexin V positive cells (%)
80%
70%
60%
50%
40%
30%
20%
10%
0%
control (5 μM PLX)
20 μM PLX
30 μM VER
5 μM CAY
PLX + 30 μM VER
PLX + 5 μM CAY
30 μM VER + 5 μM CAY

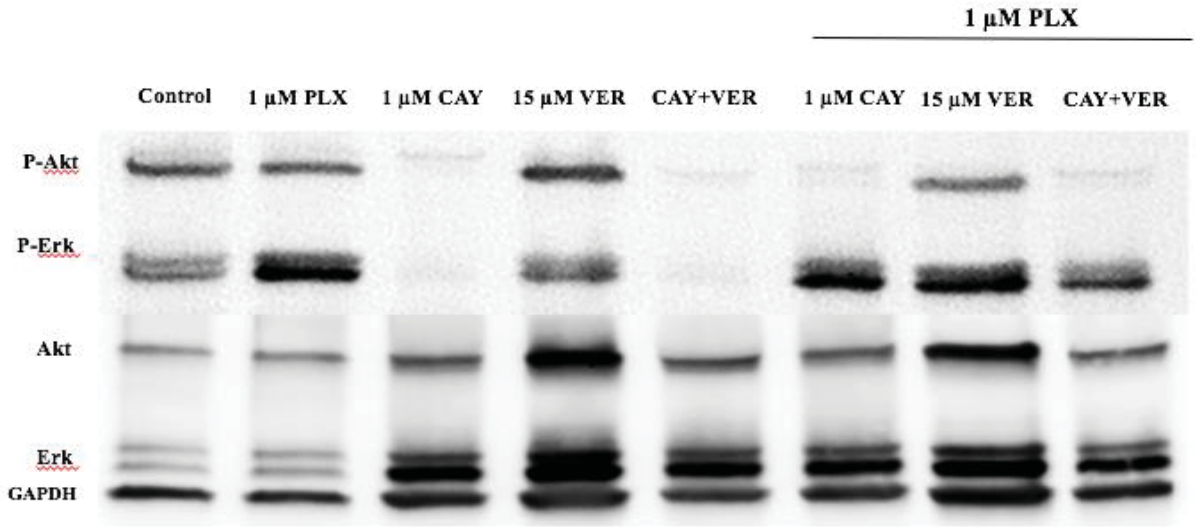
3.4 Hsp90 inhibitor CAY suppressed the activation of Akt and Erk in both the Vemurafenib sensitive melanoma cell line A375 and the resistant cell line A375VR.

Western blot assay was used to elucidate the molecular mechanism through which heat shock proteins exert cytotoxic effect on the Vemurafenib sensitive melanoma cells A375 (Figure 6A) and resistant cells A375VR (Figure 6B). Since both Akt and Erk can regulate the cell growth and proliferation, we examined whether the anti-cancer properties of Hsp inhibitors were attributed to their ability to suppress the activation of Akt or Erk. In Vemurafenib sensitive cells A375, Hsp90 inhibitor CAY alone or the combination of CAY and PLX4032 almost completely abolished the activation of Akt (Figure 6A). Activation of Erk was suppressed by CAY alone, but it was not affected by the combination of CAY and PLX4032 (Figure 6A).

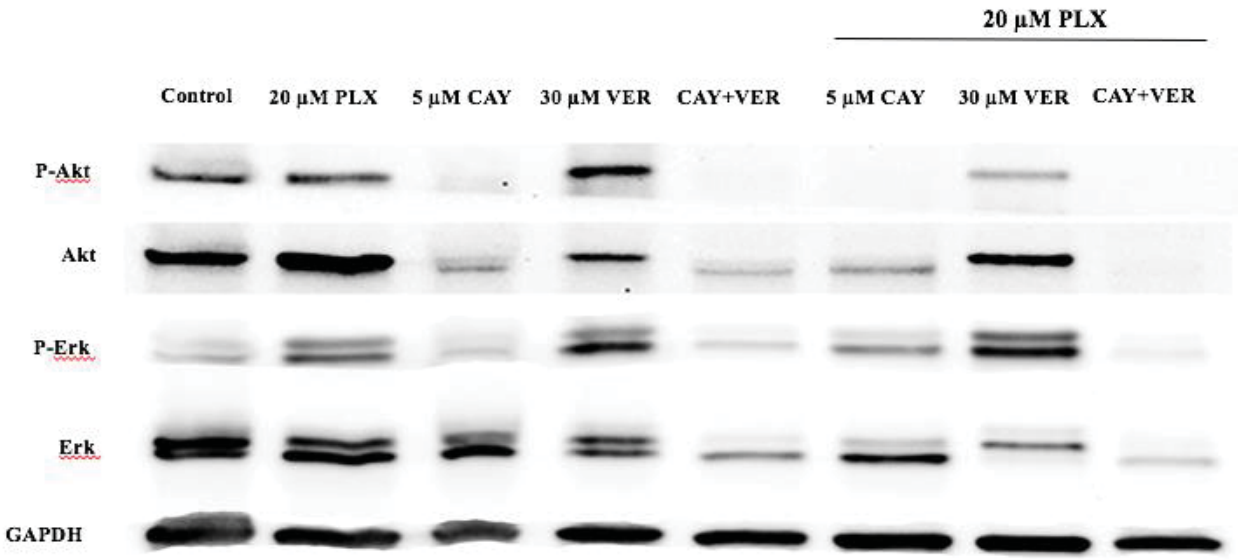
In Vemurafenib resistant cells A375VR, the Hsp90 inhibitor CAY reduced the activation of Erk, while the Hsp70 inhibitor VER activated Erk (Figure 6B). The Hsp90 inhibitor CAY showed a strong inhibitory effect on the activation of Akt, and it also inhibited the expression of non-phosphorylated Akt (Figure 6B). Thus, the Hsp90 inhibitor CAY decreased the melanoma cell viability by inhibiting the activation of Akt and Erk in both the Vemurafenib sensitive cell line A375 and the Vemurafenib resistant cell line A375VR.

