

The Combination of Olaratumab with Doxorubicin and Cisplatin Regresses a Chemotherapy-Resistant Osteosarcoma in a Patient-Derived Orthotopic Xenograft Mouse Model



Takashi Higuchi^{*,†,‡}, Norihiko Sugisawa^{*,†},
Kentaro Miyake^{*,†}, Hiromichi Oshiro^{*,†},
Norio Yamamoto[‡], Katsuhiko Hayashi[‡],
Hiroaki Kimura[‡], Shinji Miwa[‡], Kentaro Igarashi[‡],
Michael Bouvet[†], Shree Ram Singh[§],
Hiroyuki Tsuchiya[‡] and Robert M. Hoffman^{*,†}

^{*}AntiCancer, Inc., San Diego, CA, USA; [†]Department of Surgery, University of California, San Diego, CA, USA; [‡]Department of Orthopedic Surgery, Kanazawa University, Kanazawa, Japan; [§]Basic Research Laboratory, National Cancer Institute, Frederick, MD, USA

Abstract

Chemotherapy-resistant osteosarcoma is a recalcitrant disease. It is a frequent cause of death to the patients who are usually adolescent or young adults. The goal of the present study was to determine the efficacy of the combination of olaratumab (OLA), doxorubicin (DOX), and cisplatin (CDDP) on osteosarcoma, which is resistant to first-line therapy, in a patient-derived orthotopic xenograft (PDOX) model. The osteosarcoma PDOX model was randomized into six treatment groups of six mice: control; CDDP alone; DOX and CDDP; OLA + DOX; OLA + CDDP; and OLA + DOX and CDDP. Tumor size and body weight were measured during 14 days of treatment. Tumor growth was regressed only by the treatment with a combination of OLA + DOX and CDDP. Tumors treated with this three-drug combination had the most tumor necrosis and the lowest Ki-67 index. The present study demonstrates the power of the PDOX model to identify novel effective treatment strategy for chemotherapy-resistant osteosarcoma.

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Introduction

Osteosarcoma is the most common primary malignant bone tumor which accounts for 5% of all pediatric malignancies [1,2]. Osteosarcoma comprises almost 60% of all pediatric bone sarcomas [3]. Osteosarcoma is highly aggressive tumor that metastasize primarily to the lung [4]. Although advances in chemotherapy and surgery have improved the prognosis, the long-term survival rate for patients with metastatic or refractory disease remains poor [5]. Usually, the combination of doxorubicin (DOX) and cisplatin (CDDP) either with or without high-dose methotrexate is first-line treatment of osteosarcoma [1]. Adjuvant chemotherapy with these drugs has improved the survival rate. However, the survival rate is only 30–40%, if the tumor is resistant to this first-line therapy [1].

Olaratumab (OLA) is a monoclonal antibody that is directed against platelet-derived growth factor receptor alpha (PDGFR α). OLA was shown to induce tumor growth inhibition of PDGFR α -expressing sarcoma xenografts growing subcutaneously in nude mice [6–8]. OLA was approved by the FDA in 2016, it was the first new

first-line therapy. OLA showed good response in pretreated patients with or without PDGFR α -mutant metastatic gastrointestinal stromal tumor (GIST) mutations [9]. OLA was also tested in various other sarcomas [10–15]. A Phase II clinical trial revealed that the combining OLA with DOX nearly doubled median overall survival compared to DOX alone in soft-tissue sarcoma (STS) patients,

*Corresponding author at: Robert M Hoffman, AntiCancer, Inc., CA, USA E-mail: all@anticancer.com

**Corresponding author at: Hiroyuki Tsuchiya, Kanazawa University, Kanazawa, Japan. E-mail: tsuchi@med.kanazawa-u.ac.jp

***Corresponding author at: Shree Ram Singh, National Cancer Institute, Frederick, MD, USA. E-mail: singhshr@mail.nih.gov

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leading to the accelerated approval of the OLA and DOX combination on STS treatment [14]. However, little is known about the efficacy of this combination for osteosarcoma as well as the efficacy of adding OLA to the other standard chemotherapies for osteosarcoma such as CDDP or the DOX-CDDP combination.

