

PI3K inhibitor significantly enhances the antitumor activity of oncolytic virus in osteosarcoma

Ye Wang

Affiliated Hospital of North Sichuan Medical College

Xin Yang

Affiliated Hospital of North Sichuan Medical College

Hong Li

Affiliated Hospital of North Sichuan Medical College

Haiyang Xie (✉ oceanxiehy@163.com)

Affiliated Hospital of North Sichuan Medical College

Research

Keywords: osteosarcoma, PI3K inhibitor, oncolytic virus, VSV Δ 51, ZSTK474

Posted Date: February 19th, 2020

DOI: <https://doi.org/10.21203/rs.2.24004/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Osteosarcoma (OS) is a highly aggressive malignancy with less than 30% 5-year survival rate among patients with metastatic or recurrent cancer. However, the treatment for osteosarcoma has not been modified in the last three decades. Oncolytic viruses have shown encouraging results in pre-clinical trials, but have failed to translate into high therapeutic efficacy in clinical trials. In this study, we will determine the therapeutic effect of combining PI3K inhibitor with an oncolytic virus against osteosarcoma.

Material and Methods: Osteosarcoma cell lines and xenograft model were treated with ZSTK474 and/or VSV Δ 51, the tumor suppressive ability was verified by in vitro cytotoxicity experiments and in vivo antitumor activity experiments, and the antitumor mechanism was explored through the study of apoptosis-related signaling pathways.

Results: ZSTK474 sensitized the osteosarcoma cells to VSV Δ 51, and augmented apoptosis via endoplasmic reticulum stress. The combination treatment also showed greater in vivo tumor inhibition compared to either ZSTK474 or VSV Δ 51 alone, and significantly enhanced the tumor infiltration of immune cells.

Conclusion: PI3K inhibitors combined with oncolytic virus is a promising strategy against osteosarcoma.

Background

Osteosarcoma (OS) is the most common form of bone cancer in children and adolescents, and belongs to a large family of mesenchymal-derived solid tumors with considerable histological, genetic and molecular heterogeneity[1–3]. It is initiated by somatic TP53 and/or RB1 mutations that lead to chromosomal instability and malignant transformation[4]. The 5-year survival rate of patients diagnosed with metastatic or recurrent osteosarcoma is less than 30%[5]. A recent multi-center clinical trial showed that increasing the dose of chemotherapy improved prognosis of patients but did not prolong survival in high-risk populations, thus underscoring the need for novel treatment strategies[6].

Oncolytic viruses are a novel immunotherapeutic tool that has shown encouraging results in various cancer models[7]. The oncolytic herpes virus Talimogene Laherparepvec (T-VEC) was recently approved by FDA for treating metastatic melanoma[8]. Vesicular stomatitis virus (VSV), an enveloped negative sense strand RNA virus of the Rhabdoviridae family, has shown strong oncolytic activity in preclinical models and is currently undergoing clinical trials[9, 10]. Site-directed mutagenesis of VSV allows it to preferentially target tumors without infecting the healthy cells[11]. For example, VSV Δ 51 can only replicate in tumor cells and not in normal cells due to the methionine deletion at position 51[12]. Studies show that oncolytic viruses infect cancer cells through a dual mechanism of selective replication and lysis, and induce an anti-tumor immune response in the host[13]. However, these viruses cannot exert a potent "oncolytic" effect on their own, and the ensuing anti-tumor immune response is also limited[14]. To

improve their therapeutic effects against solid tumors, combination therapy or genetically engineered viruses are currently being explored[15, 16].

The PI3K/AKT/mTOR signaling pathway is constitutively active in many cancers, and promotes excessive proliferation of tumor cells while inhibiting apoptosis[17, 18]. Blocking the PI3K-Akt pathway not only mitigates tumor progression but also induces endoplasmic reticulum stress (ERS) in the tumor cells[19]. Interestingly, oncolytic virus-induced apoptosis can be significantly enhanced by aggravating ERS in the host cells through pharmacological agents[20, 21].

In this study, we determined whether the PI3K inhibitor ZSTK474 enhanced the oncolytic activity of VSV Δ 51 in osteosarcoma cells, and found that the former acted as a potent adjuvant and significantly increased viral cytotoxicity in vitro. In addition, the combination therapy also increased the infiltration of immune effector cells in the osteosarcoma xenografts in vivo, resulting in greater inhibition of tumor growth. Therefore, our findings present a novel immunotherapeutic strategy against osteosarcoma using oncolytic virus and pharmacological induction of ERS.

Materials And Methods

Cell lines and reagents

The osteosarcoma cell lines 143B, G-63, Saos-2, U2OS, SJSA-1 and HOS, and the Vero cells were obtained from the American Type Culture Collection (ATCC). RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM) were purchased from Hyclone (Logan, USA). Fetal bovine serum and L-glutamine were purchased from Gibco (Gaithersburg, USA). Penicillin and streptomycin were obtained from Sangong Biotech (Shanghai, China). HY-50847 were obtained from MCE (NJ, USA). Antibodies against IRE1 α , p-ERK and ATF6 were purchased from Santa Cruz Biotechnology (TX, USA). Antibodies against CHOP and Ki-67 were purchased from Cell Signaling Technology (MA, USA). Antibody against caspase-12 were purchased from Abcam (OR, USA). Antibody against GAPDH were purchased from Bioworld (MN, USA). CD90.2-PerCP, CD45.2-perCP, CD8a-FITC, CD4-PE, Gr1-APC, CD11b-PE, F4/80-FITC and NK1.1-PE were purchased from BioLegend (CA, USA).

Cell culture

The G-63, Vero and Saos-2 cells were cultured in RPMI-1640 medium, and 143B, U2OS, SJSA-1 and HOS cells in Dulbecco's modified Eagle's medium, each supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO₂.

VSV production, quantification, and infection

GVP expressing VSV Δ 51, a recombinant derivative of the Indiana VSV serotype, was provided by Dr. John Bell and Dr. David Stojdl (Ottawa Health Institute)[12]. VSV Δ 51 was cultivated in the Vero cells and virus titers were quantified by the standard plaque analysis method as previously described[22].

Cell viability assay

The 143B cells were seeded in a 96-well plate at the density of 3000 cells/well. Following treatment with ZSTK474, thiazole blue (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) was added to each well as the final concentration of 1 mg/mL, and the cells were incubated for 3 h at 37 °C. The medium was removed and the MTT crystals were dissolved in 100 μ L DMSO, and the absorbance at 570 nm was measured using Pickers (iMark, Bio-Rad).

