Protective Effects of the Immunopotentiator from *Pantoea agglomerans* 1 on Chemotherapeutic Agent-induced Macrophage Growth Inhibition

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Abstract. *Background: The immunopotentiator from Pantoea agglomerans 1 (IP-PA1) is an edible lipopolysaccharide (LPS) derived from symbiotic bacteria found in crops. IP-PA1 is known to ameliorate chemotherapy-induced immunosuppression; therefore, its macrophage-activating effect in the presence of chemotherapeutic agents was evaluated. Materials and Methods: Nuclear factor-κB (NF-κB) activation in IP-PA1-treated RAW264 and J774.1 cells was examined using Western blot analyses; Griess assay and ELISA were used to examine the production of nitric oxide and tumour necrosis factor α, respectively. The expression of apoptosis-related proteins was also assessed using Western blot analyses. The effect of IP-PA1 on doxorubicin-induced apoptosis was analyzed by flow cytometry after annexin-V staining. The growth of macrophages treated with chemotherapeutic agents and IP-PA1 was analyzed using an MTT assay. Results: IP-PA1 activated NF-κB and ameliorated chemotherapy induced growth inhibition in the cells. Conclusion: IP-PA1 is an edible drug that can potentially support chemotherapy by ameliorating chemotherapy-induced immunosuppression.*

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In systemic anticancer chemotherapy, various drugs with different targeting mechanisms are used. Anthracyclines such as doxorubicin inhibit DNA and RNA synthesis by intercalating between base pairs of a DNA or RNA strand (1). Platinum compounds such as cisplatin inhibit DNA replication and transcription by forming platinum-DNA crosslinks (2). 5-Fluorouracil (5-FU), an analogue of endogenous pyrimidine nucleoside, interferes with RNA and DNA synthesis (3), and methotrexate inhibits the production of folates, which are required for DNA synthesis (4). These chemotherapeutic agents all cause apoptosis despite their widely different modes of action and cellular targets (5, 6). Unfortunately, these toxic effects of currently used chemotherapeutic agents are not always selective for the target tumour cells, they often affect normal immune cells, causing bone marrow depression, lymphopenia, and immunosuppression (reviewed in 7).

Macrophages located in every peripheral tissue are the first line of defence against the occurrence and development of cancer. They recognize and kill tumour cells directly (8, 9); take in apoptotic tumour cells by phagocytosis (10); and process and present tumour-specific antigens to $CD8^+$ cytotoxic T lymphocytes (CTL), thus activating CTLs (11). These functions are essential not only for preventing the occurrence and development of tumours, but also for the elimination of tumour cells that are damaged or apoptotic because of chemotherapeutic agents; these cells can

potentially suppress antitumour immunity (12). Chemotherapeutic agents suppress these functions of macrophages partly through the induction of apoptosis (13). Therefore, rendering healthy cells with protection from chemotherapeutic agent-induced macrophage apoptosis may improve the effects of chemotherapy.

The immunopotentiator from *Pantoea agglomerans* 1 (IP-PA1) is a low molecular weight (5 kDa) lipopolysaccharide (LPS) derived from the cell wall of symbiotic gram-negative bacteria, which are found on various crops, such as cereals, fruits, and vegetables (14-17). The oral administration of IP-PA1 has immunoenhancing effects; for example, it confers protection from bacterial and parasitic infections in mice (15, 16) and aids recovery from dexamethasone-induced low antibody production in response to the *Salmonella enteritidis* vaccine or to sheep red blood cells in chickens (18). Thus, IP-PA1 is considered a potential edible immunomodulator.

The activation of nuclear factor-κB (NF-κB), an intracellular transcription factor associated with the expression of antiapoptotic proteins (19, 20), plays a role in the inhibition of apoptosis of haemopoietic cells by cytokines such as interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (21) as well as in the protection of monocytes from Fas-mediated apoptosis induced by the growth hormone (22). Natural LPS derived from pathogenic bacteria activates NF-κB *via* toll-like receptor (TLR)-4, a specific LPS receptor (23); therefore, we hypothesized that IP-PA1 protects macrophages from apoptosis *via* the activation of NF-κB. In this study, NF-κB activation and the protective effects of IP-PA1 on chemotherapeutic agent-induced inhibition of macrophage growth were evaluated.

Materials and Methods

Cell culture. Murine macrophage-like RAW264 and J774.1 cells were obtained from RIKEN BioResource Center (Tsukuba, Japan). Human breast carcinoma MCF-7 cells and human lung carcinoma A549 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All these cells were maintained in RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with L-glutamine, 10% foetal bovine serum (FBS; HyClone; Thermo Scientific, South Logan, UT, USA), and 100 μg/ml of penicillin and streptomycin (Nacalai Tesque, Kyoto, Japan).

