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Screening for Synergistic Reagents With Pazopanib Against Osteosarcoma Using a Compound Library

YUKI YADA[1], KUNIHIRO ASANUMA[1], TAKUYA KAKIMTO[1], KAZUMA OKUNO[1], TAKAYUKI OKAMOTO[2], TAKAHIRO IINO[1], TOMOKI NAKAMURA[1] and AKIHIRO SUDO[1]

[1]*Department of Orthopedic Surgery, Mie University School of Medicine, Tsu, Japan;*
[2]*Department of Pharmacology, Faculty of Medicine, Shimane University, Izumo, Japan*

Abstract. *Background/Aim: Osteosarcoma (OS) is the most common malignant bone tumor. As the same agents have been in use since the mid-1970s, new therapeutic approaches are needed to improve prognosis. Pazopanib (PZP) has already demonstrated marked antitumor activity clinically and can be effective in patients with metastatic OS. We investigated the combination treatment of candidate agents with PZP and examined effects on tumor growth using an in vivo model. Materials and Methods: A library of 324 compounds was used. MG63 OS cells were treated with PZP and each compound. Cell viability was measured. The antiproliferative effects of compound combination on four OS cell lines was tested. Cell signaling was evaluated by western blot analysis. In vivo antitumor testing was performed using 143B-bearing mice. Results: The screening process identified crizotinib (CRZ) as the most effective drug for combination with PZP. The combination of PZP and CRZ demonstrated effects compared to control or single therapy. Cell signal investigation showed that dual therapy down-regulated c-MYC, p-AKT, p-STAT3, p-cyclin D1 and survivin and up-regulated cleaved caspase-3 and cleaved PARP compared to control or single therapy. In vivo analysis showed dual therapy achieved synergic effects for tumor growth compared to control or single-treatment groups. No significant difference in the change in body weight was observed among groups. Conclusion: Combined use of PZP and CRZ offers synergic anti-tumor effects against OS, inducing apoptosis in vitro and in vivo by down-regulating AKT and STAT3. Our data suggest that these agents can be used for patients clinically.*

Correspondence to: Kunihiro Asanuma, Department of Orthopedic Surgery, Mie University School of Medicine 2-174 Edobashi, Tsu City, Mie 514-8507, Japan. Tel: +81 592315022, Fax: +81 592315211, e-mail: kasanum@gmail.com

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Osteosarcoma (OS) is a type of malignant bone tumor that is most commonly observed in children, adolescents, and young adults. The treatment of OS requires a multidisciplinary approach that includes surgery, chemotherapy, and radiotherapy (1). The discovery of effective chemotherapies for the treatment of OS has led to significant improvements in patient outcomes. The 5-year survival rate for patients with localized OS has reached 70-80% (2). However, Patients with metastatic OS have a 5-year event-free survival rate of only less than 20% (3-8). The use of a combination of chemotherapeutic agents, including doxorubicin, cisplatin, methotrexate, and ifosfamide, is widely recognized as effective against OS (9-13). However, although these agents have been used to treat OS since the mid-1970s, new therapeutic approaches are required to enhance the prognosis of OS patients (14). Molecularly targeted cancer therapies have been garnering attention for their potential advantages over conventional anticancer agents in terms of drug metabolism and accumulation, optimum doses, and side effects, for various tumors (15). Effective molecularly targeted therapies are currently expected to replace conventional OS therapies. Identifying new uses for existing drugs has been acknowledged as a more efficient approach to drug discovery than developing novel drugs, as the development of clinical agents is a costly and time-consuming process.

Pazopanib (PZP) has already demonstrated marked antitumor activity against soft tissue sarcoma clinically (16) and can be effective in treating patients with metastatic OS (17). As OS is characterized by high levels of genetic instability (18), we considered single molecularly targeted agents that have been speculated to be less effective against OS. The purpose of this study was to establish a new cocktail for OS treatment with PZP and determine its potential molecular targeting on tumor cell proliferation. We investigated the synergic effects of treatment in terms of anti-proliferative activity, sought to elucidate the intracellular mechanisms of the candidate agent with PZP and examined the inhibitory effects on tumor growth using an *in vivo* model.