Western blotting

Cells or tissues were lysed with mammalian protein extraction reagent (M-PER; Thermo Science) and separated by SDS-PAGE. The proteins bands were transferred to immunoblot membranes and probed with antibodies against IRE1 α , p-ERK, ATF6, CHOP, caspase-12 and GAPDH. The membrane was developed on a ChemiDoc XRS + system (Bio-Rad) using Immobilon Western chemiluminescence HRP substrate (Millipore), and the protein bands were quantified using Image J software.

Transmission electron microscopy

The 143B cells were infected with VSV Δ 51 at 0.001 PFU/cell for 24 h in the presence or absence of ZSTK474. The infected cells were centrifuged at 1000 g for 5 min at room temperature. After washing once with PBS, the cells were pelleted at 1500 g for 5 min, and fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice. The samples were then analyzed by standard transmission electron microscopy.

Xenograft model

Female BALB/c-nu/nu mice (4 weeks old) were each subcutaneously injected with 5×10^6 143B cells on their dorsal side. Palpable tumors ($\sim 50 \text{ mm}^3$) appeared within 3 days, and once they reached $\sim 150 \text{ mm}^3$ after 6 days, the tumor-bearing mice were injected intravenously with VSV Δ 51 (2.5×10^7 pfu/kg/d) between 6–8 days and 12–14 days with/out ZSTK474 (2 mg/kg/d) for a total of 6 times. The placebo group was injected with equal volume of PBS at the same time points. The length and width of the tumor were measured every 3 d, and the volume was calculated as $(\text{length} \times \text{width}^2)/2$. The animal experiments were approved by the Animal Ethics and Welfare Committee of affiliated hospital of north sichuan medical college

Immunohistochemistry

Tumor sections (4 μ m thick) were dewaxed in xylene, hydrated in an ethanol gradient, soaked in 0.3% H₂O₂-methanol for 30 min, washed with PBS, and incubated overnight with monoclonal antibodies against Ki-67 (9449s, Cell Signaling Technology), IRE1 α , CHOP and Cleaved-caspase-12 at 4 °C. After washing once, the sections were incubated with biotin-labeled goat anti-rabbit or anti-mouse IgG for 2 h at room temperature. Immunostaining was developed with streptavidin/peroxidase complex and diaminobenzidine, and counterstained with hematoxylin.

Flow cytometry

The tumor tissues were homogenized to single cell suspensions and stained with the following antibodies (all from BioLegend; diluted 1:100 dilution): CD90.2-PerCP, CD45.2-perCP, CD8a-FITC, CD_4-PE, Gr1-APC, CD11b-PE, F4/80-FITC and NK1.1-PE. The stained cells were acquired on a FACSCanto II, and analyzed by FlowJo 7.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Different groups were compared with Student's t test or analysis of variance (ANOVA) as appropriate. Tumor volumes were calculated by repeated measurement of single factor analysis of variance. Kaplan-Meier survival curves were compared by the log-rank test. P values less than 0.05 were considered statistically significant.

Results

ZSTK474 sensitizes osteosarcoma cells to VSVΔ51

The target cells were treated with varying doses of ZSTK474 in the presence/absence of low titer (0.001 PFU/cell) VSVΔ51 oncolytic virus (Fig. 1A). ZSTK474 decreased the viability of osteosarcoma cells in a dose-dependent manner, and also augmented the cytotoxic effect of VSVΔ51. Almost 80% of the infected cells were dead when additionally treated with 4 μM ZSTK474, indicating that the latter sensitizes osteosarcoma cells to VSVΔ51 (Fig. 1B).

The synergistic effect of ZSTK474 and VSVΔ51 depends on ERS-induced apoptosis

The surge in protein synthesis during viral replication frequently overloads the endoplasmic reticulum, resulting in ERS[23] that triggers the unfolded protein response (UPR) to restore protein homeostasis and decrease aggregates of misfolded peptides. However, persistent UPR due to severe ERS can lead to apoptosis[24]. Consistent with this, the 143B cells treated with ZSTK474 or VSVΔ51 showed markedly swollen endoplasmic reticulum within 24 h, which increased further in the dual treated cells (Fig. 2A, B). Furthermore, the ERS markers including IRE1α and p-PERK were also significantly upregulated in the cells treated with both ZSTK474 and VSVΔ51 compared to the monotherapy groups, while that of activating transcription factor 6 (ATF6) was not affected (Fig. 2C). As shown in Fig. 2D, the caspase-12 pathway was strongly activated in the combination treatment group, along with upregulation of C/EBP-homologous protein (CHOP) compared to the ZSTK474 or VSVΔ51-treated cells. Finally, Hoechst 33342 staining showed a significant increase in the number of pyknotic nuclei in the dual treated tumor cells, indicating apoptosis induction. However, karyopyknosis was not obvious in the ZSTK474 or VSVΔ51-treated cells (Fig. 2E). Taken together, ZSTK474 augmented VSVΔ51-induced ERS and apoptosis in osteosarcoma cells.

ZSTK474 and VSVΔ51 synergistically inhibit tumor growth in vivo

The in vivo therapeutic potential of ZSTK474 and VSVΔ51 was evaluated in an osteosarcoma xenograft model, as outlined in Fig. 3A. Compared to ZSTK474 or VSVΔ51 alone, the combination treatment significantly inhibited tumor growth ($P < 0.001$; Fig. 3B-D). Furthermore, the tumor weight in the combination treatment group was significantly lower than that in ZSTK474 or VSVΔ51 groups (Fig. 3E). Consistent with this, in situ Ki67 expression was significantly lower and that of IRE1a, CHOP and C-caspase-12 were higher in the tumor tissues of the combination treatment group compared to the ZSTK474 or VSVΔ51-treated tumors (Fig. 4A, B). Thus, PI3K inhibition enhanced viral infection-induced ERS and apoptosis in tumor tissues, which translated to greater therapeutic effect.

ZSTK474 and VSVΔ51 enhance immune cell infiltration into tumors

Oncolytic viruses trigger the anti-tumor immune response after infecting the tumor cells, thus converting the "cold tumor" into a "hot tumor"[25]. Since the PI3K-AKT-mTOR signaling pathway regulates immune responses, we also analyzed the immune cell infiltrates in the tumors following each treatment regimen. Compared to ZSTK474 or VSVΔ51 alone, their combination significantly enhanced the infiltration of NK cells into the tumors and decreased that of MDSCs (Fig. 5A). The number of tumor-infiltrating macrophages was lower in each treatment group compared to that in the untreated controls. Furthermore, the CD8+/CD4+ T cell ratio in the tumors was significantly increased in the combination treatment vs either monotherapy groups, indicating greater activation of anti-tumor immune responses in the former (Fig. 5B). Taken together, ZSTK474 also amplifies VSVΔ51-mediated anti-tumor immune response in vivo.

