The Tubulin Inhibitor VERU-111 in Combination With Vemurafenib Provides an Effective Treatment of Vemurafenib-Resistant A375 Melanoma

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Melanoma is one of the deadliest skin cancers having a five-year survival rate around 15–20%. An overactivated MAPK/AKT pathway is well-established in BRAF mutant melanoma. Vemurafenib (Vem) was the first FDA-approved BRAF inhibitor and gained great clinical success in treating late-stage melanoma. However, most patients develop acquired resistance to Vem within 6–9 months. Therefore, developing a new treatment strategy to overcome Vem-resistance is highly significant. Our previous study reported that the combination of a tubulin inhibitor ABI-274 with Vem showed a significant synergistic effect to sensitize Vem-resistant melanoma both in vitro and in vivo. In the present study, we unveiled that VERU-111, an orally bioavailable inhibitor of α and β tubulin that is under clinical development, is highly potent against Vem-resistant melanoma cells. The combination of Vem and VERU-111 resulted in a dramatically enhanced inhibitory effect on cancer cells in vitro and Vem-resistant melanoma tumor growth in vivo compared with single-agent treatment. Further molecular signaling analyses demonstrated that in addition to ERK/AKT pathway, Skp2 E3 ligase also plays a critical role in Vem-resistant mechanisms. Knockout of Skp2 diminished oncogene AKT expression and contributed to the synergistic inhibitory effect of Vem and VERU-111. Our results indicate a treatment combination of VERU-111 and Vem holds a great promise to overcome Vem-resistance for melanoma patients harboring BRAF (V600E) mutation.

Keywords: VERU-111, vemurafenib-resistance, melanoma, ERK, akt, skp2

INTRODUCTION

Melanoma is one of the most common skin cancers, and the five-year survival rate for metastatic melanoma is 15–20% (Patel et al., 2020). Exposure to UV radiation increases the risk of DNA damage and genetic changes, thus confers susceptibility to melanoma.

It is well-established that the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways are overactivated in melanoma since BRAF mutation leads to uncontrollable cell growth and ultimately develops into cancer (Lim et al., 2017; Faghfuri et al., 2018). BRAF mutant melanoma accounts for nearly 50% of metastatic
malignant melanoma cases, among which V600E mutant represents 84.6% of the BRAF mutations (Patel et al., 2020). Currently, targeted therapies for metastatic melanoma mainly include BRAF and MEK inhibitors, such as Vemurafenib (the first FDA-approved BRAF inhibitor), dabrafenib, encorafenib, trametinib (the first FDA-approved MEK inhibitor), cobimetinib, and binimetinib (Shirley 2018). However, although ATP-competitive BRAF (V600E) kinase inhibitor such as Vem or its combination with a MEK inhibitor has dramatically improved the treatment outcome for patients with metastatic melanoma (Spain et al., 2016; Simone et al., 2017; Trojanelli et al., 2019), over 50% of patients develop acquired drug resistance and began to show signs of tumor recurrence within 6–9 months of treatment (Torres-Collado et al., 2018).

Several mechanisms have been documented to mediate Vem-resistance, for example, overexpression of P-glycoprotein (P-gp), BRAF mutation, aberrant expression of miRNA, translocation of E3 ligase, or PI3K/AKT pathway (Johnson et al., 2014; Duggan et al., 2014) which makes the therapy more potent and less toxic in several types of tumor models. Over 50% of patients develop acquired drug resistance and began to show signs of tumor recurrence within 6–9 months of treatment (Torres-Collado et al., 2018).

Materials and Methods

Reagents and Cell Lines
Vemurafenib was purchased from LC Laboratories (Woburn, MA), and VERU-111 was synthesized as described previously (Figure 1A). The human melanoma A375 cell line was acquired from ATCC (ATCC® CRL-1619) and maintained in DMEM with 10% FBS. Vemurafenib-resistant melanoma cells were built according to literature (Su et al., 2012). Briefly, cells were chronically selected by culturing A375 cells in increasing concentrations of Vem for at least 3 months and named VR1 cells. The isolated resistant VR1 cell line steadily increased IC50 values for Vem above 10 μM and maintained in full growth medium containing 5 μM Vem. VR1-SgSkp2 cells generated from VR1 cells and Skp2 was knocked out with guide RNA sequence: 5′-atgcaaggagacaccc-3′, screened with puromycin and maintained in full growth medium containing 5 μM Vem.

