Antitumor Efficacy of the Cytotoxic RNase, Ranpirnase, on A549 Human Lung Cancer Xenografts of Nude Mice

INTAE LEE[1], ANNA KALOTA[2], ALAN M. GEWIRTZ[2] and KUSLIMA SHOGEN[3]

Departments of [1]Radiology and [2]Medicine Division of Hematology/Oncology, University of Pennsylvania, Philadelphia, PA 19104-6069; [3]Alfacell Corporation, Bloomfield, NJ 07003, U.S.A.

Abstract. *Background: The cytotoxic RNase, ranpirnase (ONCONASE®, ONC), may have promising therapeutic implication as an alternative for cisplatin for the treatment of lung cancer, due to inhibition of protein synthesis by t-RNA cleavage. Materials and Methods: A549 and NCI-H1975 human NSCLC cell lines were cultured in the presence and absence of ONC. Cytotoxicity was monitored using a clonogenic assay. Using an inverted phase and fluorescence microscope, we studied whether apoptosis was induced by ONC in gefitinib-induced apoptosis-resistant A549 tumor cells. The therapeutic effectiveness of ONC was studied via single and multiple administrations on A549 human non-small cell lung cancer (NSCLC), including tumors previously untreatable by cisplatin. ONC-induced changes in ATP levels were also monitored by non-localized phosphorus MR spectroscopy. Results: ONC significantly inhibited the cell growth of A549 tumors. Apoptosis was significantly induced by ONC in a dose-dependent manner. In animal studies, multiple small doses of ONC were more effective than one large single dose for the inhibition of tumor growth with reduced side-effects, probably due to the normalization of leaky tumor vessels. ONC in combination with cisplatin significantly reduced tumor growth of A549 tumors. In large tumors, including those unsuccessfully treated with cisplatin, ONC showed inhibition of tumor growth, while a second treatment of cisplatin did not. During monitoring by non-localized phosphorus MR spectroscopy, ATP levels decreased, likely due to ONC-induced inhibition of oxygen consumption (QO_2). Conclusion: ONC significantly inhibited tumor growth of A549 NSCLC cells in both in vitro and in vivo studies. This investigation suggests important potential clinical uses of ONC for the treatment of NSCLC cancer patients.*

Correspondence to: Intae Lee, Ph.D., Department of Radiology, University of Pennsylvania, B6 Blockley Hall, 423 Guardian Drive, Philadelphia, PA 19104-6069, U.S.A. Tel: +012156092259, Fax: +012155732113, e-mail: leeint@uphs.upenn.edu

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Ribonucleases (RNases) have great therapeutic potential as an alternative to the standard DNA-damaging chemo-therapeutic agents, to which develops resistance (1, 2). It is known that the ribonucleolytic activities of RNases are responsible for their cytotoxicity. The cytotoxic RNase, ranpirnase (trade name, Onconase®: ONC), is extracted from oocytes of *Rana pipiens*. ONC is currently being evaluated in an international confirmatory Phase IIIb trial as a treatment for unresectable malignant mesothelioma (ONC + doxorubicin *vs.* doxorubicin) (3, 4). Furthermore, ONC has promising therapeutic implications in cancer therapy due to the induction of *p53* independent apoptosis (5). Consequently, since functional *p53* is not affected by RNA damage (6), t-RNA damage cell death is a new and attractive cancer treatment modality. A Phase I/ II trial has started to evaluate the effectiveness of ONC for the treatment of non-small cell lung carcinoma (NSCLC).

We have recently reported that a single administration of ONC enhanced the radiation response of A549 human NSCLC (7), likely due to reduced O_2 consumption (QO_2), in addition to the mechanisms observed by various investigators. In this work, we studied whether ONC induced apoptosis in the gefitinib-induced apoptosis-resistant A549 human NSCLC cell lines. We investigated whether the mono-therapy with ONC *via* single and multiple administrations could inhibit tumor growth in A549 tumors *in vivo*. We further studied whether the therapeutic efficacy of ONC was enhanced when used in combination with cisplatin. The possible physiological mechanisms of ONC were studied using non-invasive magnetic resonance spectroscopy (MRS) and near infrared spectroscopy (NIRS). First, ONC-induced changes in ATP levels were monitored with non-localized phosphorus spectroscopy. Second, changes in flow parameter in tumors and skeletal muscles were monitored non-invasively using NIRS.

Materials and Methods

Tumors. Frozen A549 and NCI-H1975 human NSCLC cell lines were purchased from the American Type Culture Collection (Rockville,

MD, USA), thawed, cultured, and grown *in vitro*. Both A549 and NCI-H1975 cells were maintained at 37˚C for the duration of the experiments. The cells grew in RPMI1640 medium supplemented with 15% FBS, 10-25 mM HEPES buffer, 5 mM l-glutamine, and antibiotics (15% FBS-RPMI1640).

Preparation of ONC and cisplatin. ONC (trademark: Onconase® and generic name: ranpirnase) was supplied by the Alfacell Corporation (Bloomfield, NJ, USA). Cisplatin was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Original stock solutions of ONC at 5 mg/ml and cisplatin at 100 μM were made in sterile distilled water and frozen at –20˚C until needed. Prior to experiments, ONC and cisplatin were thawed and diluted to the proper concentrations in medium for *in vitro* studies and saline for *in vivo*.

Clonogenic survival assay for in vitro response to ONC. The appropriate cell number (from 100 to $1x10^5$) of A549 tumor or NCI-H1975 tumor cells was plated into 6-well plates (or T25 cell culture flasks). The cells were then incubated at 37˚C for 5 h for cell attachment and incubated with ONC at 0-10 μg/ml in well for 3, 24, 48 h and no rinse (exposure time=8 days). The 6-well plates were rinsed twice with RPMI1640 medium to remove the ONC from the media. After exposure to ONC, clonogenic assays were performed, as described elsewhere (8), 7 ml of 15% FBS-RPMI1640 were then added to 6-well plates and cells allowed to grow for 8-9 days. Cultures were fixed with 99.5% isopropyl alcohol, stained with 1% crystal violet and counted. Colonies with more than 50 cells were scored as positive.

