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Effect of lutein and doxorubicin combinatorial therapy on S180 cell proliferation and tumor growth

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Abstract. – OBJECTIVE: Multidrug resistance and toxicity significantly compromise the therapeutic efficacy for sarcomas. We aimed at evaluating the effect of lutein-doxorubicin (DOX) combinatorial therapy on inhibiting S180 (Sarcoma 180) cell proliferation and tumor growth.

MATERIALS AND METHODS: S180 cells in logarithmic growth phase were treated with lutein, DOX, or lutein-DOX combinatorial therapy for 48 h. The cell survival rate was determined by MTT assay. Apoptosis was detected by flow cytometry. The expression of PCNA, P53, and NF-κB was assessed by Western blot. Further, mice bearing S180 tumors received lutein, DOX, or lutein-DOX combinatorial therapy by oral gavage.

RESULTS: Lutein-DOX combinatorial therapy significantly decreased the proliferation of S180 cells ($p<0.01$) *in vitro*. Also, the expression of proliferating cell nuclear antigen (PCNA) ($p<0.05$) and the apoptosis-relevant gene p53 were decreased, which resulted in increased cell apoptosis ($p<0.05$). The level of nuclear factor kappa B (NF-κB) was also decreased by the combinatorial therapy. Lutein-DOX combinatorial therapy reduced the cytotoxicity of DOX and reduced the inflammatory response. The inhibitory effect of lutein-DOX combinatorial therapy on cell proliferation was confirmed *in vivo*. The growth rate and size of the tumor at 30 d after treatment were significantly lower than those of the control group and DOX single therapy.

CONCLUSIONS: Lutein and DOX synergistically inhibit sarcoma cell proliferation and tumor growth. This novel therapeutic regimen could potentially improve clinical outcome of sarcoma patients.

Key Words:
Lutein, DOX, S180, Cancer, p53, Xenograft mice model, Synergistic effect.

Abbreviations

DOX = Doxorubicin; FDA = Food and Drug Administration; NF-κB = nuclear factor kappa B; FBS: fetal bovine serum; MTT = methyl thiazolyl tetrazolium bromide; PBS = phosphate buffer saline; TBST = Tris-Buffered Saline Tween; ECL = Enhanced chemiluminescence; TIR = Tumor inhibition rate; PCNA = proliferation cell nuclear antigen.

Introduction

It has been increasingly realized that single chemotherapy can hardly eradicate or even retard cancer effectively[1]. Targeted chemotherapy drugs are capable of inhibiting a particular oncogenic event or pathway, but they may also contribute to the emergence of cancer cells resistant to the drugs. Activation of alternative cellular pathways compensates the inhibitory effect of the single chemotherapy, rendering its therapeutic effects less durable[2,3]. As a result, combinatorial therapy is often required. Sarcomas are tumors stemming from transformed cells of the mesenchymal origin. They are among the most difficult tumors to be treated due to their intrinsic or acquired multidrug resistance[4]. Doxorubicin (DOX), a broad-spectrum anthracycline isolated from *Streptomyces peucetius*, is widely used for the treatment of a variety of cancers, such as prostate and breast cancers, by targeting rapidly dividing cells[5]. In sarcomas, DOX is the first-line treatment in clinics. Although regarded as one of the most potent chemotherapeutics approved by the Food and Drug Administration (FDA), the use of DOX is confronted with resistance in