We previously developed the patient-derived orthotopic xenograft (PDOX) model of osteosarcoma and other malignancies [16]. In the present study, we demonstrated the efficacy of adding OLA to the DOX-CDDP combination in a PDOX model of osteosarcoma resistant to first-line chemotherapy.

Materials and Methods

Mice

Athymic nu/nu nude mice (AntiCancer, Inc., San Diego, CA, USA), 4–6 weeks old, were used. Experimental procedures and data collection were carried out as per as our previous publications [17–19]. To minimize any suffering of the animals, anesthesia and analgesics were used for all surgical experiments. The mouse investigations presented here were carried out using an AntiCancer, Inc. An institutional Animal Care and Use Committee (IACUC) protocol specifically approved for this study as previously described and the principles and procedures provided in the National Institutes of Health Guide for the Care and Use of Animals under Assurance Number A3873–1 [17–19].

Patient-Derived Tumor

A fresh biopsy sample of 14-year-old boy with osteosarcoma in the pelvis was obtained and transported immediately to the laboratory at AntiCancer, Inc., on wet ice. The sample was cut into 5 mm fragments and initially implanted subcutaneously in nude mice. The patient did not receive any chemotherapy or radiotherapy prior to biopsy. Written informed consent was obtained from the patient as part of a UCLA Institutional Review Board approved protocol (IRB#10–001857).

Establishment of An Osteosarcoma PDOX Model. Mice were anesthetized before the following procedures. After the subcutaneously-implanted tumors grew to more than 10 mm in diameter, the tumors were harvested and cut into small fragments (3–4 mm). In order to reproduce the patient osteosarcoma in the mouse as an osteosarcoma-PDOX model, the subcutaneously grown tumor was implanted into the mouse distal femur. A 10-mm skin incision was made on the right thigh of nude mice. The vastus lateralis muscle was opened and the biceps femoris muscle was split to reach the distal femur. An incision was made in the lateral patello-femoral ligament, sparing the knee joint and then the lateral condyle of the femur was resected. A single 3–4 mm tumor fragment was implanted into this space [20]. The muscle and wound were closed with a 6–0 nylon suture (UNIFY, AD Surgical, Sunnyvale, CA) (Figure 1A).

Treatment Study Design in the Osteosarcoma PDOX Model. The osteosarcoma PDOX mouse models were randomized into 6 groups of 6 mice each and treated with following drugs with intraperitoneal injection for 2 weeks (Figure 1B): G1, control (phosphate buffered salts [PBS], 0.1 ml/mouse, twice a week); G2, CDDP (6 mg/kg, once a week) alone; G3, DOX (3 mg/kg, once a week) + CDDP; G4, OLA (60 mg/kg, twice a week) + DOX; G5, OLA + CDDP; G6, OLA + DOX + CDDP. Treatment started when all tumors reached 100 mm³. Tumor length, width and mouse body weight were measured twice per week. Tumor volume was calculated with the following formula: tumor volume (mm³) = length (mm) × width (mm) × width (mm) × 1/2. Data are presented as mean ± standard error of the mean (SEM).

Histological Analysis

Fresh tumor samples were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections were deparaffinized in xylene and rehydrated in an ethanol series. Hematoxylin and eosin staining was performed according to standard protocol. Ki-67 immunohistochemical staining with anti-Ki-67 antibody (Abcam Ltd., Cambridge, MA) in combination with