Materials and Methods

Osteosarcoma cell culture. This study employed four human OS cell lines, namely 143B, MG63, HOS, and HUO9. Minimum essential medium (Gibco, Carlsbad, CA, USA) was used to culture 143B, MG63, and HOS containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). HUO9 cells were grown in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were cultured as adherent monolayers and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Chemical compounds. The Screening Committee of Anticancer Drugs (SCADS) compound library of 324 compounds in four 96-well microplates was kindly provided by a Grant-in-Aid for Scientific Research on the Priority Area 'Cancer' from the Ministry of Education, Culture, Sports, Science and Technology of Japan. A solution of dimethyl sulfoxide (DMSO) containing all compounds, which mainly consist of antitumor drugs and kinase inhibitors, was provided at a concentration of 10 mM.

Initially, PZP and Crizotinib (CRZ) were dissolved in DMSO and stored at –20°C. For the experiments, the agents were diluted with culture media to the final concentration required.

Compound screening. Human OS cells from the MG63 cell line were used. Cells were seeded on 96-well plates at 3.0×10^4 cells/well/100 μl. The total amount of medium was 100 μl/well. In 96-well plates, OS cells were exposed to either a DMSO or 10 mM of each compound from the SCADS library. The 96-well plates were incubated for another 24 h at 37°C. Cell viability was measured using a Cell-Titer 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Absorption at 492 nm was measured using a microprocessor-controlled microplate reader (Infinite F200 PRO; Tecan Group, Zurich, Switzerland).

In vitro study of PZP and CRZ. OS cells from the MG63, 143B, HOS, and HuO9 cell lines were used. Cells were seeded on 96-well plates at 3.0×10^4 cells/well/100 μl. The total amount of medium was 100 μl/well. Wells were treated with 0, 1, or 10 μM of PZP and 0, 1, or 10 μM CRZ and incubated for 24 h at 37°C. Cell viability was assessed in the same manner applied in the screening study.

Western blot analysis. Cells were seeded on 6-well plates at 8.0×10^5 cells/well/500 μl. Wells were treated with 0 or 10 μM of PZP and 0 or 10 μM of CRZ and incubated at 37°C for 30 min, 6 h, 12 h, or 24 h. After treatment, cells were lysed with radioimmunoprecipitation (RIPA) buffer (Millipore-Upstate, Temecula, CA, USA) supplemented with a protease inhibitor cocktail of 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mM Na_3VO_4. Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and samples were adjusted to the same protein concentration before loading. Proteins were transferred to a nitrocellulose membrane and blotted. Antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA) and used at the dilutions recommended by the manufacturer: phospho-STAT3 (Tyr705, D3A7) XP® (#9145) antibodies, 1:2,000 dilution; cleaved caspase-3 (Asp175; #9661); phospho-cyclin D1 (#3300); c-Myc (MYC; D85C12; #5605); survivin (#2808); phospho-AKT (AKT1; #4060); phospho-p44/42 MAPK [extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204; D13.14.4E) XP (#4370)]; and cleaved PARP antibodies (Asp214; D64E10) XP (#5625). Beta-actin protein (ab8229; 1:500) was assayed as a loading control.

Growth of 143B xenografts in athymic nude mice with in vivo PZP and CRZ treatment. All animal experiments strictly followed the guidelines of Cedars-Sinai Medical Center and the National Institutes of Health. Protocol was approved by the Institutional Committee on Animal Welfare of Mie University (approved number. 27-27). Female nude mice were subcutaneously inoculated with 5.0×10^6 cells of human OS from the 143B cell line in 200 μl of solution. The experimental groups consisted of thirty-two mice that were randomly assigned to one of the following groups: i) saline with DMSO (diluent-specific control); ii) 25 mg/kg PZP; iii) 25 mg/kg CRZ; iv) 25 mg/kg PZP + 25 mg/kg CRZ. Saline, PZP and CRZ were administered everyday orally. Body weight and tumor size were assessed twice a week, and tumor volume was calculated using the formula V=lw2/2, where l and w represent the length and width of the tumor, respectively. This formula was described previously. Treatment was discontinued after 5 weeks.