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many cancers, including sarcoma[6]. It is, therefore, imperative that combinatorial therapy is used to reduce cancer resistance and recurrence of sarcoma. Besides, the side effects associated with DOX chemotherapy, including neurological disturbances and myocardial toxicity, harm patients' quality of life and substantially compromise the clinical outcome[7]. There is a huge unmet need for novel therapeutic regimens to enhance the therapeutic efficacy of DOX, meanwhile reducing its toxicity. Recent studies on the combination of chemotherapy drugs and natural antioxidants in cancer treatment have demonstrated inspiring results in reversing multidrug resistance[8]. Lutein, a chemical found in the green vegetables, is an antioxidant that protects cells against damages from free radicals. Lutein is primarily used as a supplement in chronic diseases, such as age-related macular degeneration[9], colonic inflammation[10], etc. Lutein has also been reported to modulate cancer growth and survival[11]. Long-term intake of lutein supplements is also considered to reduce the risk of lung cancer[12]. In a recent study, lutein was used in combination with DOX to reduce DOX-induced cardiac and renal toxicity[13]. This is attributable to the effects of lutein in reducing oxidative stress and inflammation. However, clinical application of lutein-DOX combinatory therapy has not been applied to sarcomas, and whether this treatment enhances antitumor efficacy is unclear. Lutein and DOX may be useful for inhibiting sarcoma cell proliferation and tumor growth. In addition, the anti-oxidant property of lutein could ameliorate oxidative stress and inflammation from DOX administration. Herein, we aim at evaluating the effect of lutein-DOX combinatorial therapy on inhibiting the proliferation and tumor growth of murine sarcoma S180 cells. We also examined how the apoptotic genes, p53 and nuclear factor kappa B (NF-κB) are regulated by this combinatorial therapy. The results reported in this work could provide scientific ground for the clinical use of lutein and DOX to improve the therapeutic effects of sarcoma chemotherapy.

Materials and Methods

Cell Culture

S180 cells (ATCC, Manassas, VA, USA) were cultured in Roswell Park Memorial Institute 1640 (RPM 1640) medium containing 10% fetal bovine serum (FBS) and 10×10^4 μmol/L penicillin and streptomycin (Gibco, Grand Island, NY, USA). Culture plates were placed in an incubator maintained at 37°C with 5% CO_2. Cells were cultured to the logarithmic phase before being used for further study.

MTT Assay

The proliferation of cells were determined with methyl thiazolyl tetrazolium bromide (MTT) assay. S180 cells were seeded into 96-well plates (5×10^3/well) and treated with 10 μmol/L lutein (Tianben Biotechnology, Co., Ltd., Jinan, China), DOX (Wanle Pharmaceuticals, Co., Ltd., Shenzhen, Guandong Province, China) solved in water for injection, or 10 μmol/L lutein and 5 μg/mL DOX together for 24 h. Then, 20 μl MTT (5 mg/mL) was added to each well, followed by a 4-h incubation at 37°C. Next, the medium was aspirated, 150 μL dimethyl sulfoxide (DMSO) were added and shaken for 1 min; then, light absorbance of the solution was measured at 570 nm on a microplate reader (Perkin-Elmer, Waltham, MA, USA). Cell proliferation rates were calculated using the following equation: cell proliferation rate (%) = OD value of the experimental group/OD value of the control group × 100%.

Flow Cytometry

The effect of lutein/DOX combinatorial therapy on apoptosis was assessed by Annexin V-FITC (Roche, Indianapolis, IN, USA) staining, followed by flow cytometry. S180 cells were seeded in 6-well plates (5×10^5/well) and treated with different drugs as above for 24 h. After treatment, the cells were collected by centrifugation and washed with 1 × phosphate-buffered saline (PBS). The cells were then suspended in 1 × binding buffer and stained with 5 μL Annexin V-fluorescin isothiocyanate (FITC) and 5 μL propidium iodide (PI) by incubation for 30 min at room temperature in the dark. A flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to determine the apoptosis rate.

Western Blot Analysis

S180 cells were seeded in 6-well plates (5×10^5/well) and treated with different drugs as above for 24 h. Total proteins in the cells were extracted and quantified with bicinchoninic acid (BCA) assay. Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Darmstadt, Germany). Blocking was performed at room temperature for 1 h with Tris-Buffered Saline and Tween 20 (TBST) solution containing 5% nonfat

dry milk. After washing, the membranes were incubated overnight with TBST diluted primary antibodies, including PCNA, p53, NF-κB and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C. Secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted in phosphate buffered saline (PBS) and used to incubate the membranes for 1 h at room temperature. After washing, enhanced chemiluminescence (ECL) substrate (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added, followed by incubation for 3-5 min. Images were analyzed using the Quantity One gel electrophoresis imaging system.