Clonogenic survival assay for in vitro response to ONC in combination with cisplatin. The appropriate cell number (from 100 to $1x10^5$) of A549 tumor cells was plated into 6-well plates and incubated at 37˚C for 5 h to allow for cell attachment. The cells were then incubated with ONC at 0 or 10 μg/ml for 24 h and concurrently incubated with cisplatin at 0-5 μM. The 6-well plates were rinsed twice with RPMI1640 medium to remove ONC and cisplatin from the media. After exposure to ONC and cisplatin, clonogenic assays were performed. The sensitization enhancement ratio (SER) was calculated as the ratio of doses in the presence and absence of ONC and cisplatin. In this study, cisplatin concentration at $SF_{0.1}$ (surviving fraction at 0.1) derived from the no ONC-treated survival curve divided by that at $SF_{0.1}$ derived from the ONC-treated survival curve. To study the effect of drug sequence, A549 tumor cells in medium were exposed under ambient conditions to following conditions: (a) cisplatin for 24 h, rinsed, and then treated with ONC for 24 h; (b) ONC for 24 h, rinsed, and then treated with cisplatin for 24 h; (c) both drugs together for 24 h; (d) cisplatin for 24 h, rinsed, and then treated with medium containing 0.9% saline (drug vehicle) for 24 h; (e) ONC for 24 h, rinsed, and then treated with medium containing 0.9% saline for 24 h. Colonies with more than 50 cells were scored as positive.

Apoptosis assay. Apoptotic cells were detected using the In-Situ Cell Death Kit (Roche, Indianapolis, IN, USA). Briefly, A549 and NCI-H1975 tumor cells were cultured at a starting cell density of 10^4 cells/ml on 4-chamber LabTek II Chamber Slides (NUNC, Denmark) under standard conditions. After 3 days, various concentrations (0-10 μg/ml in chamber) of ONC were added. After 3 days of treatment with ONC, the cell culture medium was removed and the cells air-dried for 30 min. The cells were fixed in a para-formaldehyde solution (4% v/v in PBS, 1 h) and then incubated in a permeablization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. After rinsing twice with PBS, 50 ml of TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) reaction mixture was applied and the slides were incubated in a humidified atmosphere of 5% CO_2 in air at 37˚C for 1 h in the dark. The slides were rinsed in PBS and analyzed using an Olympus IX70 inverted phase and fluorescence (α^{exc}=488 nm, α^{em}=510-560 nm) microscope (Hitech Instrument, Inc., Edgemont, PA, USA). Digital photographs of random fields were taken with a Photometrics CoolSNAP-HQ CCD camera (Photometrics, Roper Scientific, Inc., Tucson, AZ, USA) and connected to the Macintosh computer with OPENLAB software (Improvosion, Lexington, MA, USA).

Animals bearing tumors. Animal care was in compliance with all rules as set by the University of Pennsylvania for the care and use of laboratory animals, with standards equivalent to the UKCCCR guidelines for the welfare of animals in experimental neoplasia. Eight- to ten-week-old female athymic NCR-nu/nu nude mice (purchased from the NCI, Bethesda, MD, USA) bearing human tumor xenografts of A549 human NSCLC cells were utilized. Tumors were induced by injecting viable cells ($2x10^6$) suspended in 50 μl of RPMI1640 medium subcutaneously into the right thighs of mice.

Retardation in tumor growth in vivo after treatment with single and multiple administrations of ONC. Experiments were carried out when the tumor volume was between 200 and 400 mm^3. Tumors were measured with a caliper 2-3 times a week for up to 8 weeks after treatments. Tumor volumes were calculated using the formula $V= 0.4 \times AB^2$, with A and B as the longer and shorter diameters of the tumor, respectively (8). For the *in vivo* ONC treatment, ONC was dissolved in sterile 0.9% NaCl solution before injections. The mice were given either an intravenous (*i.v.*) or intra-peritoneal (*i.p.*) injection of ONC at 5 mg/kg at a volume of 0.1 ml/ 20 g of body weight.

Animal studies with ONC in the presence of cisplatin. Based on the *in vitro* chemo-sensitization, we performed a growth delay assay using A549 human NSCLC cells growing in nude mice. We assessed the effectiveness of ONC in inhibiting the tumor growth *in vivo* in the presence and absence of cisplatin. Our experiments combining ONC and cisplatin were carried out when the tumor volume was ~250 mm^3. Tumors were measured with a caliper 2-3 times a week for up for 8 weeks after treatments. ONC at 5 mg/kg was *i.v.* injected 1 h prior to an *i.p.* injection of cisplatin. 10% lethal dose at day 30 $<LD_{10(30)}>$ of ONC alone for mice was ~12.5 mg/kg; however, the $LD_{10(30)}$ was not altered by the addition of cisplatin. In all mice, saline was *i.v.* injected every 3 days for 3 weeks following treatment to avoid cisplatin-induced dehydration of the kidney.

Biochemical analysis using non-invasive in vivo ^{31}P-MR spectroscopy (MRS). ATP levels in the tumor tissues were measured 0-2 h post-treatment with ONC using a 9.4 T 30 cm vertical bore spectrometer (400 MHz Varian INOVA, Varian NMR system, Palo Aloto, CA, USA) equipped with 55 mm, 55 G/cm gradients, and a slotted tube resonator. Experiments were carried out when the tumor volume was between 200 and 400 mm^3. ATP levels were monitored by non-localized ^{31}P-NMR spectroscopy, with the following acquisition parameters, sfrq: 162 MHz, d1 (repetition time): 1 sec, nt (number of repetition): 256, np (number of point): 4000, sw (band width): 7000 Hz, tpwr (power of the pulse): 50 dB, pw (duration of pulse): 50 μsec. Animal body temperature during the MRS experiment was maintained at 37˚C by blowing warm air through the magnet bore. Prior to measuring ONC-induced changes in ATP levels, a spin echo magnetic resonance of A549 tumors was taken (9).