Statistical analysis. Non-parametric analysis of variance (ANOVA) and the Mann–Whitney test were used to compare differences between groups. Repeated-measures ANOVA was used to compare changes in tumor volume over time. Values of $p<0.05$ were considered statistically significant.

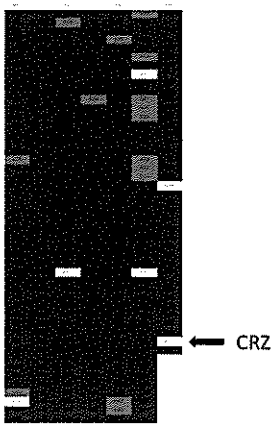
Results

Compound screening. The anti-proliferative effects of the 324 compounds were screened against the MG63 cell line. The results were color-coded according to the percentage decrease in cell viability with the compound and PZP compared to the compound without PZP, based on our previous studies (19, 20). Based on this color analysis, compounds for investigation were narrowed down to Trichostatin A, Ouabain, JAK inhibitor I, Go7874, WP1066, and CRZ. Among these candidates, we chose CRZ for further study as an agent that has already seen use in clinical situations. Subsequent experiments were therefore performed using CRZ (Figure 1).

In vitro study of PZP and CRZ. The antiproliferative effects of treatment were investigated in MG63, 143B, HOS, and Huo9 cells. PZP with CRZ demonstrated a significant, dose-dependent reduction in cell viability in all four OS cell lines. Synergistic antiproliferative effects were observed in all cells treated with 10 μM PZP and 10 μM CRZ (Figure 2).

Western blotting. MG63, 143B, HUO9, and HOS cells were treated with or without 10 μM PZP and 10 μM CRZ for 30 min, 6 h, 12 h, or 24 h. The effects of PZP and CRZ on p-STAT-3, p-Akt, p-Erk, p-cyclin D1, cleaved PARP, cleaved caspase-3, c-MYC, and survivin were analyzed by Western blotting.

Down-regulation of c-MYC was seen in MG-63, HOS, and HuO9 at 12 h and 24 h. Down-regulation of p-cyclin D1 in all cell lines and survivin at 24 h in all cell lines with dual therapy was seen compared to control or single-treatment



compound concentration 1 μM
pazopanib concentration 10 μM

$$\text{value} = \frac{\textit{compound+pazopanib OD}}{\textit{compound OD}}$$

□< 0.4 0.4 ≤ ▒ < 0.5 0.5 ≤ ■

Target	Name
HDAC	Trichostatin A
Na/K ATPase	Ouabain
JAK	JAK inhibitor I
PKC	Go7874
STAT3	WP1066
EML4-ALK	Crizotinib

Figure 1. *Color-coded analysis. A Cell-Titer 96® AQueous One Solution Cell Proliferation Assay kit was used to measure cell viability. MG-63 cells were treated with 1 μM of each compound from The Screening Committee of Anticancer Drugs (SCADS) library with or without 10 μM of pazopanib (PZP) for 24 h. Cell viability was then evaluated. The effects of compounds were categorized into color-coded groups based on cell viability. White: <40% viable cells; gray: 40-50% viable cells; dark gray: >50% viable cells. The 6 compounds that were categorized as showing <40% viable cells (white) are listed. Crizotinib (CRZ) was chosen for further analysis.*

groups. Down-regulation of p-ERK was seen at 30 min in MG63 and HOS cells compared to control or single groups. Conversely, p-ERK was up-regulated in 143B and HuO9 at 24 h. Dual therapy showed synergic apoptotic effects by inducing cleaved caspase-3 and cleaved PARP in all cell lines at all time points compared to control and single groups. The effects on cleaved caspase-3 were lost in MG63 at 24 h. Down-regulation of p-AKT and p-STAT3 was seen with dual therapy at 30 min and 6, 12, and 24 h compared to control and single groups. The effect on p-STAT3 was lost in 143B at 24 h. Down-regulation of survivin was seen at 24 h in all cell lines with dual therapy compared to control and single groups. Combination therapy with PZP and CRZ had the effect of both interrupting cell cycle progression and strongly inducing apoptosis (Figure 3A-D).