Reagents. IP-PA1 was purified to attain more than 99% purity by using previously described methods (24). LPS derived from *Escherichia coli* and doxorubicin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-IκB-α, rabbit anti-phospho-IκB-α, rabbit anti-p65, rabbit anti-Bcl-2, rabbit anti-Bcl-xL, rabbit anti-Bax, and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig) G antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Affinity-purified rat anti-mouse tumour necrosis factor (TNF)-α antibodies, biotin-labelled rat anti-mouse TNF-α antibodies, avidin-HRP, and standard recombinant mouse TNF-α were obtained from eBioscience (San Diego, CA, USA). Unless otherwise indicated, all the chemicals used, including the chemotherapeutic agents, were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Collection of cell culture supernatant. RAW264 cells (5×10^6 cells/ml) were incubated in phenol red-free RPMI-1640 containing 10% FBS and antibiotics. The cell suspension (200 μl) was applied to each well of 48-well culture plates and incubated for 4 hours. Subsequently, the cells were incubated with 0-100 ng/ml of IP-PA1 or 100 ng/ml of *E. coli*-derived LPS in phosphate-buffered saline (PBS) either for 12 hours for the detection of nitric oxide (NO) or for 150 minutes for the detection of TNF-α. After incubation, the cell culture supernatant was collected and stored at −20˚C until examination.

Griess assay. NO formation was assessed by measuring the production of nitrite (NO_2^-), one of two primary stable and nonvolatile breakdown products of NO, by using the Promega Griess Reagent System (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. In brief, culture supernatants (50 μl) were mixed with 50 μl of sulfanilamide and 50 μl of 0.1% *N*-1-napthylethylenediamine dihydrochloride. After incubation at room temperature for 10 minutes, the absorbance at 595 nm was measured using a model 550 microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). NUNC MaxiSorp 96-well ELISA plates (Nalge Nunc International, Roskilde, Denmark) were coated with anti-TNF-α antibodies (100 μl, 1 μg/ml) in 0.1 M NaHCO3 (Nacalai Tesque), incubated overnight at 4˚C, and washed with 1× PBS containing 0.05% Tween 20 (PBS-T). The plates were blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and washed with PBS-T. Standard recombinant TNF-α (100 μl) and cell supernatant diluted with 1% BSA in PBS were added to the wells, and the plates were incubated for 1 hour at room temperature; subsequently, the plates were washed with PBS-T. Biotin-labelled anti-TNF-α antibody (100 μl, 1 μg/ml) was added to the wells, after which the plates were incubated for 1 hour at room temperature and subsequently, washed with PBS-T. HRP-conjugated avidin (100 μl, 1 μg/ml) was added to the wells; this was followed by incubation for 1 hour at room temperature and washing with PBS-T. Finally, colorimetric signals were generated using TMB One Solution (Promega Corporation); the reaction was arrested with 1 N HCl after 15 minutes, and the absorbance at 450 nm was measured using a model 550 microplate reader (Bio-Rad Laboratories Inc.). The concentration of TNF-α was interpolated on the basis of standard curves.

MTT assay. RAW264, J774.1, A549, and MCF-7 cells (10^6 cells/ml) were incubated in phenol red-free RPMI-1640 containing 10% FBS and antibiotics. Each of the cell suspensions (100 μl) was applied to each well of 96-well culture plates and incubated for 4 hours. Subsequently, the cells were incubated with 0-100 ng/ml of IP-PA1 in PBS. After 1 hour, the cells were incubated with 0-10 μM of doxorubicin, cisplatin, 5-FU, or methotrexate for 72 hours. Four hours before the end of the incubation period, 10 μl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Dojindo, Kumamoto, Japan) in PBS was added to each well. The reaction was arrested by adding 100 μl of 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl. Absorbance was measured at 595 nm