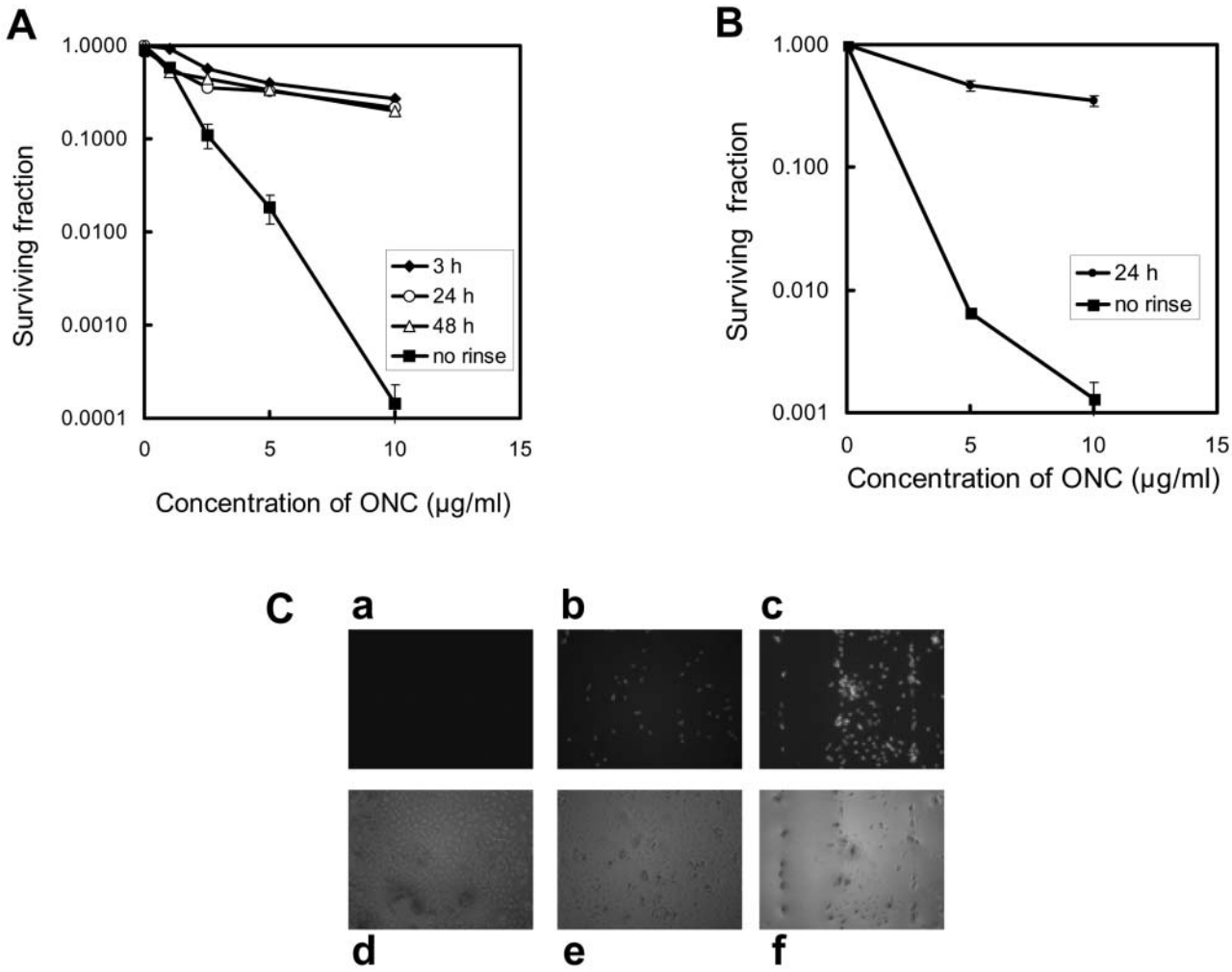

Figure 1. *A. The cytotoxic effect of ONC on A549 human NSCLC cell lines. The clonogenic assay was performed after different incubation durations with ONC. B. Cytotoxic effect of ONC on NCI-H1975 human NSCLC cell lines. The clonogenic assay was performed after different incubation durations with ONC. C. ONC-induced apoptosis in A549 tumor cell lines. No occurrence of apoptosis in untreated A549 tumor cell lines was observed in (a) fluorescent image of 0 µg/ml of ONC and (d) phase-contrast image of 0 µg/ml of ONC. However, apoptosis was induced by ONC at 2 µg/ml: (b) fluorescent image of 2 µg/ml of ONC and (e) phase-contrast image of 2 µg/ml of ONC. ONC-treated cells showed considerable induction of apoptosis: (c) fluorescent image of 10 µg/ml of ONC and (f) phase-contrast image of 10 µg/ml of ONC.*

Blood flow parameter studies using NIRS. NIRS signal intensity (arbitrary unit), a blood flow parameter, in tumor and skeletal muscle was monitored before and after treatment with ONC (10). Experiments were carried out when the tumor volume was between 200 and 400 mm^3. Since the electric field temporal autocorrelation function is explicitly related to the motion of the red blood cells, diffusion photons sometimes scatter from moving blood cells, causing the intensity of the diffusing light to fluctuate with time. The fluctuations are more rapid for faster moving blood cells. Therefore, one can derive information about tissue blood flow far below the tissue surface from measurements of temporal fluctuations impressed upon light diffusing through the tissue. Animal body temperature during the NIRS experiment was maintained at 37˚C by placing the animal in heating pads.