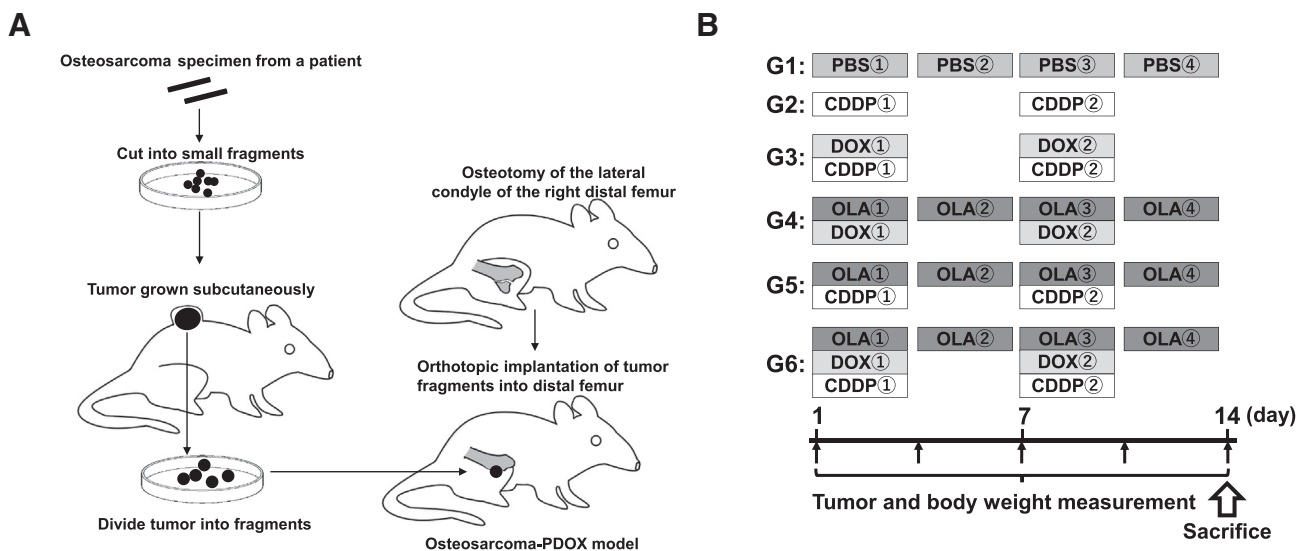


Figure 1. (A) Schematic illustration of surgical orthotopic implantation (SOI) for establishment of the osteosarcoma-PDOX mode. (B) Treatment schema of the osteosarcoma-PDOX model. The mice were randomized into 6 groups of 6 mice each: control; CDDP; CDDP and DOX; OLA combined with DOX; OLA combined with CDDP; and OLA combined with DOX-CDDP. The treatment period was for 2 weeks. PBS for control and OLA were given twice a week, while DOX and CDDP were given once a week.