Animal Model Establishment and Treatment

Animal experiments were conducted in accordance with Ethics protocols regulated by the Animal Care and Use Committee of Yantai Yuhuangding Hospital (Yantai, Shandong Province, China). A total of 30 Kunming mice (Beijing Colab Bio-Technology Ltd., Beijing, China) of equal number of males and females (20 ± 2 g) were randomly grouped into the control group, the lutein group (40 mg/kg), the DOX group (2.1 mg/kg) and the lutein-DOX combinatorial treatment group (lutein 40 mg/kg + DOX 2.1 mg/kg). Mice treated with vehicle were used as control. S180 cells diluted with sterile saline were inoculated into the left axillary subcutaneous tissue (1×10^6 cells/mouse). Drug treatment started from the 5th day after cell inoculation. Lutein was administered via oral gavage every day, and doxorubicin was administered by intravenous injection every 3 days. Tumor size was measured and recorded using the following equation: volume (mm^3) = length × width^2/2. The treatments lasted for 3 weeks, then mice were sacrificed and solid tumors were weighed to get the final tumor inhibition rate, which was calculated using the following equation: tumor inhibition rate (TIR) = (mean tumor weight of control group – mean tumor weight of experimental group)/mean tumor weight of control group × 100%.

Statistical Analysis

All data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as Mean ± SD (Mean ± standard deviation), and analyzed using one-way ANOVA followed by LSD post-test. A p-value < 0.05 were considered statistically significant.

Results

Combinatorial Therapy with Lutein and DOX Decreases S180 Cell Proliferation

We first evaluated the inhibitory effect of lutein, DOX, and lutein-DOX combinatorial therapy on S180 *in vitro*. Figure 1 shows the MTT assay of S180 cells in different treatment groups. It is clear that the use of lutein alone exerted no significant inhibition on cell survival compared to the control group. Treatment with 5 μg/mL of DOX led to moderate, albeit significant, reduction on cell proliferation (p<0.05). Strikingly, the combination of lutein and DOX markedly inhibited the cell viability with the inhibition rate of > 40% (p<0.01).

Combinatorial Therapy with Lutein and DOX Promotes Cell Apoptosis

We next examined how lutein and DOX combinatorial therapy, affect cell apoptosis. Flow cytometry demonstrated that lutein or DOX individually could induce the early apoptosis of S180 cells (p<0.05) (Figure 2). Lutein and DOX combinatorial therapy increased total apoptosis rate to 35.93%, which was higher than the summed apoptosis rate of lutein (12.57%, p<0.001) and DOX (18.95%, p<0.001). This supports the synergistic effects of this combinatorial therapy in inducing cell apoptosis.

Lutein and DOX Synergistically Regulate Expression of Apoptosis-related Proteins

To elucidate the effect of lutein-DOX combinatorial therapy on cell apoptosis, we investigated how proliferation cell nuclear antigen

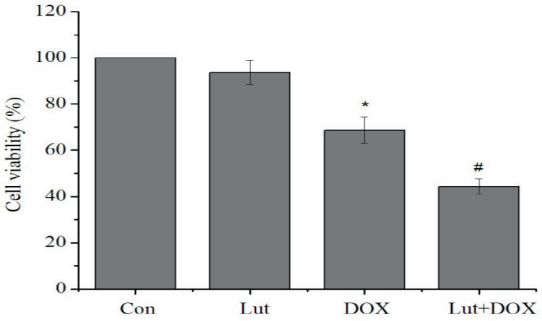

Figure 1. Cell viability assay revealed significant inhibition of cell proliferation by lutein and DOX combinatorial therapy. Data were expressed as mean ± SD. *: p>0.05 between DOX group and the control group. #: p<0.01 between Lut+DOX group and the control group, as well as Luc+DOX group and DOX group. DOX: doxorubicin; Lut: lutein.