Figure 6: Hsp90 inhibitor CAY inhibited the activation of Akt and Erk in A375 and A375VR cells. Western Blot assay was applied to examine the expression level of P-Akt and P-Erk in both A375 (Figure 6A) and A375VR (Figure 6B) melanoma cells. All treatment groups were treated with the indicated concentrations of Hsp inhibitors for 48 hrs. GAPDH served as the internal control. All experiments were repeated three times and a representative graph is shown.

A.


1 µM PLX
Control
1 µM PLX
1 µM CAY
15 µM VER
CAY+VER
1 µM CAY
15 µM VER
CAY+VER
P-Akt
P-Erk
Akt
Erk
GAPDH

B.


20 μM PLX
Control
20 μM PLX
5 μM CAY
30 μM VER
CAY+VER
5 μM CAY
30 μM VER
CAY+VER
P-Akt
Akt
P-Erk
Erk
GAPDH

4. Discussion

Heat shock proteins (Hsp), the major chaperone molecules, have been recently studied as new cancer therapy targets [44]. A number of Hsp have been shown to be involved in cancer cell proliferation, migration, differentiation, and invasion [30, 33, 44]. For example, Hsp90 maintains the malignant ability of cancer cells by binging and regulating the conformation and stability of RTKs and other kinases that are required in tumorigenesis [45-47]. At the molecular level, Hsp90 inhibitors degrade ARAF and CRAF and inhibit the activation of Akt and Erk in many acquired BRAF resistance cells [45]. Hsp70 was also found to be expressed at abnormally high levels in cancer cells and to promote carcinogenesis by acting as survival factors [48]. Besides, additional research suggests that Hsp70 inhibitors can also deregulate or upregulate some important factors that are involved in tumor development [30]. For example, a published study has shown that Hsp70 inhibitors upregulated the expression level of p53 and promoted cell apoptosis through activating the PI3K/Akt pathway [49]. This work has investigated the effects of different Hsp inhibitors on malignant melanoma cells as well as their synergistic cytotoxic effects in conjunction with PLX4032 to fight against the Vemurafenib resistant cells.

In this study, Hsp inhibitors exhibited significant cytotoxic effects against the Vemurafenib sensitive cells A375 and resistant cells A375VR. However, these two cell lines presented a different sensitivity to both the Hsp70 inhibitor and Hsp90 inhibitor. The Hsp90 inhibitor CAY significantly decreased cell viability in A375 melanoma cells (IC_{50} = 600 nM), while it displayed a much lower cytotoxicity in A375VR (IC_{50} > 10

μM), indicating that A375 melanoma cells were more sensitive to the Hsp90 inhibitor CAY. Similarly, the Hsp70 inhibitor VER (20 μM) reduced cell viability by about 60% for A375 melanoma cells but only 10% for A375VR, which means that A375 melanoma cells have a higher sensitivity to the Hsp70 inhibitor VER than A375VR melanoma cells. Notably, the Hsp70 inhibitor VER only showed cytotoxicity at concentrations no lower than 15 μM. Even though these Hsp inhibitors showed high cytotoxicity in melanoma cells when they work individually, they exhibited different cytotoxic effects when combined with PLX4032. The Hsp90 inhibitor CAY (500 nM) alleviated the inhibitory effects caused by PLX4032 in the Vemurafenib sensitive melanoma cells A375 while it showed synergistic effects with PLX in the Vemurafenib resistant melanoma cells A375VR. In addition, the combination of PLX4032 and 1 μM Hsp70 inhibitor VER showed synergistic effects in A375 melanoma cells but no effect on A375VR melanoma cells. These results indicate the differential effects of Hsp inhibitors working individually or collaboratively with PLX4032 at different concentrations. It also aligns with the anti-cancer activity of other Hsp inhibitors [50]. It is worth mentioning that PLX4032 showed a huge difference in cytotoxicity in A375 and A375VR melanoma cells. In this study, PLX4032 at a low concentration (3 μM) killed 50% of A375 melanoma cells; but A375VR melanoma cells maintained high viability even after being treated with PLX4032 at a much higher concentration.