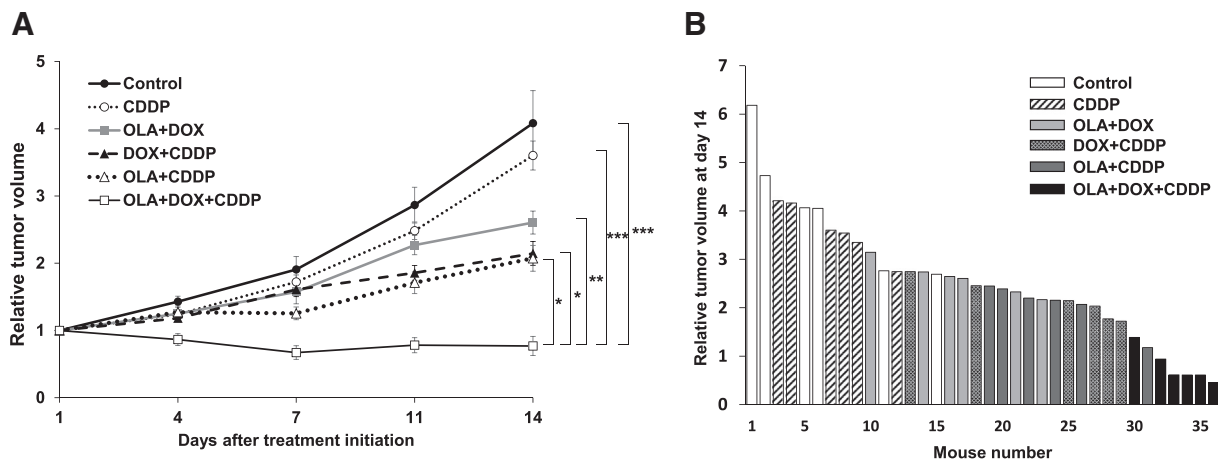


Figure 2. (A) Quantitative efficacy of drugs on the osteosarcoma-PDOX model over 14 days. Line graphs indicate tumor volume at each time point after the onset of treatment relative to the initial tumor volume for each treatment and control group. (B) Waterfall plot of relative tumor volume at day 14 relative to the initial tumor volume for each mouse. N = 6 mice/group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars: \pm SEM.

diamino-benzidine (DAB, Dako Japan Inc., Kyoto, Japan) was performed according to manufacturer's protocols. The Ki-67 labeling index, the percentage of tumor cell nuclei with positive immunostaining above the background level, was calculated semi-quantitatively.

Statistical Analysis

All statistical analyses were performed with statistical software EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan). A normal distribution was assessed with the Shapiro–Wilk test. The Bartlett's test was used to verify the homogeneity of variances between groups. One-way ANOVA with Tukey HSD for post hoc analysis was used for the parametric test for inter-group comparison. The

Student's *t*-test was used for the parametric test to compare the means between two non-related groups. The paired *t*-test was used for the parametric test to compare the means between two related groups. All *P*-values were two sided and a *P*-value of 0.05 or less was considered statistically significant.

Results

Efficacy of Chemotherapy on the Osteosarcoma PDOX Mouse Model

OLA combined with DOX and CDDP regressed the osteosarcoma PDOX tumor model, compared to all other groups (control, $P < 0.001$; CDDP alone, $P < 0.001$; OLA + DOX, $P = 0.002$;