Cell Proliferation and IC50 Measurement
Cell proliferation was determined using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt] reagents (Promega, Madison, WI) following manual instruction. Briefly, cells were seeded at a concentration of 5,000 cells/well in 96-well plate, on next day, the cell culture medium contains the vemurafenib or VERU-111 at different concentrations was added into the well with four duplications. After 72 h later, 20 μl MTS solution was added and measured at 490 nm absorbance. IC50 was calculated using Graphpad Prism software with transformed drug concentration in Log10. Compound concentrations used in vivo animal study was based on previous publication (Wang et al., 2014).

Cell-Cycle and Apoptosis Analysis
To determine apoptosis and cell-cycle distributions, treated cells (24 h) were harvested with trypsin and fixed in 70% cold ethanol for overnight, then stained with PI (50 μg/ml)/RNase (100 μg/ml) solution for 60 min at room temperature in the dark according to the manufacturer’s instructions (Sigma Aldrich, St. Louis, MO).
Cell apoptosis was monitored by using the Annexin V-FITC Apoptosis Detection Kit (Abcam) following manufacturer’s instructions, and the data was processed using the Modfit 2.0 software and analyzed by a BD LSR-II cytometer (BD Biosciences).

**Colony Formation Assays**
For colony formation assays, 1,000 cells were plated in 6-well plates with triplicates, compound with indicated concentration was added in the next day, and surviving colonies were stained with crystal violet 10 days later and counted.

**Western Blot Analysis**
At the indicated time (24 h), treated A375, VR1, VR1-SgSkp2 cells were collected to investigate levels of relevant cascade protein or apoptotic markers by Western blot analysis. The following antibodies from Cell Signaling were used: p-ERK1/2 (#9101), p44/42 MAPK (ERK1/2; #9102), p- AKT (Ser473; #9271), AKT (#9272), cleaved PARP (#9185), or GAPDH (#3683). Skp2 antibody was purchased from Santa Cruz (sc-74477).

**Endogenous Co-Immunoprecipitation Assay**
For the co-immunoprecipitation assay, A375 cells and VR1 cells were treated with 5 μM Vem for 24 h, the cell lysates were incubated with A/G beads (Millipore) with corresponding equal amount of antibody in RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and 1 mM EDTA) at 4°C overnight. After extensive washes, precipitated proteins on beads were boiled and loaded onto SDS-PAGE gel and further performed Western blotting.

**VErumafenib-Resistant Tumor Xenograft and Treatment**
6–8-weeks NSG male mice were provided by Dr Seagrous lab. VR1 cells were suspended in PBS and mixed with high concentration Matrigel (BD Biosciences) at a ratio of 2:1 right before use. 100 μl of this mixture containing 2 × 10⁶ cells were injected subcutaneously to the right-side dorsal flank of each mouse. The regimen formulation and treatment refer to (Wang et al., 2014). Briefly, VERU-111 or Vem was diluted in PEG300 (Sigma Aldrich) and administered through intraperitoneal injection once per day, 5 days per week for three continuous weeks. Tumor volume and body weight of each mouse were measured three times per week. At the end of the experiments, mice were euthanized and tumor tissues were isolated and prepared for pathogen analysis. One-way ANOVA was used to compare tumor size and body weight for in vivo xenograft study. Tumor growth inhibition (TGI) was calculated as 100 – 100 × [(T – T0)/(C – C0)], and tumor regression was calculated as (T – T0)/T0 × 100, where T, T0, C, and C0 are the mean tumor volume for the specific group on the last day of treatment, mean tumor volume of the same group on the first day of treatment, mean tumor volume for the vehicle control group on the last day of treatment, and mean tumor volume for the vehicle control group on the first day of treatment, respectively.
Developed Vem-resistant cells (VR1) from the BRAF V600E parental A375 cells (0.43 μm). The IC50 value of VERU-111 in VR1 cells only strongly support the Vem-resistant property of VR1 cells. In hallmarks of acquired Vem-resistance (Boussemart et al., 2014).