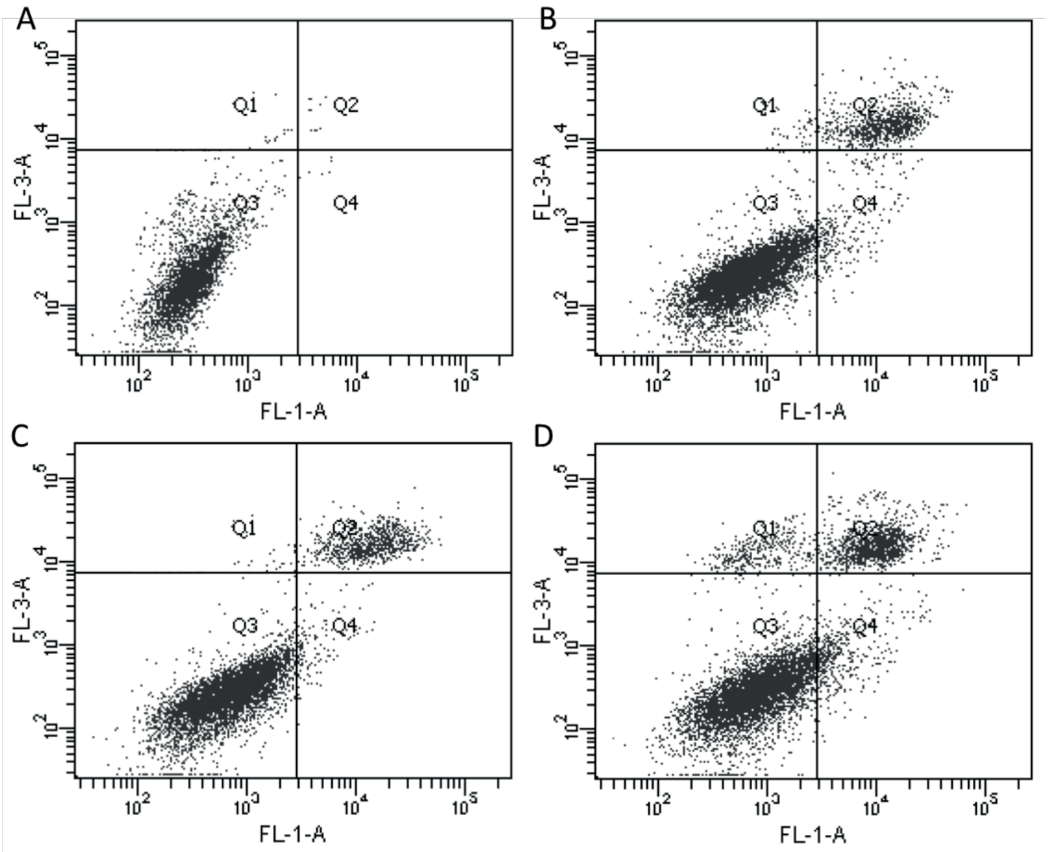

Figure 2. Lutein and DOX combinatorial therapy increased apoptosis of S180 cells. ***A,*** Control group. ***B,*** DOX group. ***C,*** Lut group. ***D,*** Lut + DOX group.

(PCNA), p53 and NF-κB responded to the treatment. PCNA is a marker of cell proliferation. As shown in Figure 3, the expression of PCNA protein was decreased by approximately 20% and 35% ($p<0.05$) in the presence of lutein and DOX, respectively. In contrast, lutein and DOX combinatorial therapy downregulated the expression of PCNA in S180 cells to 40% ($p<0.05$). Considering that PCNA expression is closely associated with cell proliferation, the prominent downregulation of PCNA under combinatorial therapy is in agreement with the substantial inhibition of cell proliferation. The expression of p53 in combinatorial therapy group was significantly higher than that in the control group ($p<0.05$), which is consistent with the increased apoptosis rate when cells were exposed to lutein and DOX combinatorial therapy. However, no significant difference in p53 was observed with DOX treatment alone. Given that, DOX also upregulated NF-κB, a factor that induces cytotoxicity in normal tissues. We were also interested whether lutein and DOX combinatorial therapy alleviated NF-κB induced by DOX. As expected, the expression of NF-κB in DOX-treated S180 cells was significantly higher than that in the control group ($p<0.05$), but lutein and DOX combinatorial therapy lowered NF-κB expression ($p<0.05$). Lutein alone was also effective in reducing NF-κB expression. Therefore, a lower cytotoxicity can be achieved by the combinatorial therapy, which is possibly mediated by reducing inflammatory responses.