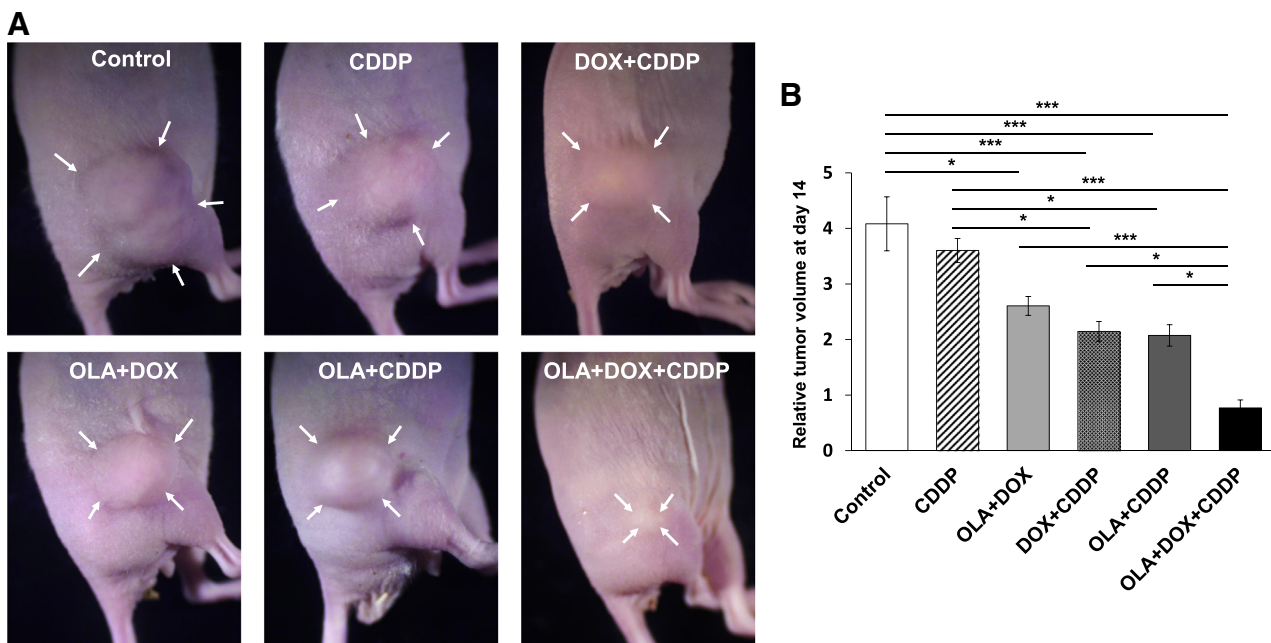


Figure 3. Efficacy of drugs on the osteosarcoma-PDOX at the end of treatment. (A) Images of representative PDOX mouse models from each treatment group at the end of treatment. Arrows indicate the apparent margin of the tumors. (B) Bar graphs show relative tumor volume of each treatment group on day 14 with respect to day 1. N = 6 mice/group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars: \pm SEM.

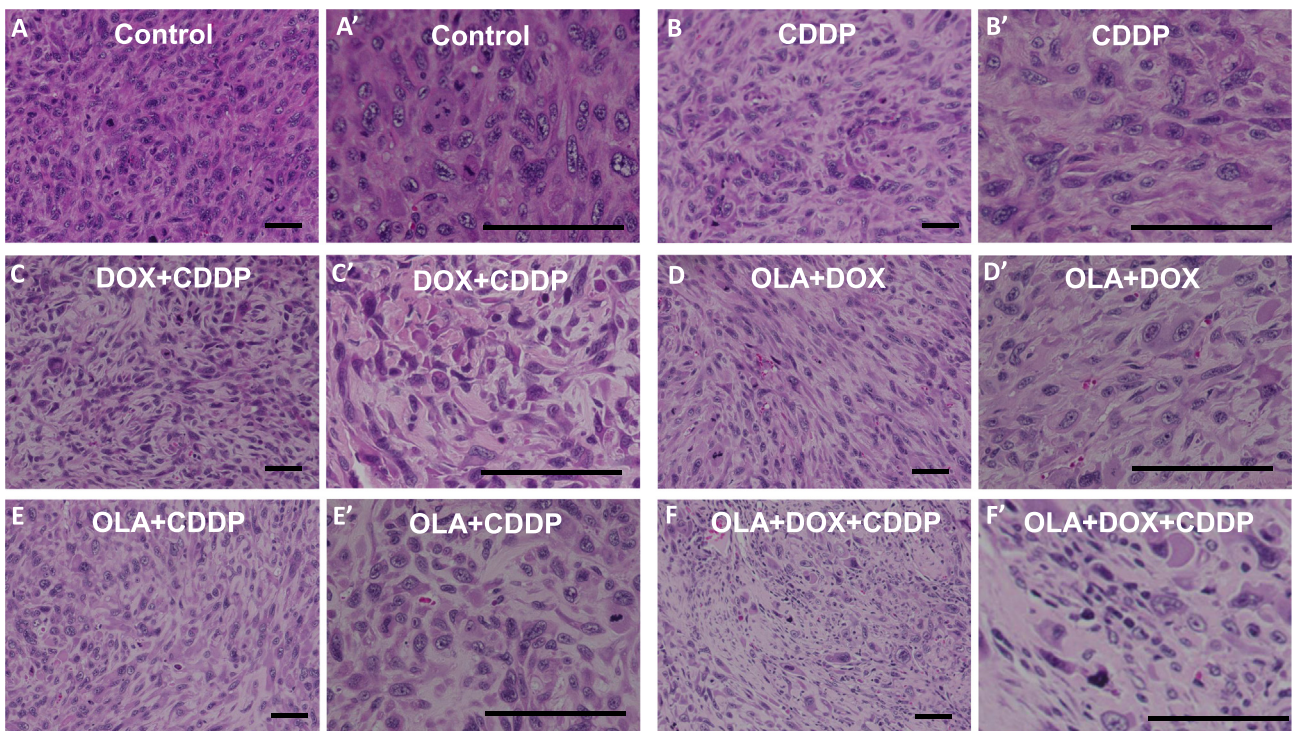


Figure 4. Tumor histology. (A-F) Low-power field (LPF). (A'-F') High-power field (HPF). Scale bars: 100 μ m.