Discussion

Osteosarcoma is a malignant tumor characterized by formation of neoplastic or neoplastic-like bone tissues, and accounts for about 20–34% of all primary malignant bone tumors[26]. The 5-year survival rate of patients with metastatic or recurrent osteosarcoma is less than 30%, thereby requiring novel treatment strategies. Oncolytic viruses are a promising immunotherapeutic tool against cancer cells and several are currently in clinical trials. The FDA approved T-Vec developed by Angen in October 2015 for the treatment of metastatic melanoma[27]. The main focus of oncolytic virus therapy is to directly kill the tumor cells as well as activate the anti-tumor immune response[28]. Genetically engineered recombinant oncolytic viruses have also been designed in order to improve antitumor activity[29]. However, this strategy is limited by the small viral genomes and technical difficulty. In addition, excessive modification of the virus skeleton reduces its infection ability, and therefore oncolytic function[30]. Thus, recent studies are focusing on "conditionally enhancing" the efficacy of oncolytic viruses through pharmacological agents[31].

The PI3K-AKT-mTOR signaling pathway is frequently dysregulated during tumor initiation and progression[32–36]. Studies show that PI3K inhibitors increase tumor cell apoptosis and modulate the tumor immune microenvironment and immune-infiltrating cells[37, 38], which can potentially affect the anti-tumor effect of oncolytic viruses. Therefore, the PI3K inhibitor ZSTK474 was tested in combination with VSVΔ51 against osteosarcoma[39]. ZSTK474 increased apoptosis in the virus-infected tumor cells by enhancing ERS in vitro, and amplified the virus-induced host immune response in the tumor-bearing mice. The therapeutic effects of this combination were significantly greater compared to either VSVΔ51 or ZSTK474 alone.

Conclusions

Our findings provide a strong experimental basis for using small molecule inhibitors of AKT and mTOR as adjuvants along with oncolytic virus to enhance therapeutic effects against solid tumors. There are however some limitations in our study that ought to be addressed. We injected the oncolytic virus via the intravenous route to ensure tumor-specific infection of the oncolytic viruses, although studies show that intra-tumoral or peritumoral injection results in better anti-tumor effects[40]. Therefore, the optimum dosage regimen needs to be further explored in the follow-up experiments. In conclusion, the combination of VSVΔ51 and ZSTK474 is a promising new strategy for treating osteosarcoma.

Abbreviations

OS: Osteosarcoma; T-VEC: Talimogene Laherparepvec; VSV: Vesicular stomatitis virus; ERS: Endoplasmic reticulum stress; UPR: Unfolded protein response; ATF6: Activating transcription factor; CHOP: C/EBP-homologous protein; TEM: Transmission electron microscopy

Declarations

Ethics approval and consent to participate

This study was approved by the Animal Ethics and Welfare Committee of affiliated hospital of north sichuan medical college.

Availability of data and materials

All data are available in the included figures.

Competing interests

The authors declare that they have no conflict of interests.

Acknowledgements

None.

Consent for publication

None.

Funding

This work is supported by Scientific Research Project of Sichuan Provincial Department of Education (18ZB0218).

Authors' contributions

Conceptualization: HX, YW; Methodology: YW, XY; Software: YW, HL; Supervision: HX; Writing original draft: YW, HX; All authors read and approved the final manuscript.

References

1. Bao Q, Shen Y, Zhang W. ASO Author Reflections: Metastatic Biopsy of Osteosarcoma with Circulating RNA. *Annals of surgical oncology* 2018; 25(3): 936-937.<https://doi.org/10.1245/s10434-018-6977-5>
2. Heymann D. Metastatic osteosarcoma challenged by regorafenib. *The Lancet Oncology* 2019; 20(1): 12-14.[https://doi.org/10.1016/S1470-2045\(18\)30821-0](https://doi.org/10.1016/S1470-2045(18)30821-0)
3. Brady SW, Ma X, Bahrami A, Satas G, Wu G, Newman S, Rusch M, Putnam DK, Mulder HL, Yergeau DA. The clonal evolution of metastatic osteosarcoma as shaped by cisplatin treatment. *Molecular Cancer Research* 2019; 17(4): 895-906.<https://doi.org/10.1158/1541-7786>
4. Song K, Song J, Lin K, Chen F, Ma X, Jiang J, Li F. Survival analysis of patients with metastatic osteosarcoma: a Surveillance, Epidemiology, and End Results population-based study. *International orthopaedics* 2019; 43(8): 1983-1991.<https://doi.org/10.1007/s00264-019-04348-4>
5. Bajpai J, Chandrasekharan A, Simha V, Talreja V, Karpe A, Pandey N, Singh A, Rekhi B, Vora T, Ghosh J. Outcomes in Treatment-Naïve Patients With Metastatic Extremity Osteosarcoma Treated With OGS-12, a Novel Non-High-Dose Methotrexate-Based, Dose-Dense Combination Chemotherapy, in a Tertiary Care Cancer Center. *Journal of global oncology* 2018; 4: 1-10.<https://doi.org/10.1200/JGO.17.00137>
6. Kopp LM, Malempati S, Krailo M, Gao Y, Buxton A, Weigel BJ, Hawthorne T, Crowley E, Moscow JA, Reid JM, Villalobos V, Randall RL, Gorlick R, Janeway KA. Phase II trial of the glycoprotein non-metastatic B-targeted antibody-drug conjugate, glebatumumab vedotin (CDX-011), in recurrent osteosarcoma AOST1521: A report from the Children's Oncology Group. *Eur J Cancer* 2019; 121: 177-183.<https://doi.org/10.1016/j.ejca.2019.08.015>
7. Zou P, Tang R, Luo M. Oncolytic virotherapy, alone or in combination with immune checkpoint inhibitors, for advanced melanoma: A systematic review and meta-analysis. *International Immunopharmacology* 2020; 78: 106050.<https://doi.org/10.1016/j.intimp.2019.106050>