In vivo Evaluation of the Antitumor Effect of Lutein-DOX Combinatorial Therapy

As shown in Figure 4, both lutein and DOX showed inhibition of tumor growth compared to tumor-bearing control mice ($p<0.05$). The tumors of the DOX treatment group were about 50% smaller than that of the control group. Tumor growth rate of the control group increased substantially on the 20th day ($p<0.05$). The tumor size of mice treated with lutein-DOX combinatorial therapy was significantly smaller than that of the control group ($p<0.05$). Consistently, al-

Table I. Tumor weight in different treatment groups.

	Tumor weight (g)	Tumor inhibition rate (%)
Control	2.42 ± 0.12	--
Lutein	1.98 ± 0.13[a]	18.21
DOX	1.47 ± 0.10[a,b]	39.17
Lutein + DOX	1.13 ± 0.11[a,b,c]	53.10

[a], $p<0.05$ vs. control group; [b], $p<0.05$ vs. Lutein group; [c], $p<0.05$ vs. DOX group.

though lutein and DOX alone could significantly decrease tumor weight compared to the control group ($p<0.05$), lutein-DOX combinatorial therapy reached 53% tumor inhibition rate, which was significantly higher than that of DOX or lutein alone ($p<0.05$) (Table I).

Discussion

The combinatorial therapy, where lutein and DOX were used to treat S180 cells, demonstrated a superior efficacy in reducing S180 proliferation and inducing cell apoptosis *in vitro*. In line with this, the inhibition of tumour growth was also significantly higher than that of lutein or DOX single therapy. Therefore, lutein and DOX synergistically enhanced the antitumor effects in sarcomas. Multidrug resistance of cancer significantly aggravates the clinical outcome of cancer, which demands novel strategies to effective inhibit resistant cancer. Indeed, carotenoids, including lutein, have long been used as a chemopreventive drugs in cancer; natural anti-oxidants, including ascorbic acid and lutein, were shown to assist in overcoming multidrug resistance in sarcoma[14,15]. Uptake of fruits and vegetables rich in lutein is associated a lower risk of a variety of cancers, such as prostate cancer[16]. Our study is preceded by efforts in using lutein to promote antitumor effects of some chemotherapy drugs, presumably by overcoming drug resistance[17]. However, data are lacking concerning the therapeutic efficacy of lutein in the treatment of sarcomas. To our knowledge, our research represents one of the first studies demonstrating the potential of lutein in the treatment of sarcomas. Previously, the antitumor efficacy of lutein was thought to originate from its effect in inducing cancer cell apoptosis[18]. However, the exact molecular mechanism of lutein in cancer still needs to be elucidated. Here, we were particularly interested at evaluating how lutein, DOX, or the combination of both, induced changes in p65 and NF-κB expression, which are important regulators of cell apoptosis[18]. Previous studies suggested that the transcriptional activation of p53 is crucial to DOX-induced apoptosis[19] and suppressed transcription of p53 in tumour cells accounts for resistance of many cancers to DOX[20,21]. Indeed, in our work neither DOX nor lutein single therapy induced an upregulation of p53, thereby resulting in no change in PCNA expression, which is characteristic of cancer resistance. This is a clear indication that DOX alone cannot suffice in suppressing S180 proliferation and progression. Importantly, an increase in p53 expression and a decrease in the PCNA expres-