DOX + CDDP, $P = 0.03$; OLA + CDDP, $P = 0.03$). OLA plus CDDP significantly, but moderately, inhibited the osteosarcoma PDOX growth compared with the control ($P < 0.001$) or CDDP alone ($P = 0.01$). Similarly, DOX plus CDDP moderately inhibited the PDOX growth compared with the control ($P = 0.001$) or CDDP alone ($P = 0.03$). OLA plus DOX had significant difference only with the control ($P = 0.02$). (Figs. 2, 3). A waterfall plot indicating the change in tumor volumes for the individual tumors is presented in Figure 2B, which showed the tumor volume at day 14 relative to the initial tumor volume for each mouse.

Histology of the Osteosarcoma PDOX

The tumor tissue of the control group comprised viable high-grade malignant cancer cells. Tumors treated with either CDDP alone or combined with DOX, and OLA combined either with DOX or CDDP comprised spindle-shaped viable cells, but the cancer-cell density was lower than the control. The cancer-cell density was lowest, and a degenerative scar change in the stroma was detected, in the tumor treated with OLA combined with DOX and CDDP. Cancer cells in this group had aggregated nuclei or no nuclei suggesting tumor necrosis and/or apoptosis. The strong anti-tumor efficacy of OLA combined with DOX and CDDP on the osteosarcoma PDOX tumor was thus also demonstrated histologically (Figure 4).

Ki-67 Immunohistochemical Staining

Ki-67 immunohistochemical staining was performed on tumor sections to evaluate the proliferative capacity of tumor cells after treatment. Tumors treated with OLA combined with DOX and CDDP (mean Ki-67 labeling index, 2.6%), had a significantly lower Ki-67 labeling index compared to the control (24.0%) ($P = 0.005$) (Figure 5).

Effect of Treatment on Body Weight

Mouse body weight was measured pre-treatment and post-treatment. Final body weight of mice in control ($P = .01$), OLA combined with DOX ($P < .001$) or with CDDP ($P = .02$) significantly increased compared with initial body weight. There was no significant difference in body weight among the other groups (Figure 6). There were no other observable side effects or animal deaths in any group.

Discussion

In the present study, we found that the OLA combined with DOX and CDDP regressed tumor growth in the osteosarcoma PDOX model. This is the first study which shows that the OLA-DOX-CDDP combination is active in osteosarcoma, in this case a tumor resistant to CDDP and partially resistant to DOX-CDDP, OLA-DOX, and OLA-CDDP.

OLA elicits anti-tumor activity by inhibiting ligand-binding and receptor activation of PDGFR α [13]. OLA has been tested as a single agent in patients with advanced sarcomas where it was well tolerated [15,21]. Since OLA itself only causes mild adverse effects, OLA is often preferred to be used in combination with other chemotherapeutic drugs [14,15,22]. OLA alone or together with DOX inhibited the growth of various sarcomas such as osteosarcoma, uterine leiomyosarcoma, malignant rhabdoid tumor, and other cancers such as pretreated GIST and lung cancer [6,9,23–25]. However, OLA together with paclitaxel/carboplatinum, [26], liposomal DOX [27], mitoxantrone and prednisone [28] was ineffective for untreated advanced NSCLC [26], platinum-resistant or platinum-refractory ovarian cancer [27], and metastatic castration-resistant prostate cancer [28], respectively.