8. Coffin R. Interview with Robert Coffin, inventor of T-VEC: the first oncolytic immunotherapy approved for the treatment of cancer. *Immunotherapy* 2016; 8(2): 103-106.<https://doi.org/10.2217/imt.15.116>
9. Velazquez-Salinas L, Naik S, Pauszek SJ, Peng K-W, Russell SJ, Rodriguez LL. Oncolytic recombinant vesicular stomatitis virus (VSV) is nonpathogenic and nontransmissible in pigs, a natural host of VSV. *Human Gene Therapy Clinical Development* 2017; 28(2): 108-115.<https://doi.org/10.1089/humc.2017.015>
10. Zhang N, Du B. Reply to Rubinstein: Is Lgr4 essential for VSV– and VSV-G–pseudotyped lentiviral vector entry to cells? *Journal of Biological Chemistry* 2018; 293(1): 113-113.<https://doi.org/10.1074/jbc.L117.000471>
11. Kimpel J, Urbiola C, Koske I, Tober R, Banki Z, Wollmann G, Von Laer D. The oncolytic virus VSV-GP is effective against malignant melanoma. *Viruses* 2018; 10(3): 108.<https://doi.org/10.3390/v10030108>
12. Stojdl DF, Lichty BD, Paterson JM, Power AT, Knowles S, Marius R, Reynard J, Poliquin L, Atkins H, Brown EG. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer cell* 2003; 4(4): 263-275.[https://doi.org/10.1016/S1535-6108\(03\)00241-1](https://doi.org/10.1016/S1535-6108(03)00241-1)
13. Melcher A. Oncolytic Virotherapy: Single Cycle Cures or Repeat Treatments?(Repeat Dosing Is Crucial!). *Molecular Therapy* 2018; 26(8): 1875-1876.<https://doi.org/10.1016/j.ymthe.2018.07.002>
14. Malinzi J, Ouifki R, Eladdadi A, Torres DF, White K. Enhancement of chemotherapy using oncolytic virotherapy: mathematical and optimal control analysis. arXiv preprint arXiv:1807.04329 2018.<https://doi.org/10.3934/mbe.2018066>
15. Berg DR, Offord CP, Kemler I, Ennis MK, Chang L, Paulik G, Bajzer Z, Neuhauser C, Dingli D. In vitro and in silico multidimensional modeling of oncolytic tumor virotherapy dynamics. *PLoS Comput Biol* 2019; 15(3): e1006773.<https://doi.org/10.1371/journal.pcbi.1006773>
16. Atherton MJ, Stephenson KB, Tzelepis F, Bakhshinyan D, Nikota JK, Son HH, Jirovec A, Lefebvre C, Dvorkin-Gheva A, Ashkar AA, Wan Y, Stojdl DF, Belanger EC, Breau RH, Bell JC, Saad F, Singh SK, Diallo JS, Lichty BD. Transforming the prostatic tumor microenvironment with oncolytic virotherapy. *Oncoimmunology* 2018; 7(7): e1445459.<https://doi.org/10.1080/2162402X.2018.1445459>
17. Rahmani F, Ziaemehr A, Shahidsales S, Gharib M, Khazaei M, Ferns GA, Ryzhikov M, Avan A, Hassanian SM. Role of regulatory miRNAs of the PI3K/AKT/mTOR signaling in the pathogenesis of hepatocellular carcinoma. *J Cell Physiol* 2019.<https://doi.org/10.1002/jcp.29333>
18. Roncolato F, Lindemann K, Willson ML, Martyn J, Mileskin L. PI3K/AKT/mTOR inhibitors for advanced or recurrent endometrial cancer. *Cochrane Database Syst Rev* 2019; 10: CD012160.<https://doi.org/10.1002/14651858.CD012160.pub2>
19. Kanaizumi H, Higashi C, Tanaka Y, Hamada M, Shinzaki W, Azumi T, Hashimoto Y, Inui H, Houjou T, Komoike Y. PI3K/Akt/mTOR signalling pathway activation in patients with ERpositive, metachronous, contralateral breast cancer treated with hormone therapy. *Oncology letters* 2019; 17(2): 1962-1968.<https://doi.org/10.3892/ol.2018.9759>

20. Mahoney DJ, Lefebvre C, Allan K, Brun J, Sanaei CA, Baird S, Pearce N, Grönberg S, Wilson B, Prakesh M. Virus-tumor interactome screen reveals ER stress response can reprogram resistant cancers for oncolytic virus-triggered caspase-2 cell death. *Cancer cell* 2011; 20(4): 443-456.<https://doi.org/10.1016/j.ccr.2011.09.005>
21. McConkey DJ. RAIDDing ER stress for oncolytic viral therapy. *Cancer cell* 2011; 20(4): 416-418.<https://doi.org/10.1016/j.ccr.2011.10.002>
22. Ben Yebdri F, Van Grevenynghe J, Tang VA, Goulet ML, Wu JH, Stojdl DF, Hiscott J, Lin R. Triptolide-mediated inhibition of interferon signaling enhances vesicular stomatitis virus-based oncolysis. *Mol Ther* 2013; 21(11): 2043-53.<https://doi.org/10.1038/mt.2013.187>
23. Stepanenko AA, Chekhonin VP. Recent Advances in Oncolytic Virotherapy and Immunotherapy for Glioblastoma: A Glimmer of Hope in the Search for an Effective Therapy? *Cancers (Basel)* 2018; 10(12).<https://doi.org/10.3390/cancers10120492>
24. Yuan P, Dong L, Cheng Q, Wang S, Li Z, Sun Y, Han S, Yin J, Peng B, He X, Liu W. Prototype foamy virus elicits complete autophagy involving the ER stress-related UPR pathway. *Retrovirology* 2017; 14(1): 16.<https://doi.org/10.1186/s12977-017-0341-x>
25. SM OB, Mathis JM. Oncolytic Virotherapy for Breast Cancer Treatment. *Curr Gene Ther* 2018; 18(4): 192-205.<https://doi.org/10.2174/1566523218666180910163805>
26. Kiyuna T, Tome Y, Miyake K, Murakami T, Oshiro H, Igarashi K, Kawaguchi K, Hsu J, Singh M, Li Y, Nelson S, Bouvet M, Singh SR, Kanaya F, Hoffman RM. Eribulin Suppressed Cisplatin- and Doxorubicin-resistant Recurrent Lung Metastatic Osteosarcoma in a Patient-derived Orthotopic Xenograft Mouse Model. *Anticancer Res* 2019; 39(9): 4775-4779.<https://doi.org/10.21873/anticancer.13661>
27. Blake Z, Marks DK, Gartrell RD, Hart T, Horton P, Cheng SK, Taback B, Horst BA, Saenger YM. Complete intracranial response to talimogene laherparepvec (T-Vec), pembrolizumab and whole brain radiotherapy in a patient with melanoma brain metastases refractory to dual checkpoint-inhibition. *J Immunother Cancer* 2018; 6(1): 25.<https://doi.org/10.1186/s40425-018-0338-6>
28. Bommareddy PK, Patel A, Hossain S, Kaufman HL. Talimogene Laherparepvec (T-VEC) and Other Oncolytic Viruses for the Treatment of Melanoma. *Am J Clin Dermatol* 2017; 18(1): 1-15.<https://doi.org/10.1007/s40257-016-0238-9>
29. Cassidy T, Craig M. Determinants of combination GM-CSF immunotherapy and oncolytic virotherapy success identified through in silico treatment personalization. *PLoS computational biology* 2019; 15(11).<https://doi.org/10.1371/journal.pcbi.1007495>
30. Harrington KJ, Puzanov I, Hecht JR, Hodi FS, Szabo Z, Murugappan S, Kaufman HL. Clinical development of talimogene laherparepvec (T-VEC): a modified herpes simplex virus type-1-derived oncolytic immunotherapy. *Expert Rev Anticancer Ther* 2015; 15(12): 1389-403.<https://doi.org/10.1586/14737140.2015.1115725>
31. Denton NL, Chen CY, Hutzen B, Currier MA, Scott T, Nartker B, Leddon JL, Wang PY, Srinivas R, Cassady KA, Goins WF, Cripe TP. Myelolytic Treatments Enhance Oncolytic Herpes Virotherapy in