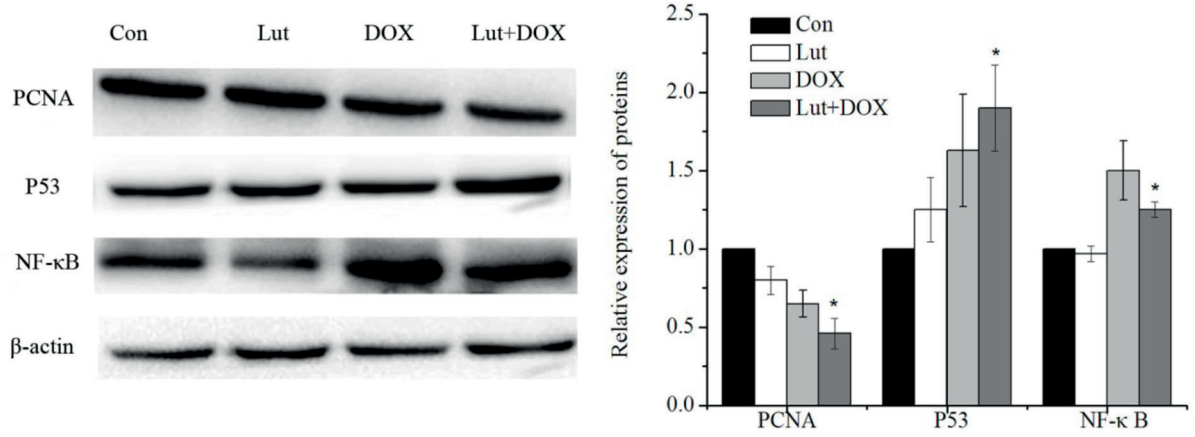

Figure 3. Effect of lutein, DOX, and lutein-DOX combinatorial therapy on PCNA, p53 and NF-**κB** expression. The level of β-actin was used as a loading control. *: $p<0.05$ compared to all other groups. DOX: doxorubicin; Lut: lutein.

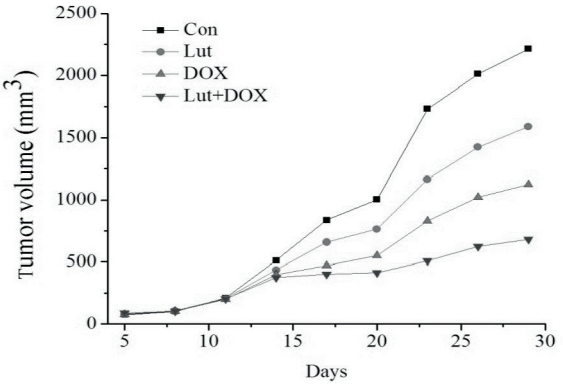

Figure 4. Effect of lutein, DOX, and lutein-DOX combinatorial therapy on tumor growth in xenograft model. DOX: doxorubicin; Lut: lutein.

sion was seen in S180 cells treated with the lutein-DOX combinatorial therapy, which is a solid evidence that resistance to DOX was reversed by combining lutein. Cancers resistant to chemotherapy and radiotherapy are characterized by NF-κB activation[22]. While single DOX treatment led to upregulation of NF-κB, lutein-DOX therapy reduced NF-κB expression. Strikingly, single lutein treatment also resulted in a decrease in NF-κB. Currently, researchers are in search for NF-κB inhibitors as novel chemotherapy drugs to counterfeit the cancer resistance[23] and our results potentiated lutein as a potent NF-κB inhibitor. These data point towards the conclusions that lutein augment the therapeutic efficacy of DOX by regulating p65/NF-κB pathway. The other important advantage of this combinatorial therapy is the reduced cytotoxicity and inflammation. Chemotherapy-enhanced inflammation in kidney, liver and cardiovascular systems is a main reason for compromised clinical outcome, which is also a growing concern for the clinical use of cytotoxic drugs such as DOX[24-26]. NF-κB signaling pathway is known to regulate inflammatory responses and immune responses[22]. The anti-inflammatory effect of lutein, which has been frequently employed in treating diseases originated from dysregulated inflammation, has also been proven useful for counterfeiting inflammation induced by DOX, as evidenced by the reduction of NF-κB. In support of this, lutein was also reported to reduce cisplatin-induced acute renal failure[27], which is also attributable to its anti-inflammatory and anti-oxidative properties. Similar to our approach, other anti-inflammatory agents, such as curcumin[28] and resveratrol[29], have also been used in combination to DOX for cancer treatment, in which their anti-inflammatory effects ameliorated the side effects of DOX to improve the treatment outcome.

Conclusions

We provided a new modality for cancer therapy based on the combination of lutein and DOX. This combinatorial therapy effectively increases the DOX sensitivity in S180 cells. Although further validation of inflammatory factors are necessary to consolidate the strength of this therapy in reducing systemic inflammation, and a comprehensive evaluation of genes modulated by the lutein-DOX combinatory therapy is still needed to be done, our work could potentially give rise to a new combinatorial therapy that is worthy of being tested in sarcoma patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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