DOX, an essential component of most regimens for osteosarcoma as well as STS, in combination with OLA showed a prolongation of

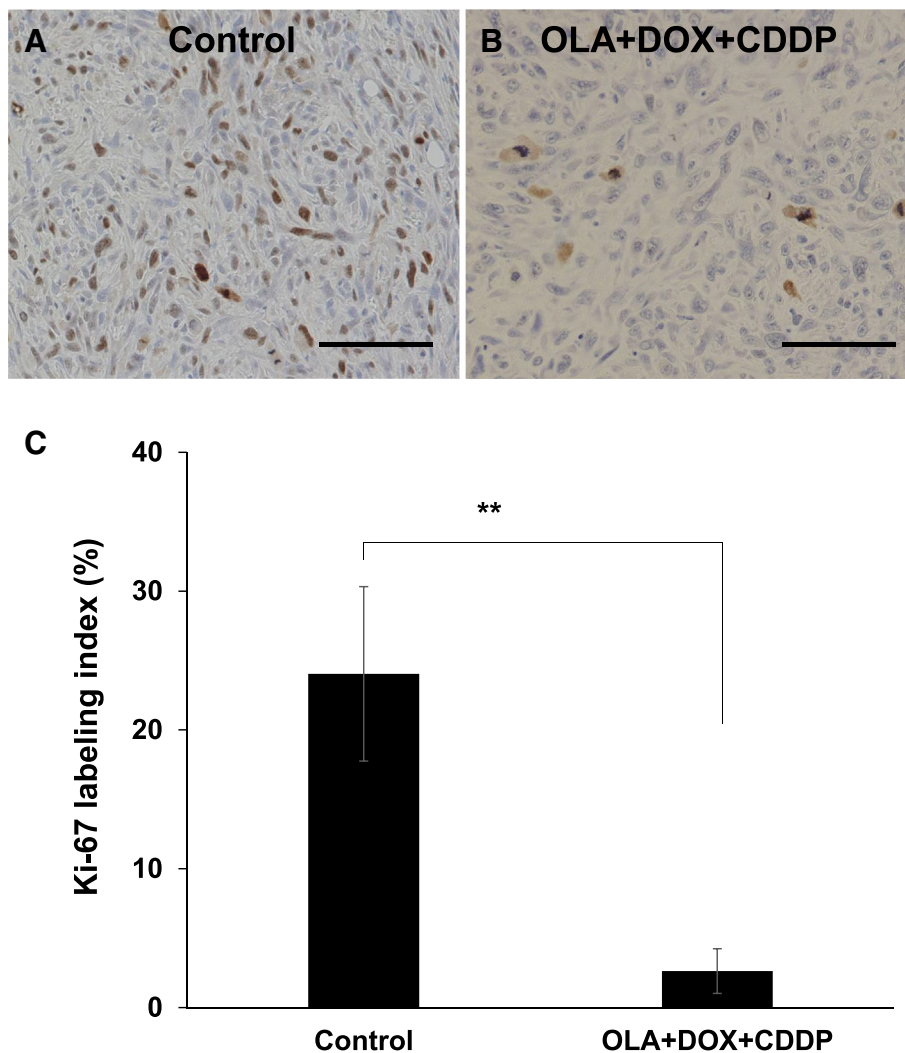


Figure 5. Ki-67 immunohistochemical staining. (A) Control. (B) OLA combined with DOX and CDDP for treatment. Scale bars: 100 μ m. (C) Ki67 labeling index. Bar graphs show the percentage of cancer-cell nuclei with positive immunostaining. N = 8 fields /group. $**P < .01$.