- Models of Ewing Sarcoma by Modulating the Immune Microenvironment. *Mol Ther Oncolytics* 2018; 11: 62-74.<https://doi.org/10.1016/j.omto.2018.10.001>
32. Yao W, Lin Z, Shi P, Chen B, Wang G, Huang J, Sui Y, Liu Q, Li S, Lin X, Liu Q, Yao H. Delicaflavone induces ROS-mediated apoptosis and inhibits PI3K/AKT/mTOR and Ras/MEK/Erk signaling pathways in colorectal cancer cells. *Biochem Pharmacol* 2019; 171: 113680.<https://doi.org/10.1016/j.bcp.2019.113680>
33. Tateishi K, Nakamura T, Juratli TA, Williams EA, Matsushita Y, Miyake S, Nishi M, Miller JJ, Tummala SS, Fink AL. PI3K/AKT/mTOR pathway alterations promote malignant progression and xenograft formation in oligodendroglial tumors. *Clinical Cancer Research* 2019; 25(14): 4375-4387.<https://doi.org/10.1158/1078-0432.CCR-18-4144>
34. Haines CN, Klingensmith HD, Burd CJ. GREB1 regulates proliferation of estrogen receptor positive breast cancer through modulation of PI3K/Akt/mTOR signaling. *bioRxiv* 2019: 704825.<https://doi.org/10.1101/704825>
35. Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X. Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Molecular cancer* 2019; 18(1): 26.<https://doi.org/10.1186/s12943-019-0954-x>
36. Conway JR, Herrmann D, Evans TJ, Morton JP, Timpson P. Combating pancreatic cancer with PI3K pathway inhibitors in the era of personalised medicine. *Gut* 2019; 68(4): 742-758.<https://doi.org/10.1136/gutjnl-2018-316822>
37. Lastwika KJ, Wilson W, Li QK, Norris J, Xu H, Ghazarian SR, Kitagawa H, Kawabata S, Taube JM, Yao S. Control of PD-L1 expression by oncogenic activation of the AKT–mTOR pathway in non–small cell lung cancer. *Cancer research* 2016; 76(2): 227-238.<https://doi.org/10.1158/0008-5472.CAN-14-3362>
38. Larabi A, Barnich N, Nguyen HTT. New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. *Autophagy* 2020; 16(1): 38-51.<https://doi.org/10.1080/15548627.2019.1635384>
39. Namatame N, Tamaki N, Yoshizawa Y, Okamura M, Nishimura Y, Yamazaki K, Tanaka M, Nakamura T, Semba K, Yamori T, Yaguchi SI, Dan S. Antitumor profile of the PI3K inhibitor ZSTK474 in human sarcoma cell lines. *Oncotarget* 2018; 9(80): 35141-35161.<https://doi.org/10.18632/oncotarget.26216>
40. Killock D. Skin cancer: T-VEC oncolytic viral therapy shows promise in melanoma. *Nat Rev Clin Oncol* 2015; 12(8): 438.<https://doi.org/10.1038/nrclinonc.2015.106>