Growth of 143B xenografts in athymic nude mice with in vivo PZP and CRZ treatment. To determine the effects of PZP and CRZ on tumor growth inhibition *in vivo*, we evaluated changes in 143B xenografts following PZP and CRZ treatment. In the absence of PZP or CRZ, growth of the 143B tumor was highly aggressive, but treatment with 25 mg/kg of PZP or CRZ significantly inhibited tumor growth. Dual therapy with both PZP and CRZ achieved significant synergistic effect on inhibition of tumor growth compared to control or single-treatment groups. Tumor volume at day 35 with combination therapy was significantly lower than with single therapy or control according to the Mann–Whitney test. Repeated-measures ANOVA showed that the change in tumor volume over time was significantly reduced with combination therapy compared to that with single-agent therapy (Figure 4). In addition, weight loss due to tumor progression was mildly suppressed in the group treated with both PZP and CRZ (Figure 5), but no significant difference in body weight was evident between groups.

Discussion

Progress in the development of therapeutic agents for primary musculoskeletal malignant tumors including OS has been much slower than that with molecularly targeted agents for other cancers. This is because the prevalence of these pathologies is lower and tumor tissues are formed heterogeneously by various cells. Although screening compound libraries to detect potentially useful agents for tumor treatment is not difficult, few such screening procedures have been performed for rare malignancies such as OS (21). The rarity of these tumors may result in economic disadvantages for pharmaceutical companies and difficulty in performing clinical trials of a size sufficient to offer statistical validity. While various molecularly targeted agents have been developed for use as monotherapies, combination therapy may induce unexpected synergies in antitumor effects.

PZP is a tyrosine kinase inhibitor with high affinity for vascular endothelial growth factor receptor (VEGFR) and activity against both platelet-derived growth factor receptor (PDGFR) and c-KIT (22). PZP has been approved for second-line treatment of soft tissue sarcomas after the failure of standard chemotherapy. PZP for soft tissue sarcomas improved median progression-free survival (mPFS) to 4.6