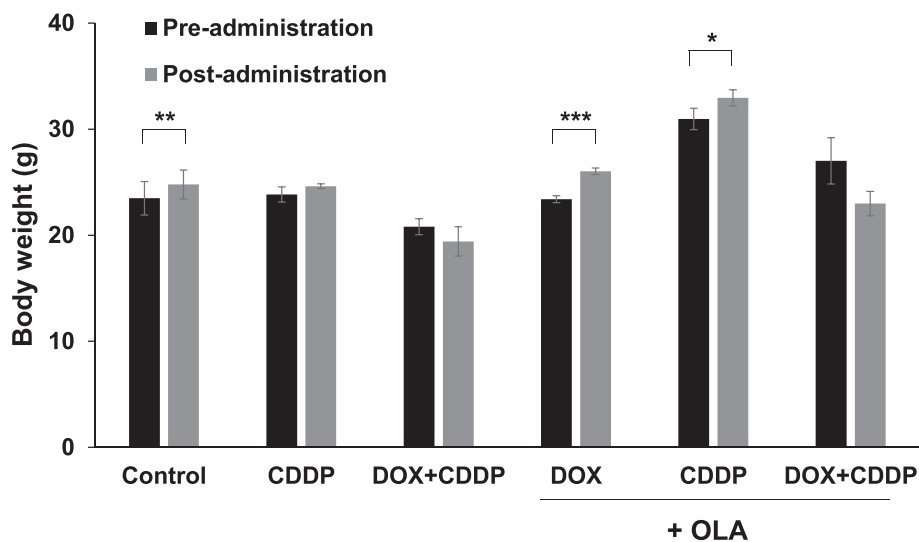


Figure 6. Effect on drugs on mouse body weight. Bar graphs show mouse body weight in each treatment or control group at pre- and post-treatment times. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Error bars: \pm SEM.

survival compared with DOX alone in patients with advanced STS [14]. OLA plus DOX showed an acceptable safety profile in Japanese patients with STS [29]. The combination of OLA with gemcitabine and docetaxel arrested the tumor growth in drug-resistant STS [30]. In addition, OLA together with DOX and ifosfamide arrested the growth of DOX- and OLA-resistance undifferentiated STS [31]. Recently, a Phase III trial of the OLA-DOX combination for advanced STS did not meet the primary end points of overall survival [32] and was withdrawn from the market [33]. However, little is known about the effect of this combination on osteosarcoma. In the present study, we found that the OLA and DOX combination had significant, but only moderate efficacy against the osteosarcoma PDOX model, suggesting a need of different strategy.

CDDP, the second most commonly used drug for osteosarcoma, in combination with OLA inhibited osteosarcoma cell growth both in vitro and in vivo and was superior to the OLA and DOX combination [23]. In the present study, OLA and CDDP combination significantly inhibited osteosarcoma PDOX growth compared to the control or CDDP alone, suggesting synergy effect of adding OLA to CDDP. However, this combination did not arrest or regress the PDOX tumor growth.

The combination of DOX and CDDP, well-known as the AP regimen, is widely used as a contemporary combination chemotherapy for osteosarcoma [2]. In the present study, the DOX-CDDP combination significantly inhibited, but did not arrest or regress, osteosarcoma PDOX growth. However, if OLA was added to this combination, the osteosarcoma PDOX tumor growth was regressed. Although adding OLA to the other chemotherapeutics is considered to be safe [10,14,15], the tolerance of OLA combined DOX and CDDP should be investigated in the future clinical studies. Our histological analysis indicated unambiguously that the combination treatment induced necrosis, suggesting cancer-cells were killed by apoptosis. Future studies will focus on other markers of necrosis and apoptosis as well as analysis of synergy among the three drugs in the highly effective combination.

In conclusion, this study uniquely demonstrates the power of the PDOX model to identify novel effective therapy using OLA combined with DOX and CDDP for osteosarcoma resistant to first-line chemotherapy. The data presented here suggest that combination of OLA with DOX and CDDP could be a promising novel therapy for osteosarcoma and that combination should be tested in a co-clinical trial with each patient on the clinical trial also having a PDOX model.

Conflicts of interest

TH, NS, KM, HO, NY, KH, HK, SM, KI, and RMH are or were unsalaried associates of AntiCancer Inc. AntiCancer Inc. uses PDOX models for contract research. The authors declare that they have no competing interests.

Acknowledgments

This paper is dedicated to the memory of Reese Imhoff.

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