Figures

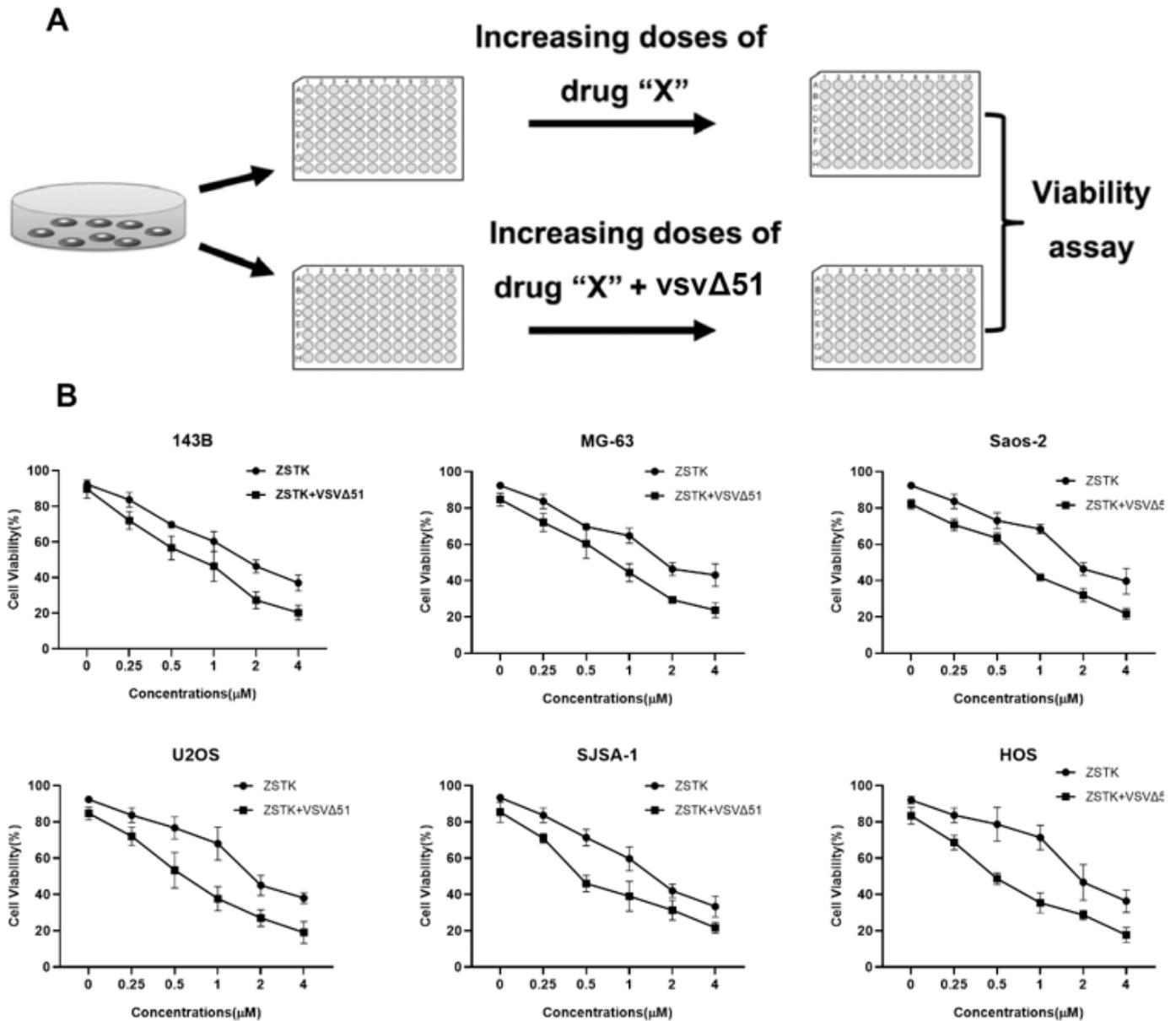


Figure 1

ZSTK474 sensitizes osteosarcoma cells to VSVΔ51. A. Experimental outline: 143B, G-63, Saos-2, U2OS, SJSA-1 and HOS cells were treated with varying doses of ZSTK474 with/out VSVΔ51 (MOI = 0.001). B. Percentage of viable cells following different treatments.

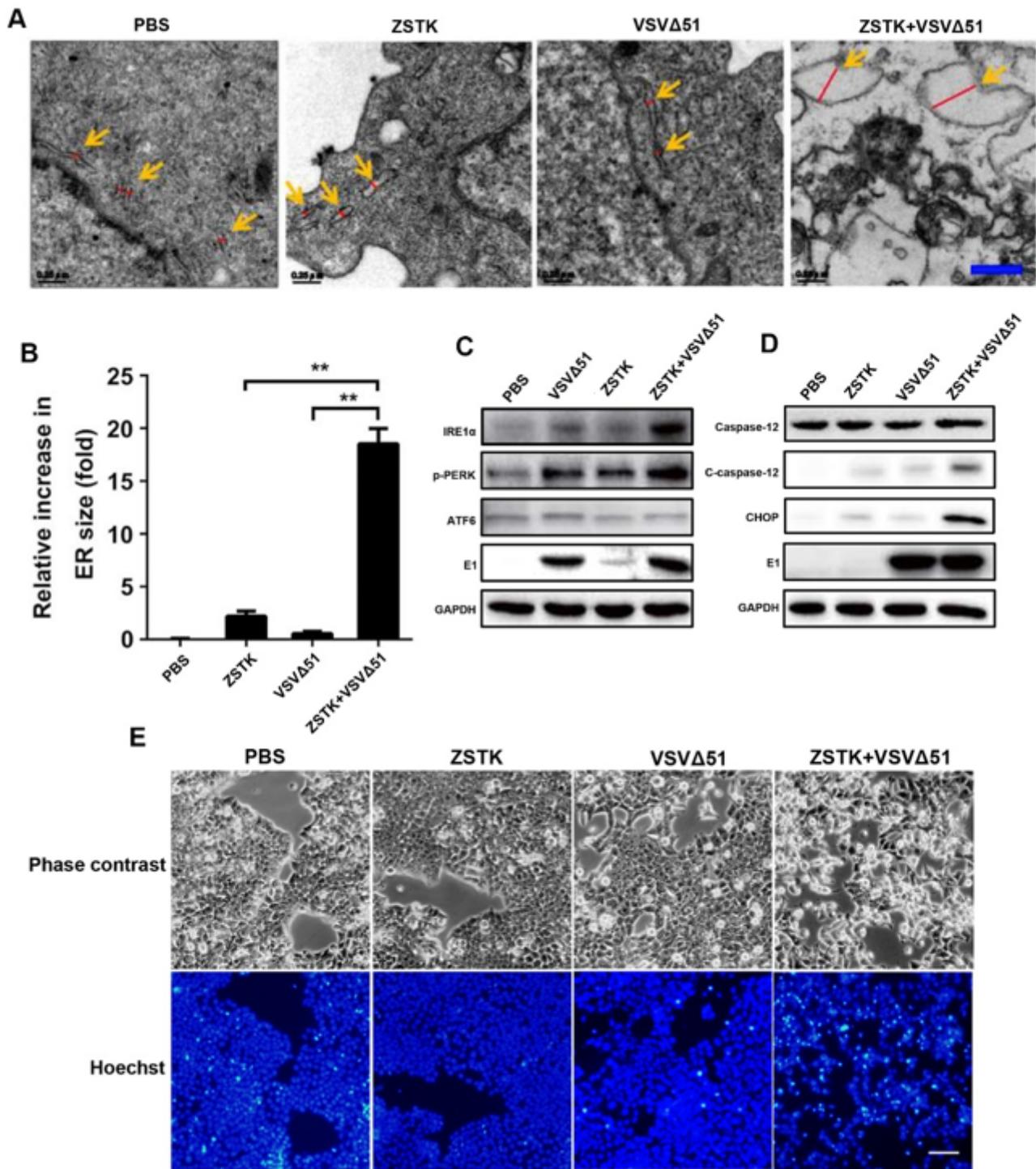


Figure 2

PI3K inhibition aggravated ERS-induced apoptosis in VSVΔ51-infected cells. **A.** Transmission electron microscopy (TEM) images of PBS, ZSTK474, VSVΔ51 and ZSTK474 + VSVΔ51-treated 143B cells in vitro. Orange arrows indicate the ER and the red line trace the relative size. Scale bar = 0.5 μm. **B.** ER swelling as quantified from TEM images. Data is expressed as mean ± SD (n = 4, ** P < 0.01). **C-D.** Immunoblot showing expression levels of (C) IRE1α, p-PERK and ATF6, and (D) CHOP, caspase-12 and E1

proteins in the differentially treated 143B cells in vitro. E. Representative images of Hoechst 33342-stained images of the differentially treated cells. Scale bar = 100 mM, n = 3.