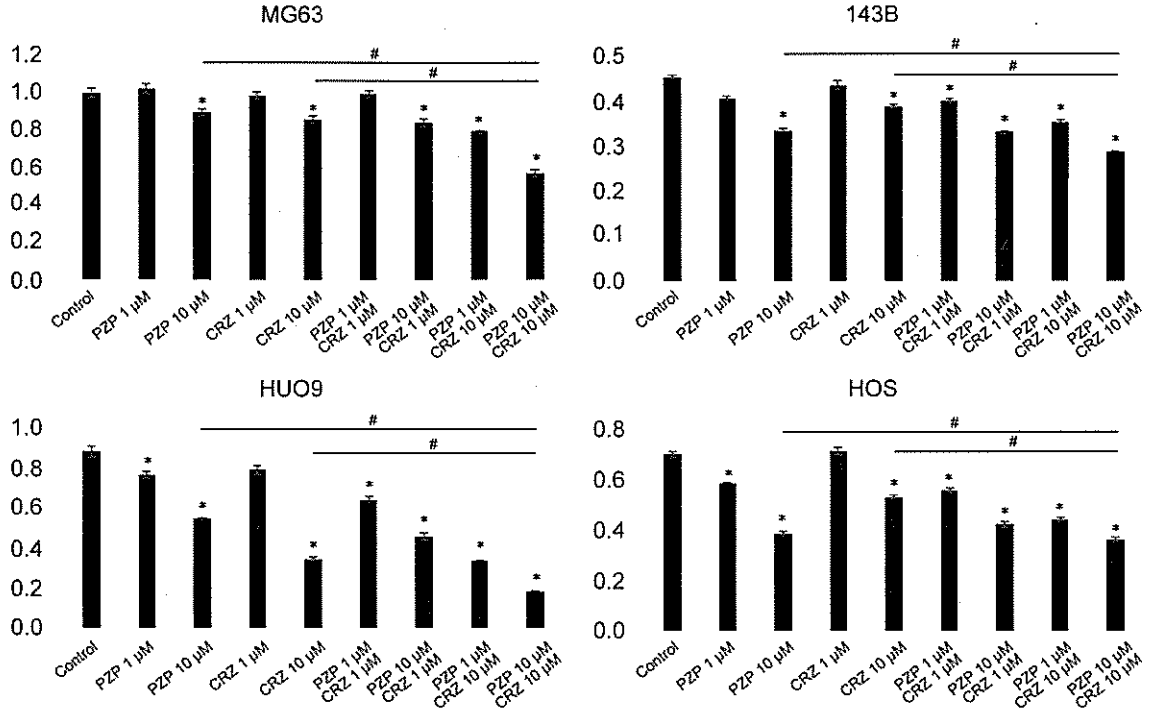


Figure 2. *Cell viability assay in four osteosarcoma (OS) cell lines. The same assay as screening was used (Figure 1). Pazopanib (PZP) and crizotinib (CRZ) were used at concentrations of 1 or 10 μM. The effect of combination therapy was evaluated in MG-63, 143B, HuO9, and HOS cells. *p<0.05 compared to control. #p<0.05.*

months compared to 1.6 months with placebo (23). However, data on the efficacy of PZP in patients with OS remains limited and based on case reports and small retrospective analyses on the off-label use of the drug (24). Longhi *et al.* reported a 60% disease control rate (DCR) with 1 partial response among 15 patients with relapsed OS treated using PZP (25). Similar outcomes were reported by Aggerholm-Pedersen *et al.* in 19 patients with bone tumors, including eight patients with OS, treated using PZP. Four patients with OS showed partial response to treatment (26). They reported that mPFS for the whole group was 5.5 months, with an overall response rate of 32% and a DCR of 68% (26). Efficacy of PZP has also been shown in some case reports for both pediatric (27) and adult patients (28-30), with a PFS of approximately 6 months. Given such findings, we focused on utilizing PZP in the treatment of OS, including combination with other drugs. Our study showed that PZP alone has anti-proliferative effects on OS cell lines. Furthermore, we attempted to identify a new combination therapy for OS through cell-based screening using the SCADS inhibitor kit and four OS cell lines. We selected CRZ from among many candidates based on its ability to inhibit cell viability in OS cell lines when administered with PZP.

CRZ is an ATP-competitive small-molecule inhibitor of the receptor tyrosine kinases C-Met, ALK, and ROS1 (31). CRZ shows robust effectiveness against non-small-cell lung cancers (NSCLCs) harboring EML4-ALK-rearrangements resulting in constitutional activation of ALK. The drug has been approved for use against this entity, which represents no more than 3-5% of all NSCLCs (30). CRZ is thus already in clinical use. In addition, CRZ reportedly inhibits the proliferation of OS cells. Qing *et al.* reported that CRZ could inhibit the proliferation of OS cells by increasing apoptosis and causing G_0/G_1 arrest by targeting MTH1 and activating oxygen reactive·species (ROS). In addition, CRZ could inhibit the migration of OS cells (32). Our study showed that CRZ alone exerted anti-proliferative effects on OS cell lines.

Some reports have described the combined use of PZP and CRZ. Piha-Paul *et al.* reported that the anti-angiogenic properties of CRZ could augment the clinical efficacy of PZP, since the combination of PZP and CRZ would augment clinical efficacy through dual inhibition of angiogenic pathways in patients (33). Subbiah *et al.* reported a case of gastrointestinal neuroectodermal tumor harboring an EWSR1-CREB1 fusion in a patient who showed very good response to combination therapy with CRZ and PZP, with