Results

Pathology and Immunohistochemistry Analysis
Tumor tissues fixed in formalin buffer for more than 1 week were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC) analysis, the excised tumor tissues were collected in 10% formalin and embedded in paraffin. The following primary antibodies were used with rabbit anti-Ki67 (#9027, Cell Signaling Technology), rabbit anti-cleaved-caspase 3 (#9664, Cell Signaling Technology), rabbit anti-phospho-ERK1/2 (#4376, Cell Signaling Technology), rabbit anti-AKT (#4691, Cell Signaling Technology), p-AKT (#4060) following HRP-DAB-methods with signal boost reagents (#8114, Cell Signaling Technology). Slides were imaged with BZ-X700 microscope and analyzed by image J.

Statistical Analysis
Data were analyzed using Prism Software 5.0 (GraphPad Software, Inc.). The statistical significance (p < 0.05) was evaluated by student t test, and one-way ANOVA.

Combination of VEM with VERU-111 Inhibits Cell Proliferation and Increases Apoptosis in Both A375 and VR1 cells by Inhibiting AKT Expression
Next, we investigated whether VERU-111 has any synergistic interaction with Vem on melanoma cell lines, by comparing the single-agent treatment efficacy with their combination in both A375 and VR1 cells. Colony formation assays unveiled that proliferation of both parental A375 cells and Vem-resistant VR1 cells were inhibited following the entire regimen (Figures 2A,B). Moreover, Vem did not change cell cycle distribution of both cell lines, while addition of tubulin inhibitor bypassed G0-G1 cycle phase and arrested cell cycle at G2-M phase in both A375 and VR1 cells (Figure 2C). As Figure 2C showed that in VR1 cells, there is 41.3, 27.8, 10.9% of cells distributing in the G0-G1, S or G2-M phase, respectively. Vem single treatment produced similar cell cycle phase distribution. In VERU-111 single treatment group, the percentage of cells distributed in the G2-M phase had accumulated up to 88.6%. The combination of Vem and VERU-111 strongly arrested VR1 cells in both G0-G1 (3.7%) and G2-M (78.6%) phases while the combination regimen arrested parental A375 cells in both G0-G1 (47.9%) and G2-M (35.6%) phases, which indicated VERU-111 could capture Vem-resistant cells leaking from G0-G1 arrest, and thus produce a strong synergistic effect with Vem. Correspondingly, there are 0.8, 0.7, 10.5, 18.5% of apoptotic cells were detected in DMSO, Vem, VERU-111, combination treatment groups in VR1 cells respectively, and 0, 5.5, 8.9, 10.6% of apoptotic cells were observed in the indicated treatment groups in A375 cells. All these data suggested combination regimen has stronger efficiency in arresting the cell cycle and inducing apoptosis than a single treatment.

VR1 has sustained expressions of p-ERK upon single-agent Vem treatment (Figure 2D), similarly, sustained p-MEK expression was noted in VR1 cells after Vem treatment, consistent with the cross-resistance to MEK inhibitors in these Vem-resistant VR1 cells compared with the parental A375 cells (Figure 2D). In contrast, when treated with the combination of Vem and VERU-111, both A375 and VR1 cells had significantly more apoptosis (cleaved-PARP, Figure 2D), together with additional decreased expression of AKT expression and p-AKT activation (Figure 2D). In VR1 cells, the combination of VERU-111 and Vem reduced the level of AKT to 67 and 75% (0.6/0.9 × 100%, 0.6/0.8 × 100%) compared with single treatment, whereas the p-AKT expression level inhibited to 75 and 60% (0.3/0.4 × 100%, 0.3/0.5 × 100%) compared with a single treatment (Figure 2D). AKT is a serine/threonine kinase activated downstream of PI3K, which is a receptor for various pro-proliferation and bioactive substances. To our knowledge, the activation of AKT often contributes to tumorigenesis and plays a role in regulating cell motility, local invasion, and metastasis. Furthermore, our previously published outcomes proved that the synergistic anti-proliferation might be mediated by simultaneously targeting both ERK and AKT pathways (Wang et al., 2014).

Recently, F-box protein S-phase kinase-associated protein 2 (Skp2) was reported to be involved in drug resistance, including paclitaxel resistance (Kajiyama et al., 2007; Yang et al., 2014; Yang
et al., 2016; Huang et al., 2017; Byun et al., 2018; Cui et al., 2020), PI3K inhibitor resistance (Liu et al., 2013; Jia et al., 2014; Clement et al., 2018; Tian et al., 2018; Wang et al., 2018), and vemurafenib resistance (Feng et al., 2020), et al.