Hsp90 inhibitors showed different abilities to affect cell migration in different melanoma cells. When combined with PLX4032, CAY mitigated the suppression of cell migration by PLX4032 in the Vemurafenib sensitive cells A375, but it increased the

inhibitory effect of PLX4032 on cell migration in the Vemurafenib resistant cells A375VR even at a low concentration. In addition, Hsp90 inhibitor CAY significantly inhibited cell migration in A375VR when it worked individually, and the Hsp90 inhibitor VER showed a synergistic effect with PLX4032 in inhibiting cell migration in A375 melanoma cells. These results suggest that Hsp90 could be a potential chemotherapy for inhibiting cancer cell migration and cancer development. This result is also validated by a published work that studied other Hsp90 inhibitors (17-AAG) [50].

Studies show that Hsp are critical in protecting cancer cells from undergoing cell apoptosis [51]. Thus, Hsp inhibitors kill cancer cells by inducing cell apoptosis [52]. For instance, it is reported that Hsp90 inhibitor SY-016 induced cell apoptosis in paclitaxel resistant human ovarian cancer cells [53]. In contrast to that study, our study revealed that Hsp90 inhibitor CAY did not induce cell apoptosis when it worked alone. Therefore, CAY did not exert cytotoxicity in both Vemurafenib sensitive and resistant cells by inducing apoptosis. However, when it was combined with PLX, Hsp90 inhibitor CAY attenuated the apoptosis induced by PLX4032 in the Vemurafenib sensitive melanoma cells A375 but enhanced the apoptosis caused by PLX4032 in the Vemurafenib resistant melanoma cells A375VR. Moreover, CAY also shows a synergistic effect with Hsp70 inhibitor VER in promoting cell apoptosis in both A375 and A375VR cell lines. Unlike the Hsp90 inhibitor CAY, the Hsp70 inhibitor VER was more potent in inducing cell apoptosis in both A375 and A375 VR cell lines when it worked alone. In addition, VER displayed a strong cytotoxic effect in A375VR when combined with PLX. These pro-apoptosis properties of Hsp70 inhibitors suggest that, similar to some other Hsp

inhibitors, VER may cause cytotoxicity by inducing apoptosis in both drug sensitive and drug resistant cells [45].

Since A375 and A375VR melanoma cell lines exhibited different responses when they were treated with the same Hsp inhibitors, we investigated the effect of Hsp on the activation of intracellular signaling pathways in both the Vemurafenib sensitive and resistant cell lines. In the Vemurafenib sensitive A375 cell line, the activation of Akt can be significantly inhibited by the Hsp90 inhibitor CAY alone or the combination with PLX4032. Furthermore, the activation of Erk can also be inhibited when cells are treated with CAY alone or in combination with CAY and VER. This is especially encouraging since it suggests that the Hsp90 inhibitor CAY decreased cell viability by blocking the PI3K/Akt and Ras-Raf-Mek-Erk pathways. These results are in agreement with the previous studies showing that Hsp inhibitors kill cancer cells by regulating the expression level of specific molecules [2, 10, 13]. Compared with the Hsp90 inhibitor, the Hsp70 inhibitor VER seemed not to work as well as the Hsp90 inhibitor in affecting signaling pathway. VER did not affect the phosphorylation of Akt and Erk but increased the expression level of Akt and Erk, indicating that VER may upregulate some translation factors which are responsible for increasing the expression of Akt and Erk. It is worthy of mention that in the Vemurafenib resistant cells A375VR, the Hsp90 inhibitor CAY not only inhibited the activation of Akt but also decreased the expression of Akt, indicating that CAY could be a highly effective anti-cancer drug in both drug sensitive and resistant cell lines.

In summary, this study shows that Hsp inhibitors exhibit strong anti-tumor ability in both the Vemurafenib sensitive and resistant cell lines. The Hsp90 and Hsp70 inhibitors decreased cell viability by inhibiting cell migration in both A375 and A375VR cells. In addition, the Hsp90 and Hsp70 inhibitors induced cytotoxicity by regulating the activation and expression of Akt and Erk. Additionally, the Hsp70 inhibitor inhibited cancer development by inducing cell apoptosis.

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Date Received