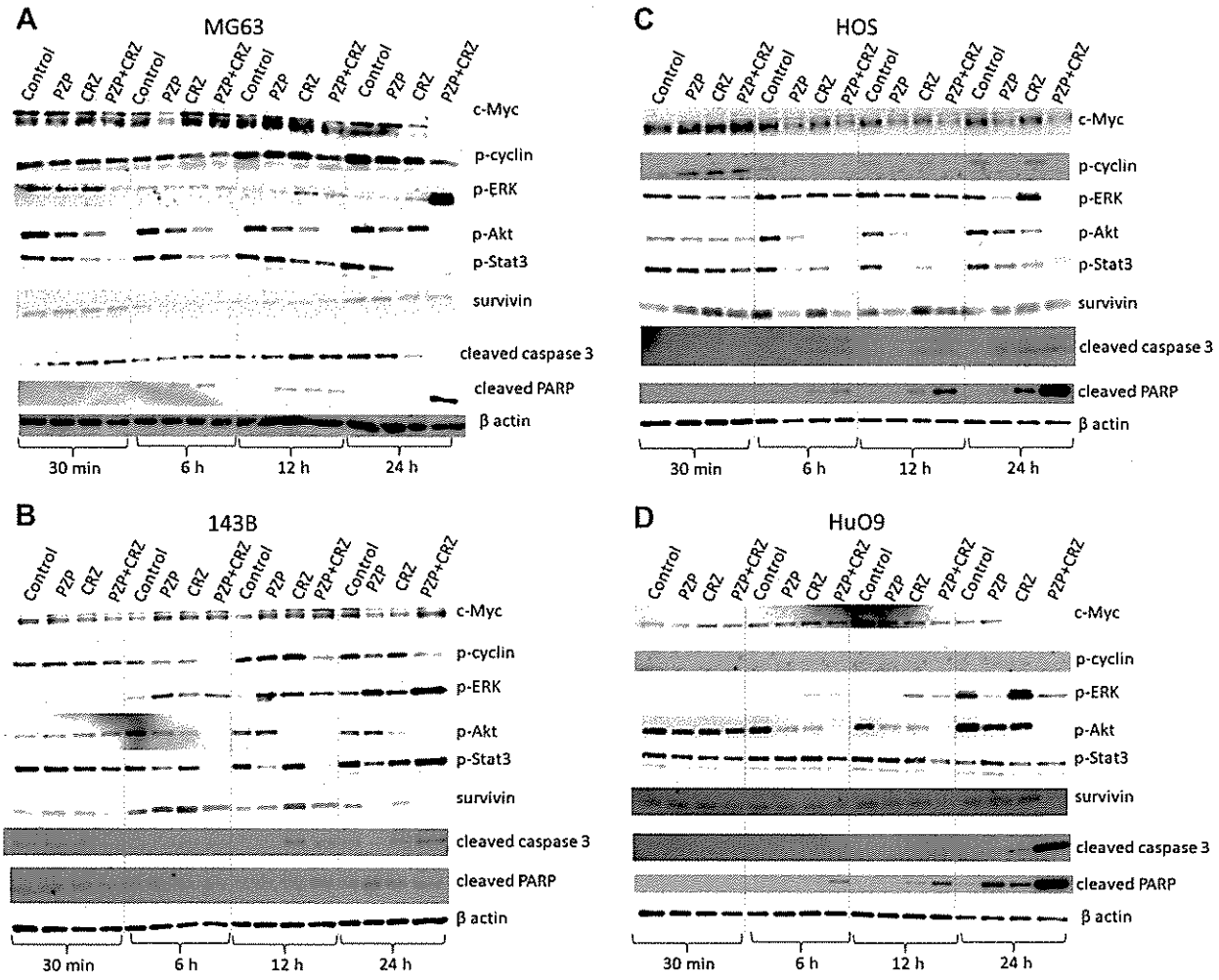


Figure 3. *Intracellular signaling investigation. MG63 (A), 143B (B), HOS (C), and Huo9 (D) cells were analyzed by western blot at 30 min, 6 h, 12 h, and 24 h after treatment with 0 or 10 μM of pazopanib (PZP) and 0 or 10 μM of crizotinib (CRZ) and incubated at 37˚C.*

clinical benefit for over 1.5 years (34). However, those reports did not show the effects on OS.