Interestingly, we also observed the overexpressed S-phase kinase-associated protein 2 (Skp2) in VR1 cells when treated with different concentrations of Vem compared with parental A375 cells (Figure 1B). However, the mRNA level of Skp2 did not increase in VR1 cells (Data not shown). Additionally, in the Vem-resistant cells, the expression of Skp2 dramatically reduced to 67 and 47% after the combination treatment compared with vem treatment (lane density normalized with GAPDH, 0.8/1.2 × 100%) and VERU-111 treatment (0.8/1.7 × 100%) (Figure 2D), indicating that Skp2 plays a role in the Vem-resistance. In our experiment, we also noticed Skp2 inhibition induced by Vem (Figures 2D,E) in parental A375 cells, which might be dependent on c-Myc transcriptional regulation (Feng et al., 2020). In malignant melanoma, Skp2 is highly expressed and correlates with tumor malignancy. It is noteworthy that Skp2 E3 ligase binds to AKT and is responsible for AKT degradation, and Skp2 is also required for AKT activation and membrane recruitment (Chan et al., 2012). Conversely, phosphorylation of Skp2 on Ser72 by AKT promotes its stabilization (Song et al., 2015). In line with these studies, a dramatic reduction of AKT levels and p-AKT expression was also seen in the combination treatment group (Figure 2D). Skp2 binds with AKT (Figure 2E), and the interaction was increased in parental A375 cells while decreased in VR1 cells after Vem treatment. Collectively, the result highlighted that Skp2 is involved in Vem-resistance, and it may contribute to the synergistic effect of Vem and VERU-111. It is worth noting that p53 expression increased upon combination treatment, which is consistent with our recent finding that VERU-111 could inhibit tumor growth and migration in cervical cancer cells by promoting DNA damage response mediated by p53 (Kashyap et al., 2020).

Skp2 involved in mechanisms of Vem-resistance and contributes to the effect of combination treatment

To further clarify the role of Skp2 in the indicated treatment, we knocked out Skp2 in VR1 cells using CRISPR-Cas9 technique. Expectedly, IC_{50} of Vem and VERU-111 improved approximately 2- and 5-fold (Vem from 33.92 to 16.74 μM and VERU-111 from 0.056 to 0.01 μM) respectively, which indicated that knockout of Skp2 not only restored compound sensitivity of VR1 cells to Vem, but also increased drug sensitivity to VERU-111 (Figure 3A). Interestingly, increased apoptosis was observed in VR1-SgSkp2 (Figure 3B). Indeed, Skp2 may inhibit apoptosis and contribute to drug resistance (Schüler et al., 2011; Wang et al., 2011). In line with these observations, we also found highly expressed Skp2 in Vem-resistant melanoma cells (Figures 1B, 2D), and decreased AKT
expression and AKT phosphorylation in two single clones of VR1-SgSkp2 cells (VR1-SgSkp2-No.1 and No.2), which might be the reason to increased cell apoptosis and cell growth arrest caused by the combination regimen (Figures 2, 3C). Of note, in order to keep the resistant feature, VR1-SgSkp2 cells were still cultured in the medium with Vem, which explains minor alteration about of IC50 of Vem. Further analysis demonstrated that knockout of Skp2 compromised AKT activation, as indicated by decreased phosphorylation of AKT (Figure 3C).

**Combination of VEM and VERU-111 Synergistically Suppress Vemurafenib-resistant Tumor Growth in vivo**

The combination of dabrafenib (BRAF inhibitor) and trametinib (MEK inhibitor) is approved to treat Braf V600E mutant melanoma patients (Robert et al., 2015; Long et al., 2017; Hauschild et al., 2018). To evaluate our in vivo xenograft mouse model, we compared the inhibitory effect of dabrafenib and its combination with trametinib. Based on previous research, the doses of 30 mg/kg dabrafenib and 0.3 mg/kg trametinib were selected (Kawaguchi et al., 2017; Yanagihara et al., 2018). Figure 4A showed no significant toxic effect in all three groups as no much change of body weight was observed. Importantly, dabrafenib plus trametinib regimen has a stronger tumor inhibitory effect (TGI at 28.6%) with statistical significance ($p < 0.05$, compared with vehicle control and dabrafenib alone) (Figures 4B,C and Table 1), demonstrating the efficacy of our in vivo animal model.