Statistical evaluation. All measured values are presented as the mean±standard error (SE) of each group. Significant differences within a group before and after ONC treatment were evaluated using a paired *t*-test, and those between treatment groups were evaluated with an independent (unpaired) *t*-test. Significance was set at 95% ($p<0.05$) for all analyses.

Results

Significant growth inhibition of two NSCLC cell lines by ONC. In order to determine the cytotoxic effect of ONC on A549 tumor cell lines, a clonogenic assay was performed after incubation with ONC (Figure 1A). We determined the ONC

concentration required for 10% and 50% cell survival (surviving fraction (SF), $SF_{0.1}$ and $SF_{0.5}$, respectively). $SF_{0.5}$ after 24 h was approximately 2 μg/ml. $SF_{0.5}$ values were similar for incubation times of 3 to 48 h. $SF_{0.1}$ was >10 μg/ml and beyond the used ONC dosages in our cell culture study. With no rinse (8 days of continuous incubation with ONC), the surviving fraction was dramatically decreased ($SF_{0.1}$ was 2.5 μg/ml. As shown in Figure 1B, a similar effect by ONC was found on NCI-H1975 NSCLC cell lines ($SF_{0.1}$ was 3 μg/ml).

ONC-induced apoptosis in NSCLC cell lines. We examined whether ONC-induced apoptosis occurred in gefitinib-induced apoptosis-resistant A549 tumor cell lines. In untreated A549 tumor cell lines, cells were confluent in more than 85% of the chamber, and apoptotic cells were not found when observed with Phase-contrast and Fluorescence microscopes (Figure 1C-a <fluorescent image> and Figure 1C-d <phase-contrast image>). 3 days after incubation with ONC at 10 μg/ml, ONC-induced apoptosis was found (Figure 1C-c <fluorescent image> and Figure 1C-f <phase-contrast image>). Additionally, cells at the center of the chamber were completely detached after exposure to ONC at 10 μg/ml. Since our method measured attached cells only, it likely underestimated the ONC-induced apoptosis.

Retardation in tumor growth in vivo after treatment with single and multiple administrations of ONC. Based on our *in vitro* studies, we tested the effectiveness of ONC in A549 tumors of nude mice. Figure 2A shows the effect of a single administration of ONC on the growth of A549 tumors. Tumor growth delay is defined as the time for a treatment group to reach a four fold volume increase minus the time for the controls to reach a four fold volume increase. Saline-treated control tumors took 11.5 days for a four-fold volume increase, establishing the baseline measurement. When the animals were *i.v.* injected with ONC at 2.5 mg/kg, a four-fold increase in tumor volume took 12.7 days, resulting in a growth delay of 1.2 days. When the animals were *i.v.* injected with ONC at 5 and 10 mg/kg, the tumor volume increased four times in 31 and 34 days, respectively. An *i.v.* injection of ONC at 2.5 mg/kg alone did not effectively retard the tumor growth of A549 tumors. However, ONC at 5-10 mg/kg was significantly and effectively tumoricidal in A549 tumors, reaching a growth delay of 20-22 days (*vs.* control, $p<0.05$). Thus, in reducing the tumor volume, ONC was more effective in a dose-dependent response from 2.5 mg/kg to 10 mg/kg. As shown in Figure 2B, multiple *i.v.* administrations of ONC significantly inhibited the tumor growth of A549 without any reduction of body weight compared to the saline-treated control group. When the animals were *i.v.* injected with ONC at 2 x 2.5 mg/kg per week, the tumor volume increased four times in 38.3 days, giving us a growth delay of 27 days (*vs.* control, $p<0.05$). When the animals were *i.v.* injected with ONC at 2 x 5 mg/kg per week, the tumor volume increased four times in 36 days, giving us a growth delay of 24 days (*vs.* control, $p<0.05$). Interestingly, it was more effective to administer ONC by multiple small-dosage injections compared to a single equivalent dose injection (ONC-5 mg/kg x1 *vs.* ONC-2.5 mg/kg x2, $p<0.05$).

ONC-induced changes in ATP and flow parameter using noninvasive methods. Blood flow parameter was monitored in A549 tumors and in counterpart skeletal muscles before and after treatment with ONC (Figure 3A). There was a significant increase in the flow parameter (NIRS signal intensity, arbitrary unit) in A549 tumors at 1.5 h from 13.5±0.5 to 18.6±0.8 (n=20 measurements, $p<0.05$), but not in skeletal muscles where the increase was minimal from 33.0±1.2 to 35.0±3.5 (n=20 measurements, $p=0.58$).

ONC-induced changes in ATP levels in the tumor tissues were measured using non-localized phosphorus MRS. ATP levels were decreased (Figure 3B).

In vitro studies with ONC combined with treatment of cisplatin using a clonogenic assay. The $SF_{0.5}$ and $SF_{0.1}$ values for cisplatin alone were 1.2 μM and 3.5 μM, respectively (Figure 4A). The sensitization enhancement ratio at $SF_{0.1}$ was approximately 1.6. As shown in Figure 4B, the greatest enhancement in cytotoxicity of cisplatin occurred when ONC was exposed simultaneously to A549 tumor cell lines. The enhanced sensitization of cisplatin was also observed before and after exposure to ONC.

