Improvements of Doxorubicin-Induced Antitumor Activity and Adverse Reaction by Combined Citrulline

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Citrulline is an amino acid with antioxidant activity. In this study, effects of citrulline on the adverse effects of doxorubicin (DOX) and DOX-induced antitumor activity were examined. Citrulline significantly inhibited DOX-induced increases in lipid peroxide (LPO) in the heart as adverse reaction. Combined treatment with DOX and citrulline increased DOX levels in tumor cells and enhanced cytotoxicity in vitro by significantly increasing DOX uptake compared with DOX treatment alone. In simultaneous in vivo treatments, combination treatment with DOX and citrulline decreased tumor weight and increased DOX concentrations in tumors. Promotion of DOX uptake by citrulline enhanced the antitumor activity of DOX through the action of energy-independent and Na⁺-independent transporters. This effect of citrulline on DOX influx is identical to that of S-(4-nitrobenzyl)-6-thioinosine, promoting DOX influx through the equilibrative nucleoside transporter 1. Therefore, it is anticipated that citrulline as a food component may enhance DOX efficacy.

Key words  citrulline; doxorubicin; adverse reaction; antitumor activity; doxorubicin permeability

Currently, cancer therapies include surgery, radiation therapy, and chemotherapy, which is appropriate for hematological tumors and general metastases. Among cancer chemotherapies, alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics platinum compounds, and molecular targeting agents are used. Furthermore, because mechanisms of antitumor agents vary, combined therapies offer clinical potential to enhance antitumor activity and reduce adverse reactions.¹⁻⁵ In contrast, while some biochemical modulators without antitumor activity enhance the pharmacological properties of antitumor agents, these can improve the therapeutic indexes or ameliorate adverse reactions by antitumor agents. However, these therapies often enhance antitumor agent induced adverse reactions with the increase in antitumor activity. Hence, developments and discoveries of novel modulators that enhance antitumor activities and reduce adverse reactions to antitumor agents are required.

The anthracycline doxorubicin (DOX) is widely used to treat a variety of malignancies, despite the severity of associated adverse reactions, such as cardiotoxicity. Although many studies report enhancement of DOX antitumor activity in tumor cells by direct inhibition of DOX efflux, no agents or combinations have been found to increase the therapeutic index of DOX by expression of these efflux pumps in normal tissues. Previously, we reported improved DOX-induced antitumor activity using the food components caffeine, theanine, and cucurbitacins.⁶⁻¹⁵ It appeared that these effects were mediated by increased DOX concentration in the tumors, which suppress DOX efflux from tumor cells. In contrast, linalool promoted DOX influx into tumor cells and enhanced its activity by acting on the Na⁺-dependent nucleoside transporter (CNT) 3.¹⁶ Furthermore, decreases in DOX levels following combined therapy with these dietary modulators in normal tissues could reduce adverse reactions to DOX, leading to enhanced antitumor activity, reduced patient burden, and increased quality of life.

Citrulline is an amino acid found in watermelon that has been shown to suppress arteriosclerosis¹⁷,¹⁸ and recovery from fatigue¹⁹ and ameliorate ischemic heart disease,²⁰ thereby offering potential as a novel health food. Moreover, citrulline acts as an antioxidant²¹ and may reduce adverse reactions to DOX by inhibiting lipid peroxide (LPO) accumulation in the heart. However, this mechanism may decrease the antitumor activity of DOX because DOX acts by producing oxygen radicals. In this study, the effects of citrulline on cardiotoxicity and the antitumor activity of DOX were examined.

MATERIALS AND METHODS

Materials  DOX, at 10 mg/vial (Adriaclin³), was purchased from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). Citrulline, sodium azide, and S-(4-nitrobenzyl)-6-thioinosine (NBMPR) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). 2-Deoxy-D-glucose was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). RPMI 1640 medium was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). WST-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Drugs were dissolved in sterile isotonic saline. All other chemicals used in this study were of the highest purity available.

Animals  Male CDF₁, DBA/2, and BDF₁ mice (5 weeks old, 20–25 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Animals were housed in a room that was maintained at 25±1°C and 55±5% relative humidity and were allowed free access to regular chow pellets and water. Animal experiments were approved by the institutional animal care and use committee at Iwate Medical University.