Next, we evaluated whether there was a strong synergistic interaction between Vem and VERU-111 to counteract Vem-resistance in vivo. We inoculated VR1 cells in the right flank of NSG mice and treated them either with a single compound or the combination treatment strategy to assess the inhibitory effect on tumors. Based on our previous research on ABI-274, the dose of 10 mg/kg VERU-111 was selected in the current (Wang et al., 2014). As depicted in Figure 5A, no significant change was noted in body weight in all the groups. At the end of the experiment, we euthanized all the mice and examined their major organs, and no injure was found. This indicated that no general toxicity was induced by VERU-111 in vivo. Notably, the combination treatment strategy dramatically inhibited tumor growth compared with a single treatment or control group (Figures 5B,C), in which the tumor size in the combination group was within 100 mm³, while it reached 1,000 mm³ in vehicle group after 4 weeks of treatment. As shown in Figures 5B,C and Table 2, Vem (30 mg/kg) single treatment achieved minimal (40.6%) TGI and VERU-111 (10 mg/kg) resulted in slightly better TGI at 76.6%, whereas combination treatment significantly enhanced tumor inhibition to 96.1% after 4 weeks treatment to Vem-resistant xenograft model. Hematoxylin and eosin (H&E) staining of the tumor tissue showed that the tumor cell lost intact shape, nuclei shrank, and even some cells lost membranes, highlighting the
antitumor effect of tubulin inhibitor (Figure 5D). Immunohistochemistry (IHC) staining revealed that decreased proliferation (Ki67 staining), increased cell apoptosis (cleaved-caspase three staining), and remarkably reduced expressions of $\text{p}-\text{ERK}$, total AKT and $\text{p}-\text{AKT}$ (Figure 5D). Overall, the above-mentioned findings demonstrated that the tubulin inhibitor had a strong inhibitory effect on Vem-resistant tumor growth either as a single candidate or combined regimen with Vem. Additionally, VERU-111 showed a giant potential to overcome Vem-resistance in melanoma cancer cells (Figure 5D), which may be advantageous for melanoma patients harboring BRAF(V600E) mutation.

**DISCUSSION**

Recently, the combination of BRAF inhibitor dabrafenib with MEK inhibitor trametinib was approved by FDA to treat patients harboring BRAF (V600E) mutation in NSCLC (non-small cell lung cancer) or melanoma. Although this regimen has exhibited great success in clinical therapy, patients may eventually acquire resistance after a couple of months (Robert et al., 2015; Long et al., 2017). We have developed a series of tubulin inhibitors that bind to the colchicine site in tubulin and have shown their anti-tumor effect and potential in overcoming Vem-resistance, paclitaxel-resistance in nude mice xenograft model (Lu et al., 2014; Wang et al., 2018; Wang et al., 2019; Chen et al., 2020). VERU-111 (ABI-231) is an orally available tubulin inhibitor that disrupts tubulin polymerization, promotes microtubule fragmentation, inhibits cancer cell migration, and is currently in phase 1b/2 clinical trials for men with metastatic castration and androgen-blocking agent resistant prostate cancer (ClinicalTrials.gov Identifier: NCT03752099). Tubulin inhibitor is less prone to develop resistance, therefore bearing potential to cure cancer and to sensitize drug-resistance cancer patience (Wang et al., 2014; Guan et al., 2017; Arnst et al., 2018; Deng et al., 2020; Kashyap et al., 2020; Mahmud et al., 2020).

In this study, we investigated whether the orally derivative of ABI-274, VERU-111, has synergistic effect with Vem. VERU-111 has been tested in many cancer cell lines and its IC_{50} is 5.6 nM in M14 cell, 7.2 nM in WM164 melanoma cell (Wang et al., 2019), 8.2 nM in MDA-MB-231 breast cancer cell (Chen et al., 2020), 55.6 nM in NSCLC A549 cell and 102.9 nM in A549-Paclitaxel resistant cells (Mahmud et al., 2020). In agreement with outcomes of previous research, it was confirmed that by synergistically arresting cancer cells at G0-G1 and G2-M phases, the combined treatment regimen of Vem and VERU-111 could overcome the Vem-resistance through enhanced apoptosis and compromised Skp2-AKT signaling pathway. In a tumor xenograft model, the combined regimen displays a better inhibitory efficiency against tumor progression than either single treatment. Further IHC analysis of tissue sections confirmed decreased tumor proliferation and the diminished expression of AKT and p-AKT. Several studies reported an association between inhibition of AKT and tubulin polymerization (Zhang et al., 2009; Krishnegowda et al., 2011; Viola et al., 2012). Inhibition of AKT-mediated survival signaling pathway has been shown to increase sensitivity to microtubule-targeted tubulin-polymerizing agents (MTPAs)-induced apoptosis in cancer cells (Bhalla 2003). The results of the present research are consistent with findings of these studies, highlighting a close interaction between tubulin polymerization inhibitors and downregulation of AKT in melanoma.