On the other hand, the side effects of this combination must be considered. In this study, no significant *in vivo* differences in body weight change were seen among mouse groups. Subbiah *et al*. also reported on the toxicity of PZP and CRZ, finding at least one adverse event that was possibly drug related in 77 patients (94%) (34). Those events were mostly grade 1 or 2 and reversible. Piha-Paul *et al*. conducted clinical trials using PZP and CRZ, dosing PZP at 200-800 mg/day and CRZ at 200-250 mg either daily or twice daily. Grade 3 or 4 toxicities at all doses were seen in 32% of patients, with fatigue (n=9, 11%), diarrhea (n=6, 7%), vomiting (n=3, 4%), anemia (n=2, 2%) and increased Alanine Aminotransferase (ALT) (n=2, 2%) as the most common. They concluded that a dose of PZP at 600 mg/day and CRZ at 200 mg twice daily was the most tolerable regimen for this combination (33).

The tyrosine kinase receptors ALK, c-kit, PDGFR, and VEGFR show crosstalk with each other and mediate signals to PIK3, RAS, and STAT (35). With those signals, AKT plays a quite important role in transferring signals, such as proliferation, migration, apoptosis and protein synthesis and function to exacerbate tumor progression (36). In our study, p-AKT decreased markedly with combination therapy compared to control or single therapy in all cell lines at all time points. This inhibition led to decreases in MYC, cyclin D, and survivin and increases in caspase-3 and cleaved PARP. STAT3 is another key factor in malignant tumors and is related to migration, invasion, proliferation, and angiogenesis (37). We have previously reported that the STAT3 inhibitor cucurbitacin I successfully inhibited proliferation of OS cells *in vitro* and *in vivo* (38). In

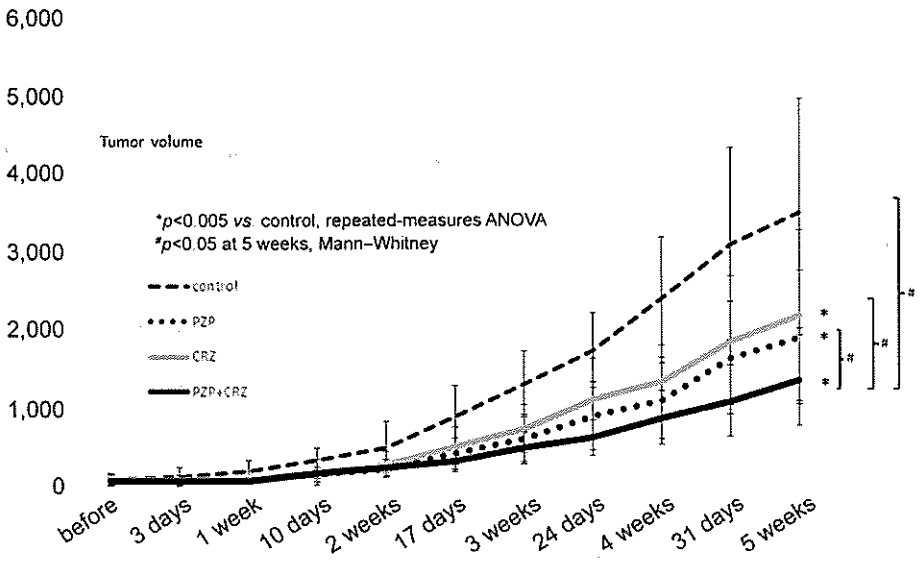


Figure 4. *Antitumor effect investigation in a mouse model. Athymic nude mice with a 143B xenograft were treated with vehicle (Cont), single therapy [pazopanib (PZP) or crizotinib (CRZ)], or combination therapy (PZP+CRZ). Effects on tumor growth were evaluated as the mean tumor volume±standard error. *p<0.05, repeated-measures ANOVA (combination therapy versus control or single therapy). #p<0.05 at 5 weeks, Mann–Whitney test (combination therapy versus control or single therapy).*