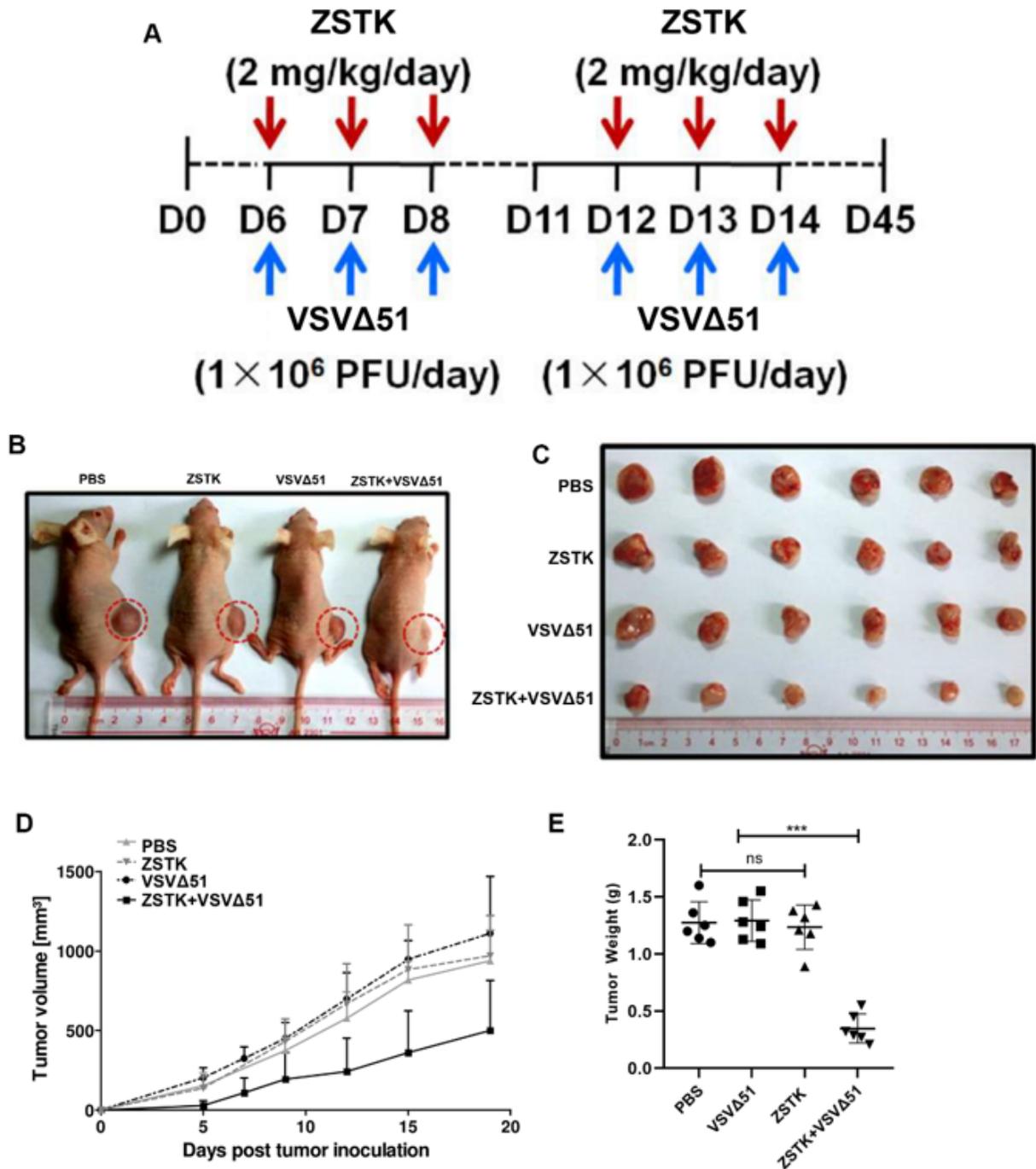


Figure 3

ZSTK474 improved the therapeutic effect of VSVΔ51 in the mouse model of osteosarcoma. A. Experimental flowchart. B-C. Representative images of tumors from the PBS, ZST K474, VSVΔ51 and combination treatment groups. D. Changes in tumor volume in the different groups (n = 5). E. Tumor weight in each treatment group at the end of the experiment (n = 5, *** P < 0.001).