Determination of LPO in Hearts and Livers of CDF₁, Mice in Vivo DOX (15 mg/kg) was intraperitoneally injected into CDF₁ mice. Citrulline (10 or 100 mg/kg/d for 5 d) was intraperitoneally injected daily starting from the day before DOX administration. Mice were killed by cervical dislocation on the day after the last citrulline injection (the 4th day after DOX administration), and their hearts and livers were immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate buffer (pH 7.4) immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10 mM phosphate buffer (pH 7.4)
LPO concentrations in each sample were assayed using the thiobarbituric acid method.22

**Effect of Citrulline on DOX-Induced Cytotoxicity in P388 Leukemia Cells** P388 leukemia cells (1.0×10^6 cells/animal) were intraperitoneally transplanted into male DBA/2 mice. Ascites fluid was collected 7d after transplantation. Leukemia cells were washed twice and then resuspended in RPMI 1640 medium containing 10% fetal bovine serum.

P388 leukemia cell suspensions (1.0×10^6 cells/mL medium) were seeded into 96-well plates (Falcon) and incubated at 37°C for 24h. After incubation, DOX (90 or 900 nm) was added to the cell suspension and incubated at 37°C for 48h in the presence or absence of citrulline (1.0, 10, or 100 µM). Subsequently, WST-8 (10 µL) was added and the suspension was incubated at 37°C for 3h. Absorbance at 450nm was determined, and the probability of cell survival was expressed as a percentage of that of untreated control cells. Experiments were repeated at least 3 times.

**Effects of Citrulline on DOX Concentrations in P388 Leukemia Cells**

**In Vitro** To examine DOX influx into P388 leukemia cells, cells (5.0×10^6 cells/mL) were incubated with 9.0 µM DOX at 37°C for 60min in the presence or absence of citrulline (0.1 or 1.0 µM).

To examine the effect of citrulline on DOX efflux from P388 leukemia cells, cells were preincubated with 9.0 µM DOX at 37°C for 20min. After incubation, the medium was cooled on ice and then centrifuged at 150×g for 3min. Cells were washed and then resuspended in fresh medium. The resulting cell suspension (5.0×10^6 cells/mL) was incubated at 37°C for 30min in the presence or absence of citrulline (0.1 or 1.0 µM).

In both systems, the cell suspension was cooled on ice after incubation and was then centrifuged at 150×g for 3min. Cells were washed and resuspended in ice cold phosphate buffer (10mM, pH 7.8). Cell suspensions were mixed for 30s with 5.0mL of chloroform–methanol (4:1, v/v) and then centrifuged at 1200×g for 15min. The concentration of DOX in the organic phase was determined using a fluorescence spectrophotometer (excitation wavelength, 470nm; emission wavelength, 580nm).8,23)

**Effect of Citrulline on DOX-Induced Antitumor Activity in Vivo** P388 leukemia cells (5.0×10^5 cells/animal) were subcutaneously transplanted into the backs of BDF1 mice. DOX (2.0mg/kg/d) was intraperitoneally injected on days 3, 5, 7, and 9 after inoculation. Citrulline (20 or 100mg/kg/d) was intraperitoneally injected on days 4, 6, 8, and 10 post inoculation (alternate treatment), or at 100 mg/kg/d 3h before each DOX administration (simultaneous treatment). The mice were sacrificed by cervical dislocation on day 11 post inoculation. Tissue samples were immediately removed and weighed. Tissue samples were homogenized in 10 volumes (w/v) of 10mM phosphate buffer (pH 7.8). Each suspension was mixed for 60s with 5.0mL of chloroform–methanol (4:1, v/v) and then centrifuged at 1200×g for 15min. DOX concentrations were determined as described above.

**Effects of Citrulline on DOX Permeability. Effects of ATP Depletion on DOX Permeability** To examine the effect of citrulline on DOX influx into P388 cells under ATP-depleted conditions, cells (5.0×10^6 cells/mL medium) were preincubated with sodium azide (10mM) and 2-deoxy-d-glucose (10mM) in RPMI medium at 37°C for 20min. After incubation, cells were incubated with 0.9 µM DOX at 37°C for 10min in the presence or absence of 1.0µM citrulline. After incubation, the cell suspension was examined as described above.

**Effects of Na^+ on DOX Permeability** To examine the effect of sodium ions on citrulline-induced changes in DOX influx in P388 leukemia cells, cells (5.0×10^6 cells/mL) were suspended in Hanks’ balanced salt solution (HBSS; pH 7.4) or choline-replaced HBSS. Cells were preincubated with sodium azide (10mM) and 2-deoxy-d-glucose (10mM) in HBSS, or choline-replaced HBSS, at 37°C for 20min. After incubation, cells were incubated with 0.2 µM DOX at 37°C for 3min in the presence or absence of 1.0 µM citrulline. After incubation, cell suspensions were examined as described above.