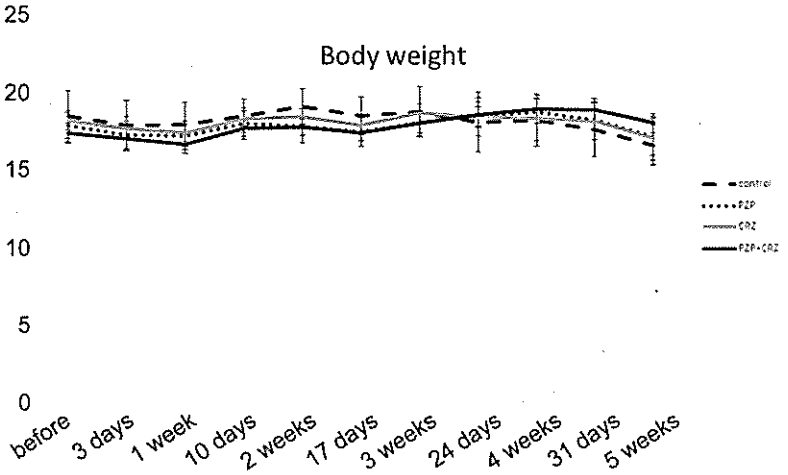


Figure 5. *Body weight of mice. Body weight changes over the 5-week period are shown. No significant differences are apparent between groups.*

the present study, STAT-3 decreased with combination therapy compared to control or single-agent therapies in all cell lines at all time points except for 24 h in 143B cells. The ERK pathway mediates proliferation, differentiation, motility, and survival (39). Conversely, ERK has been associated with apoptosis dependent on dual-specificity phosphatases. Various anti-cancer agents induce ERK activation and are involved in the suppression of tumor growth (40, 41). In the present study, p-ERK was decreased in MG63 and HOS at 30 min in combination therapy compared to control or single therapy. However, p-ERK in MG63 and 143B at 24 h was up-regulated with combination therapy compared to control or single therapy. The response of p-ERK differed between cell lines and the anti-proliferative effects were unclear.

Conclusion

In conclusion, combined use of PZP and CRZ appears to offer synergic anti-tumor effects against OS both *in vitro* and *in vivo*, by down-regulating AKT and STAT3 (Figure 6). In addition,

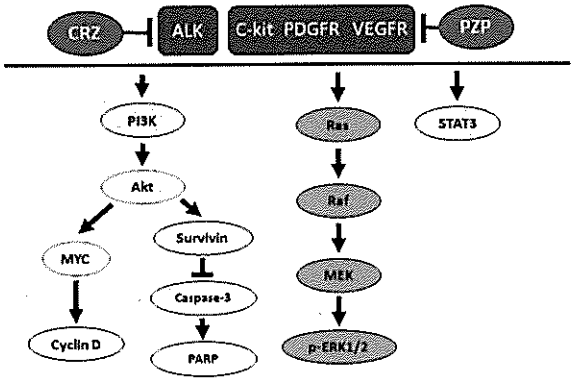


Figure 6. *Scheme of intracellular signaling Blockade of intracellular signaling by pazopanib (PZP) and crizotinib (CRZ) is shown. PZP inhibits c-kit, platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR); CRZ inhibits ALK.*

no significant differences in body weight change were noted among groups. Therefore, it is thought that no strong side effects were noted in the PZP or CRZ administration groups. Piha-Paul *et al*. suggested doses of PZP at 600 mg/day and CRZ at 200 mg twice daily as the most tolerable dosing regimen for this combination (33). Because the dose of agents we used is below these doses, it is considered possible to use.

Combined use of PZP and CRZ is expected to be effective in the treatment of OS patients. Our data indicate that the combination of PZP and CRZ can be used for patients clinically and use for OS patients may be beneficial.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

YY: Project administration, Writing – original draft. KA: Conceptualization, Writing – review & editing, Supervision. TK, KO, TI: Investigation. TO: Methodology. TN: Formal analysis. AS: Supervision. All Authors discussed the results and commented on the manuscript.

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