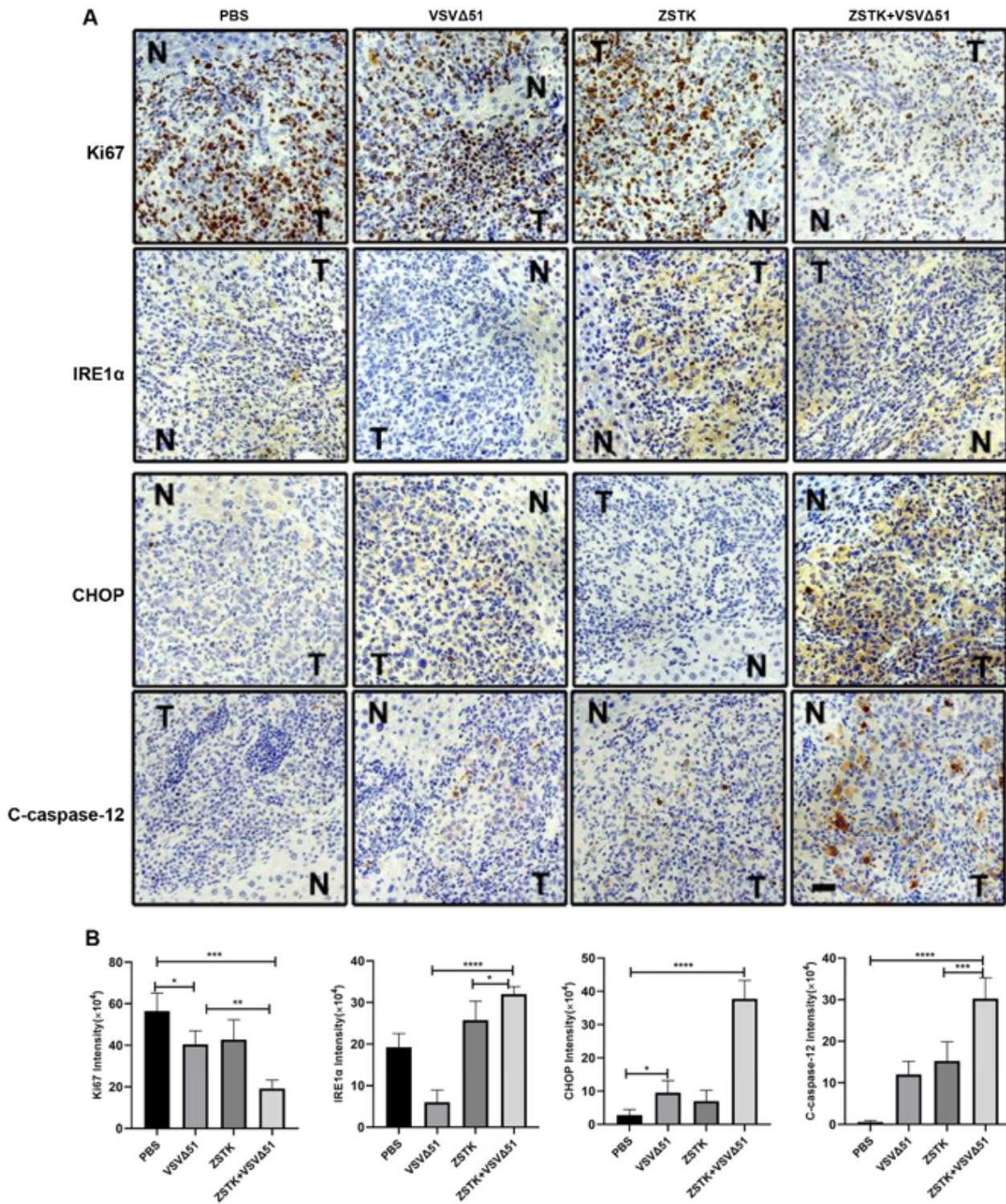


Figure 4

ZSTK474 and VSVΔ51 inhibited osteosarcoma growth xenograft growth in vivo. A. In situ expression of Ki-67 (proliferation marker), IRE1α, CHOP and C-caspase-12 in the tumor tissues. Scale bar = 50 μm. B. Quantification of immunohistochemistry results.

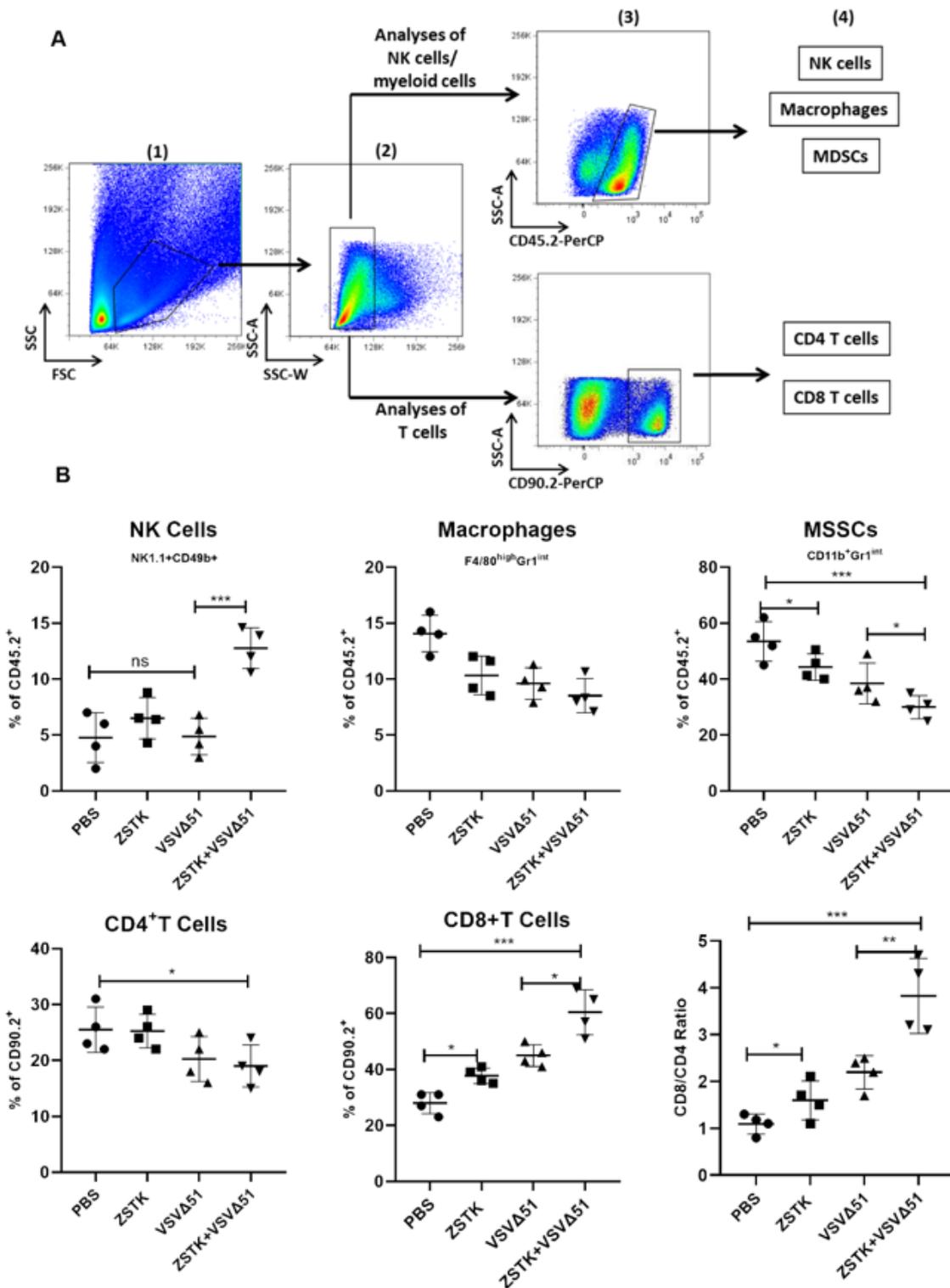


Figure 5

ZSTK474 and VSVA51 enhanced immune cell infiltration in tumor tissues. A. Flow cytometry plots showing various immune cell subtypes in the tumor tissues. B. The proportion of tumor infiltrating NK cells, macrophages and MDSCs in the differentially treated mice relative to the CD45.2⁺ white blood cells. The frequency of CD8 and CD4 T cells was calculated as the percentage of CD90.2⁺ lymphocytes (n = 3, * P < 0.05, ** P < 0.01, *** P < 0.001).