**Effects of NBMPR or Citrulline on DOX Permeability in the Absence of Na^+** To examine the effects of NBMPR or citrulline on DOX influx into P388 leukemia cells in choline-replaced HBSS, cells (5.0×10^6 cells/mL HBSS) were preincubated with sodium azide (10mM) and 2-deoxy-d-glucose (10mM) in HBSS at 37°C for 20min. After incubation, cells were incubated in medium containing 0.5 µM DOX at 37°C for 5min in the presence or absence of 1.0 µM citrulline. After incubation, cell suspensions were examined as described above.

**Effects of NBMPR or Citrulline on DOX Permeability** To examine the effect of citrulline on DOX influx into P388 leukemia cells in the presence and absence of NBMPR in RPMI medium, cells (5.0×10^6 cells/mL medium) were preincubated with sodium azide (10mM) and 2-deoxy-d-glucose (10mM) in RPMI medium at 37°C for 20min. After incubation, cells were incubated in medium containing 0.5 µM DOX at 37°C for 5min in the presence or absence of 1.0 µM citrulline. After incubation, cell suspensions were examined as described above.

**Statistical Analysis** Statistical analysis was performed using Student’s t-test and ANOVA. These statistical analyses were performed using Stat View 5.0 statistical software (ASA Institute Inc., Cary, NC, U.S.A.).

**RESULTS**

**Effects of Citrulline on DOX-Induced Changes in LPO Levels in Vivo** The effects of citrulline on DOX-induced changes in LPO levels in the heart and liver are shown in Fig. 1. LPO concentrations were significantly increased by 1.3-fold in heart after DOX treatment (4.29±0.45 nmol/mg protein, p<0.01) compared with that in control heart (3.43±0.15 nmol/mg protein; Fig. 1A). In contrast, citrulline (100mg/kg) inhibited DOX-induced increases in LPO levels (p<0.05). After combined citrulline and DOX treatment, LPO levels in the liver also tended to decrease (Fig. 1B).

**Effect of Citrulline on DOX-Induced Cytotoxicity in P388 Leukemia Cells** The cytotoxic effects of DOX in the presence of citrulline in P388 leukemia cells are shown in Fig. 2. Single treatments with DOX (900nM) induced cytotoxicity in a concentration dependent manner, with survival ratios of 67% (p<0.05). Survival ratios of tumor cells after single treatments with citrulline (<1000µM) did not differ from those of the control group (data not shown). The cytotoxicity of DOX at 90nM (no effect concentration) increased with increase in citrulline concentration. At 10 µM citrulline the effect of DOX...
at its IC$_{50}$ (0.73 µM) was increased to that of DOX at 2.7-fold this concentration (1.93 µM).

**Effect of Citrulline on DOX Concentrations in P388 Leukemia Cells in Vitro** The effects of citrulline on DOX influx and efflux in P388 leukemia cells are shown in Fig. 3. Citrulline promoted DOX influx into tumor cells in a concentration dependent manner with a significant 1.2-fold increase in intracellular DOX concentrations after co-treatment with citrulline for 60 min. In contrast, citrulline had no effect on DOX efflux.

**Effect of Citrulline on DOX-Induced Antitumor Activity in Vivo** The effects of citrulline on DOX-induced antitumor activity in P388 leukemia-bearing mice were examined. In alternate treatments (Fig. 4), tumor weights did not change after combination treatment with citrulline (100, 20 mg/kg) and DOX (0.52±0.04, 0.53±0.12 g, respectively) compared with the DOX alone treated group (0.53±0.11 g). In simultaneous treatments (Fig. 5), tumor weights in DOX alone treated mice
(0.72±0.23 g) were decreased to 71% of the control (1.02±0.16 g), whereas in combination with 100 mg/kg citrulline, DOX decreased tumor weights to 52% (0.53±0.09 g, p<0.001; Fig. 5A). DOX concentrations in tumors of citrulline co-treated animals increased by 2.8-fold (p<0.05) compared with those in the DOX alone treated group, whereas co-treatment with citrulline did not cause increased DOX concentrations in normal tissues (Fig. 5B).

Effects of Citrulline on DOX Permeability The effect of ATP depletion on DOX influx into P388 leukemia cells is shown in Fig. 6A. DOX influx into tumor cells after co-treatment with citrulline for 6 min significantly increased by 57% (p<0.05) compared with that in DOX alone treated cells, indicating that ATP depletion did not affect the promotion of DOX influx by citrulline.