Animal studies with ONC in the presence of cisplatin. ONC increased the cisplatin-induced tumor growth delay of A549 tumors in nude mice, as shown in Figure 5A. Saline-treated tumors took 15.4 days for a 4-fold volume increase, thus, establishing the baseline measure. When the animals were *i.v.* injected with ONC at 5 mg/kg, a four-fold increase in tumor volume took 31 days, giving us a growth delay of 15.6 days (*vs.* control, $p<0.05$). When the animals were *i.p.* injected with cisplatin at 5 mg/kg, a four-fold increase in tumor volume took 22.5 days, resulting in a growth delay of 7 days (*vs.* control, $p<0.05$). An *i.v.* injection of ONC at 5 mg/kg alone effectively retarded the tumor growth of A549 tumors. This effectiveness was similar to an *i.p.* injection of cisplatin at 5 mg/kg. When the animals were *i.v.* injected with ONC at 5 mg/kg and *i.p.* injected with cisplatin at 5 mg/kg, a four-fold increase in tumor volume took 48.5 days, giving us a growth delay of 33 days (*vs.* ONC or cisplatin alone, $p<0.05$). The combined treatment with ONC and cisplatin dramatically inhibited tumor growth in A549 tumors. The effect of a combination of ONC and cisplatin

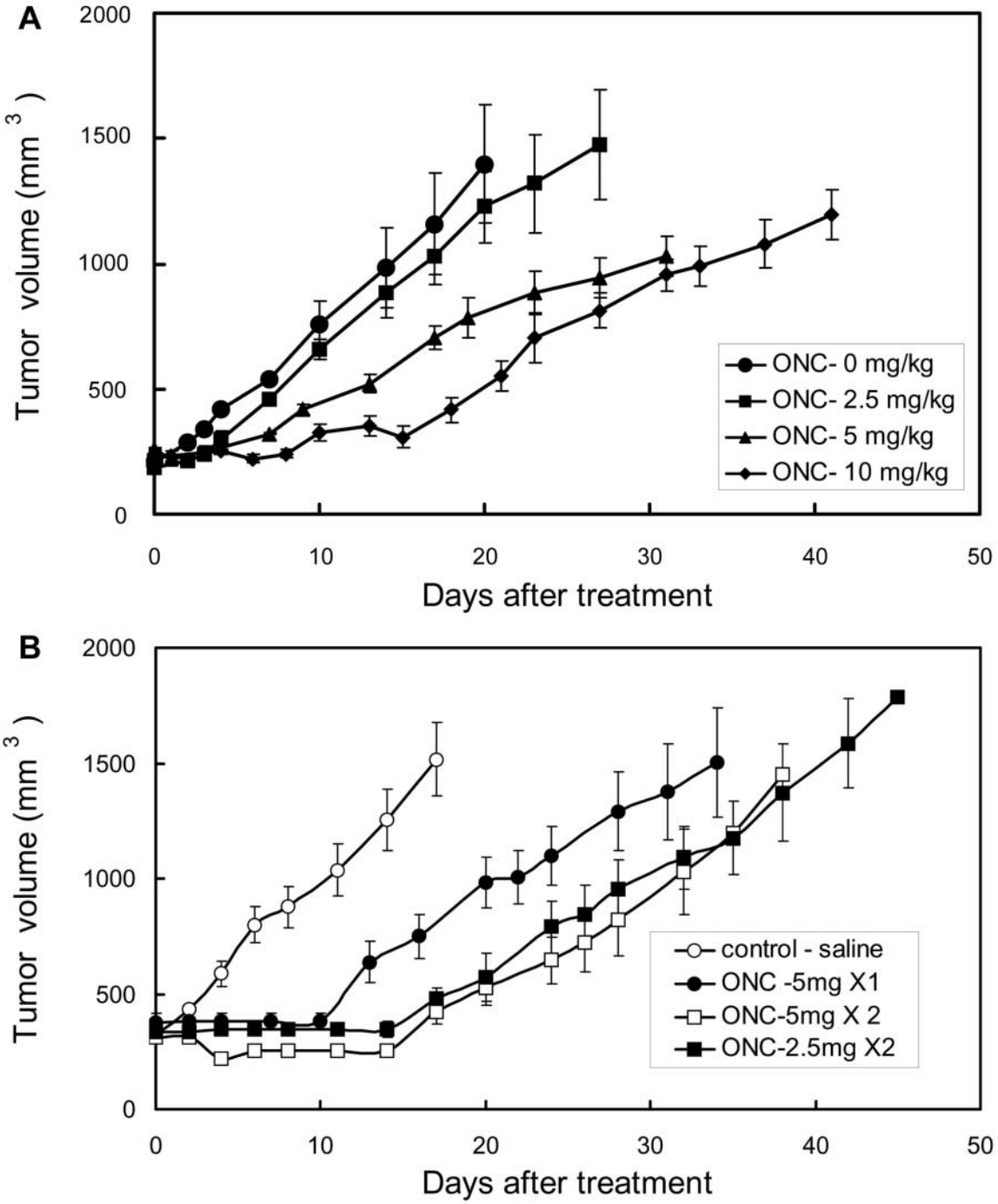

Figure 2. *Mono-therapy using single and multiple i.v. injections of ONC on A549 NSCLC (mean volume of 5-10 tumors, bars, ±SE). A. The volume of A549 tumors in the right hind legs of athymic nude mice are shown as a function of days after dosage of ONC at 2.5 mg/kg (closed squares), 5 mg/kg (closed triangles) or 10 mg/kg (closed diamonds). B. The volume of A549 tumors are shown as a function of days after the multiple small dosage injections of ONC at 2.5 mg/kg x 2 (closed squares) and 5 mg/kg x 2 (open squares).*

was greater than the additive effects of ONC and cisplatin alone in inhibiting tumor growth of A549 *in vivo*.