Remarkably, Skp2 E3 ligase was also involved in the mechanisms of Vem-resistance and synergistic effect of combination regimen. Recent studies reported that overexpressed Skp2 was found in paclitaxel-resistant prostate cancer cells (Yang et al., 2016; Byun et al., 2018;
Gong et al., 2018), and knockdown of Skp2 restored the sensitivity of paclitaxel in prostate cancer cells (Byun et al., 2018). Skp2 also plays a pivotal role in mitosis and spindle checkpoint by triggering ubiquitination and activation of Aurora-B (Nakayama et al., 2004; Sugihara et al., 2006; Hu and Aplin, 2008; Wu et al., 2015). Skp2 depletion in melanoma cells resulted in a G2-M phase arrest (Hu and Aplin 2008), and suppression of both BRAF (V600E) and Skp2 inhibited cell growth and invasion in melanoma cell lines (Sumimoto et al., 2006). Since Skp2 was reported to interact with AKT (Chan et al., 2012), we also tested the interaction and found decreased AKT expression and AKT phosphorylation in VR1-SgSkp2 cells (Figures 2E, 3D), thereby leading to cell apoptosis and cell growth arrest caused by the combination treatment (Figure 3). Meanwhile, BRAF inhibitor dabrafenib combination with MEK inhibitor trametinib present a mild synergistic effect in inhibition of tumor growth, as shown in Figure 4 and Table 1. By contrast, our in vivo xenograft tumor model demonstrated that combination regimen of Vem and VERU-111 has more potent tumor inhibitory effect than single administration (Figure 5 and Table 2). When administated in combination with Vem, VERU-111 has a tumor growth inhibitory rate (TGI) of 96.1%, which was better than ABI-274 (TGI 88.6%) [Table 2 and reference (Wang et al., 2014)].

Collectively, based on our study, VERU-111 overcome Vem-resistance through the following mechanisms: 1) As a tubulin destabilizing agent, disrupt tubulin polymerization, promote microtubule fragmentation, inhibit cancer cell migration; 2) Combined with Vem, arresting cell both in G0-G1 and G2-M phase; 3) Compromised Skp2-AKT signaling pathway. Our study showed that VERU-111bears inspiring potential in synergistically combination with BRAF inhibitor Vem to overcome drug resistance in melanoma. Furthermore, this synergistic effect might through regulating Skp2-AKT, as evidenced by increased apoptosis and drug sensitization when skp2 was knocked out, which suggested that silencing skp2 might be an effective way to overcome Vem-resistance.

**CONCLUSION**

In conclusion, our findings provide direct evidence and a reasonable explanation for giving a combination of a tubulin

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**TABLE 2** | TGI comparison for in vivo combination of vemurafenib (30 mg/kg) and VERU-111 (10 mg/kg) in the Vem-resistant VR1 xenograft model.

| Treatment group | TGI (100%) |
|-----------------|------------|
| Vehicle control | —          |
| Vem (30 mg/kg)  | 40.6 ± 11.6|
| VERU-111 (10 mg/kg) | 76.6 ± 18.4 |
| Vem + VERU-111  | 96.1 ± 4.8*|

* p < 0.05, compared with single-agent treatment groups.
inhibitor VERU-111 with a BRAF inhibitor to overcome Vem-resistant in melanoma patients.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the University of Tennessee HSC.

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**AUTHOR CONTRIBUTIONS**

HC and WL designed the study, HC and QW performed the experiments; HC, QW, DM, and WL wrote the manuscript.

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**Conflict of Interest:**世界级的专家咨询委员会成员和Veru公司授权发行VERU-111用于商业化开发。WL和DM也报告接受过相关研究协议的资助。Veru公司。

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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