The effects of Na⁺ on the initial uptake rates of DOX into P388 leukemia cells are shown in Fig. 6B. Co-treatment with citrulline significantly promoted DOX influx in the presence and absence of Na⁺ (p<0.01), indicating that citrulline-enhanced DOX uptake is independent of Na⁺.

The effects of citrulline and NBMPR on the uptake of DOX into P388 leukemia cells were examined. In the absence of NBMPR, DOX uptake was significantly increased by 1.5-fold (p<0.05) in the presence of citrulline. However, this enhance-
ment of DOX uptake was disappeared in the presence of NBMPR (data not shown), indicating that citrulline-enhanced DOX uptake was inhibited by NBMPR.

The effects of citrulline and NBMPR on DOX uptake ratios in the absence of Na$^{+}$ are shown in Fig. 7. DOX uptake was significantly increased in the presence of citrulline at 0.01–1 µM, but was decreased at 10 µM citrulline (Fig. 7A). Furthermore, DOX uptake was also increased in the presence of NBMPR at 1–30 µM whereas decreased at 100 µM NBMPR (Fig. 7B). Both citrulline and NBMPR promoted DOX influx at low concentrations, but inhibited DOX influx at high concentrations in the absence of Na$^{+}$.

**DISCUSSION**

Among various physiological activities in health foods, the amino acid citrulline may be recognized as a novel dietary antioxidant.21) In this study, we showed that citrulline acts as a novel modulator of DOX efficacy that reduces the adverse reactions by DOX, and examined the effects of citrulline on DOX-induced antitumor activity.

It has reported that severe cardiotoxicity of DOX is caused by LPO accumulation in the heart.24,25) Thus, we examined the effects of citrulline using LPO as an indicator of DOX cardiotoxicity, and observed significant inhibition of DOX-induced LPO accumulation in the heart. It is suggested that these effects were related to antioxidant activities, involving scavenging of hydroxyl radicals by citrulline.21) Therefore, citrulline was expected to reduce DOX cardiotoxicity by inhibiting LPO accumulation in the heart.

However, inhibition of DOX-induced increases in LPO levels may prevent the antitumor activity of DOX. Hence, the cytotoxic effects of DOX were examined in the presence and absence of citrulline in vitro to clarify the effects of citrulline on DOX-induced antitumor activity. Combined treatment with DOX and citrulline increased DOX cytotoxicity depending on the concentration of citrulline, indicating that citrulline may enhance the antitumor activity of DOX rather than decreasing it.

To clarify the mechanisms by which citrulline increases the cytotoxicity of DOX, the effects of citrulline on DOX influx and efflux were examined in P388 leukemia cells. Although citrulline had no effect on DOX efflux, it significantly enhanced DOX influx at low concentrations, but inhibited DOX influx at high concentrations in the absence of Na$^{+}$.
enhanced DOX permeability on Na+-dependent system. Subsequently, the dependence of citrulline-citrulline promoted DOX influx via a Na+-independent system. Subsequently, the dependence of citrulline-enhanced DOX permeability on Na+ was examined in the absence of Na+. In these experiments, Na+ had no effect on DOX influx in the presence of citrulline. Taken together, these data indicate citrulline promoted DOX influx was mediated by energy- and Na+-independent DOX transporter.

Members of the nucleoside transporter family, such as CNT and equilibrative nucleoside transporters (ENT), are energy-independent,26 and are reported that the transporters appear in cancer cells.27 While CNT transports DOX in an Na+-dependent manner, ENT is Na+-independent.27 Because citrulline appears to enhance DOX influx via an energy- and Na+-independent transporter, we examined the effects of the ENT inhibitor NBMPR on DOX uptake in the presence of citrulline. In these experiments, the effect of citrulline on DOX uptake was inhibited in the presence of NBMPR, suggesting that citrulline acts via ENT1. In the absence of Na+, low concentrations of NBMPR promoted DOX influx, whereas at high concentrations it inhibited DOX influx. Interestingly, citrulline showed similar behavior to NBMPR, indicating similar modes of action, presumably involving ENT1.

CONCLUSION

Citrulline ameliorates adverse reactions of DOX by inhibiting LPO accumulation in the heart, and by increasing DOX concentrations in tumor cells by enhancing the activity of DOX influx transporters, and therefore increased antitumor activity. Thus, we anticipate the recognition of citrulline as a food component that modulates DOX efficacy.

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REFERENCES