We investigated whether tumors, previously treated with cisplatin, could be effectively treated with ONC. ONC showed inhibition of tumor growth in large-size tumors unsuccessfully treated by cisplatin as shown in Figure 5B. This inhibition in tumor growth was ONC dose-dependent. When the tumors grew to a large size after treatment with

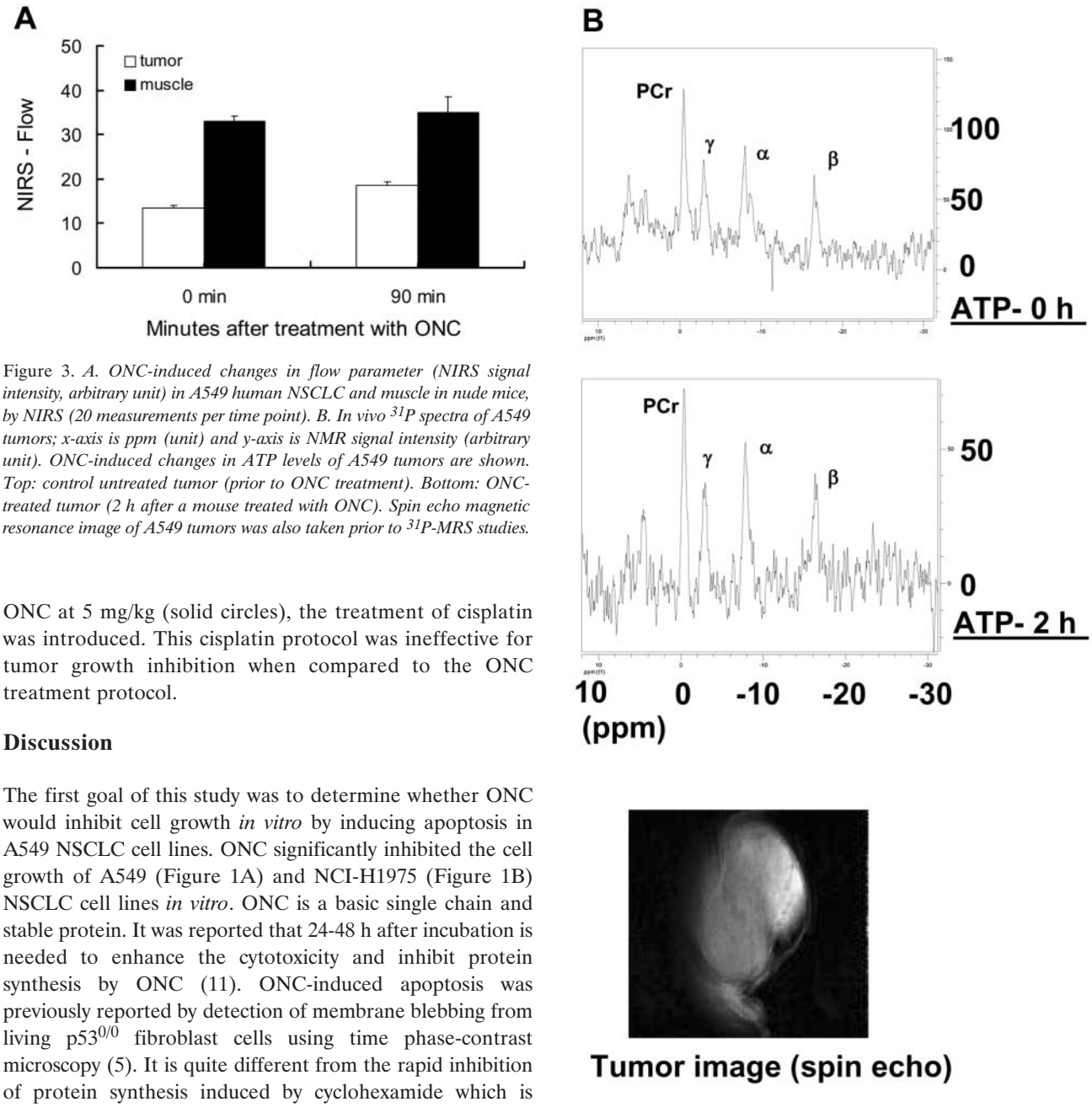

Figure 3. *A. ONC-induced changes in flow parameter (NIRS signal intensity, arbitrary unit) in A549 human NSCLC and muscle in nude mice, by NIRS (20 measurements per time point). B. In vivo ^{31}P spectra of A549 tumors; x-axis is ppm (unit) and y-axis is NMR signal intensity (arbitrary unit). ONC-induced changes in ATP levels of A549 tumors are shown. Top: control untreated tumor (prior to ONC treatment). Bottom: ONC-treated tumor (2 h after a mouse treated with ONC). Spin echo magnetic resonance image of A549 tumors was also taken prior to ^{31}P-MRS studies.*

ONC at 5 mg/kg (solid circles), the treatment of cisplatin was introduced. This cisplatin protocol was ineffective for tumor growth inhibition when compared to the ONC treatment protocol.

Discussion

The first goal of this study was to determine whether ONC would inhibit cell growth *in vitro* by inducing apoptosis in A549 NSCLC cell lines. ONC significantly inhibited the cell growth of A549 (Figure 1A) and NCI-H1975 (Figure 1B) NSCLC cell lines *in vitro*. ONC is a basic single chain and stable protein. It was reported that 24-48 h after incubation is needed to enhance the cytotoxicity and inhibit protein synthesis by ONC (11). ONC-induced apoptosis was previously reported by detection of membrane blebbing from living $p53^{0/0}$ fibroblast cells using time phase-contrast microscopy (5). It is quite different from the rapid inhibition of protein synthesis induced by cyclohexamide which is needed incubation for 2-3 h. Our clonogenic results confirm previous findings on ONC-enhanced apoptosis and the significant reduction of the clonogenic survival fraction (5, 11).

Based on our apoptosis analysis using a TUNEL assay of A549 tumor cells after treatment with ONC, no apoptosis was observed in untreated A549 tumor cells (Figure 1C). However, ONC-treated cells showed considerable induction of apoptosis in a dose-dependent manner. A similar ONC-induced apoptosis was observed in NCI-H1975 (data not shown). ONC selectively damaged tRNA degradation which resulted inhibition of protein synthesis and induced cell death *via* caspase activation (11).

As suggested in earlier studies, gefitinib mono-therapy in general was not well correlated with tumor expressions of EFGR (12). A549 tumor cell lines are known to be $EFGR^{WT}$ and gefitinib-resistant (*i.e.*, no change in apoptosis), but H1666 cell lines were $EFGR^{WT}$ and gefitinib-sensitive *in vitro* (10). Sordella *et al.* reported that NCI-H1975 cell lines are $EFGR^{mutant(L858R)}$ and gefitinib-sensitive using their cell growth curve studies *in vitro* (13). We observed that NCI-H1975 cell lines were sensitive to ONC using a clonogenic assay. Therefore, our findings show

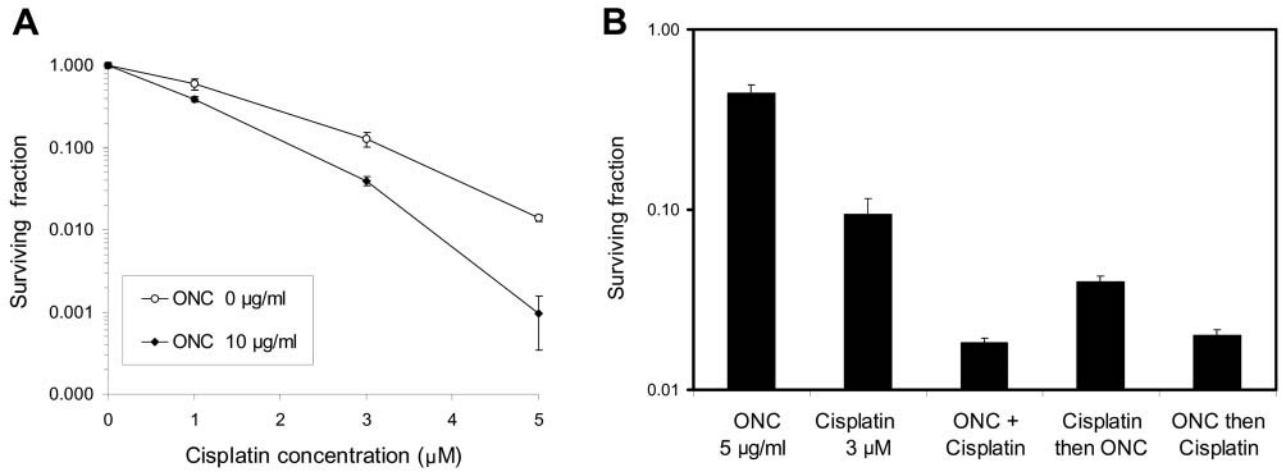

Figure 4. *A. The cytotoxic effect of ONC in the presence of cisplatin on A549 tumor cells. The clonogenic assay was performed after 24 h of concurrent exposure to ONC and cisplatin. B. Cell surviving fractions of A549 tumor cells after the treatment with ONC and cisplatin, singly and in combination.*

that ONC could be a good candidate for treatment of gefitinib-resistant NSCLC patients with $EFGR^{WT}$. NSCLC cell lines should be examined to see whether the EFGR status is a main contributor to the sensitivity to ONC treatment using tumor growth delay assays *in vivo*.

The second goal was to determine whether the mono-therapy of ONC would inhibit tumor growth *in vivo*. As shown in Figure 2B, it is more effective to administer ONC by multiple small-dosage injections compared to a single equivalent dose injection. This effectiveness is probably due to the normalization of leaky tumor vessels during the ONC therapy. Our preliminary histopathology reports using caspase-3 antibody labeling and TUNEL labeling assay *in vivo* showed remarkable increases in apoptosis after small-multiple injections of ONC (Lee *et al.*, unpublished data, September, 2006). Additionally, multiple small-dosage injections (*i.e.*, 2.5 mg/kg, two injections per week, ONC treated with 4 injections for two weeks) did not show a reduction in body weight (data not shown). To our knowledge, this is the first study of anti-tumoral effects of mono-therapy with ONC on human NSCLC *in vitro* and *in vivo*.

The third goal was to elucidate the physiological mechanism of ONC. Using a NIRS method, we found that the blood flow in A549 tumors at 1.5 h was significantly increased, but unchanged in skeletal muscle, shown in Figure 3A. This is in agreement with our previous observations, using a laser Doppler flowmetry method, of increased tumor blood flow in MCaIV murine mammary tumors (8, 14). We previously reported that ONC significantly inhibited cellular oxygen consumption rates in various tumor cell lines (7, 15). This led to ONC-induced reduction in ATP levels *in vivo*, observed non-invasively using an *in vivo* non-localized phosphorus MRS method in A549 tumors (Figure 3B). Based on our physiological observations, we speculated that the enhanced tumor oxygenation was mainly due to the reduction in QO_2 and in part, to an increase in tumor blood flow after treatment with ONC. It is well-documented that increases in viscous and geometric resistance to blood flow leads to elevated tumor interstitial fluid pressure (TIFP) in solid tumors (16, 17). The acute effect of ONC may be due to an ONC induced reduction in viscous resistance (lowering of TIFP) in A549 tumors. In addition, due to inhibition of the cell proliferation and clonogenic cell survival of endothelial cells by ONC, the QO_2 was significantly reduced, making O_2 more available to the peripheral tissues.

The fourth goal of the study was to determine whether ONC enhanced the effectiveness of cisplatin in A549 NSCLC *in vitro* and *in vivo*. ONC-induced-chemo (cisplatin) enhancement likely involved several cellular mechanisms including the inhibition of QO_2, induction of apoptosis, and inhibition of sub-lethal damage repair (SLDR). ONC significantly increased the cisplatin-induced tumor growth delay of A549 tumors in nude mice, with no noticeable side-effects (Figure 5A). Interesting observations occurred when a second treatment was performed in tumors that had an unsuccessful first treatment (Figure 5B). A second cisplatin treatment was not effective for large tumors with an unsuccessful initial treatment with cisplatin. In contrast, ONC was very effective in treating tumors with an unsuccessful initial treatment with cisplatin. This was due in part to the reduced TIFP improved the penetration of ONC into the tumor regions (17). Thus, it may be therapeutically worthwhile for this treatment protocol to proceed to clinical trials, particularly if it induces significant tumor volume reduction in NSCLC patients.

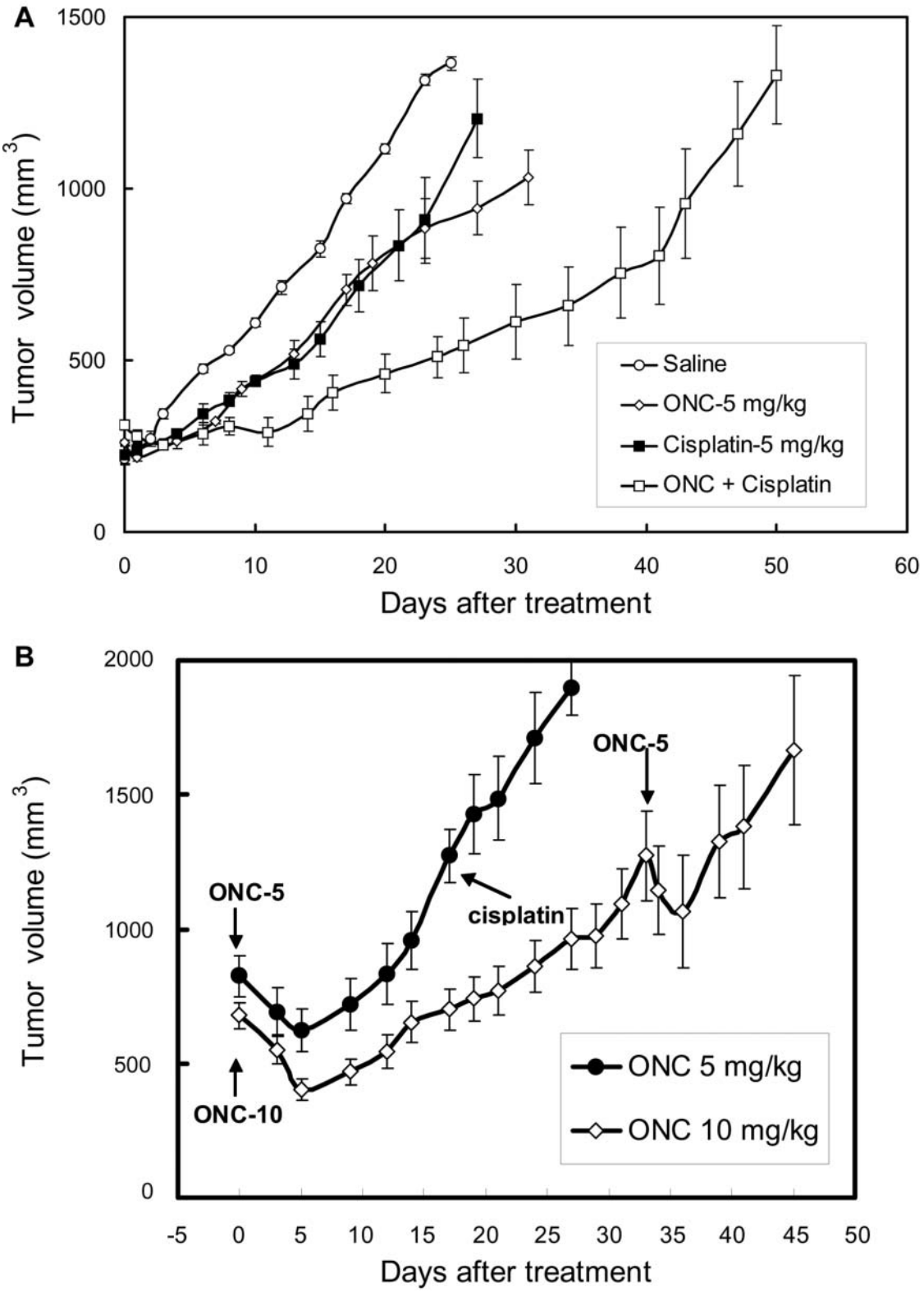

Figure 5. *The combined therapy with ONC and cisplatin (mean volume of 5-10 tumors, bars, ±SE.). A. The volume of A549 tumors in the right hind legs of athymic nude mice are shown as a function of days after treatment with ONC±cisplatin. ONC at 5 mg/kg was i.v. injected 1 h prior to an i.p. injection of cisplatin. In all mice, saline was i.v. injected every 3 days for 3 weeks following treatment. ONC increased the cisplatin-induced tumor growth delay of A549 tumors in nude mice. B. For large-size tumors unsuccessfully treated by cisplatin, ONC showed inhibition of tumor growth.*

Conclusion

ONC significantly inhibited tumor growth of A549 NSCLC cells both *in vitro* and *in vivo*. ONC reduced tumor hypertension and increased tumor blood flow. Multiple small-dosages of ONC significantly increased tumor growth delay of A549 NSCLC xenografts with manageable toxicity, probably due to the normalization of leaky tumor vessels during the ONC therapy. This investigation suggests important potential clinical uses of ONC for the treatment of NSCLC patients.

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