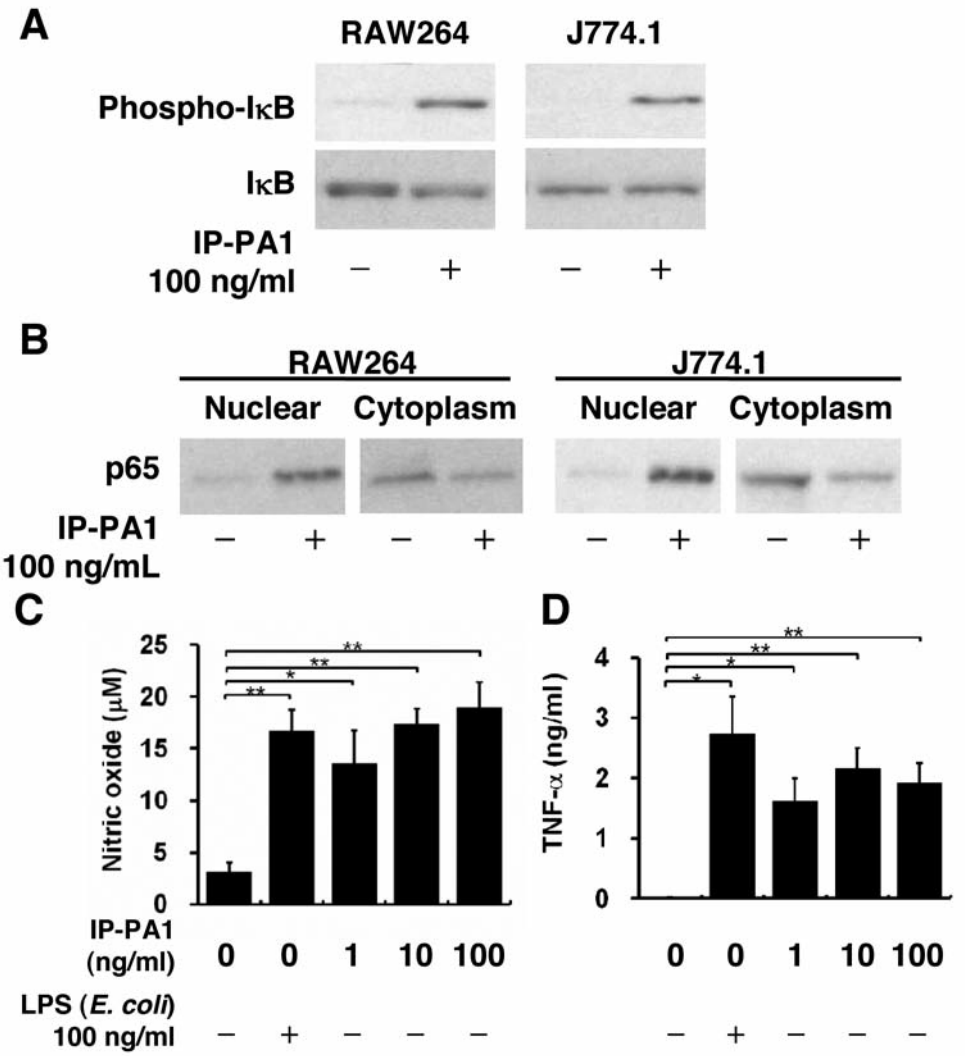

Figure 1. *NF-κB activation by IP-PA1. RAW264 and J774.1 cells were treated with 100 ng/ml of IP-PA1, and IκB phosphorylation (A) or nuclear and cytoplasmic expression of p65 (B) were detected by Western blot analyses. Representative data of 3 independent experiments are shown. RAW264 cells were treated with 0, 1, 10, or 100 ng/ml of IP-PA1 or 100 ng/ml of LPS (derived from E. coli) and incubated for 24 hours (C) or 150 minutes (D). Production of nitric oxide (NO) (C) and tumour necrosis factor (TNF)-α (D) were evaluated at the end of the incubation period by using the Griess assay (C) and ELISA (D), respectively. In (C) and (D), data are presented as the mean±SEM of 3 independent experiments. Significant differences between groups are indicated by *p<0.05 and **p<0.01.*

by using a model 550 microplate reader (Bio-Rad Laboratories Inc.). The relative number of viable cells was calculated as the ratio of the optical density (OD) of the treated cells to that of the cells at the beginning of the treatment.

Cell lysate collection. RAW264 and J774.1 cells (2.5×10^7 in 5 ml) were seeded in 6-well culture plates and incubated for 4 hours. Subsequently, they were incubated with PBS or 100 ng/ml of IP-PA1 for 1 hour. In order to detect caspase-3 activation, the cells were treated with PBS or 5 μM of doxorubicin after pre-incubation with IP-PA1 and were further incubated for 24 hours. The cells were washed with PBS prior to protein extraction. For the detection of proteins in whole-cell lysate, cells were lysed in 200 μl of M-PER mammalian protein extraction reagent (Thermo Scientific Pierce, Rockford, IL, USA) supplemented with 10 μl/ml of Halt protease inhibitor cocktail (Thermo Scientific Pierce) according to the manufacturer's instructions. For the detection of p65 localization, nuclear and cytoplasmic extracts were separated using NE-PER (Thermo Scientific Pierce) according to the manufacturer's instructions.

Western blot analysis. Supernatants were collected by centrifuging the cell lysates, and protein concentrations therein were measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories Inc.). Samples containing 20 μg of proteins were mixed with the same volume of 2× sample buffer [20% glycerol, 10% 2-mercaptoethanol (2-ME), 4% SDS, and 125 mM Tris-HCl; pH 6.8], boiled at 95˚C for 7 minutes, and subjected to SDS-PAGE on 12.5% PAGEL (ATTO, Tokyo, Japan). The separated proteins were transferred onto

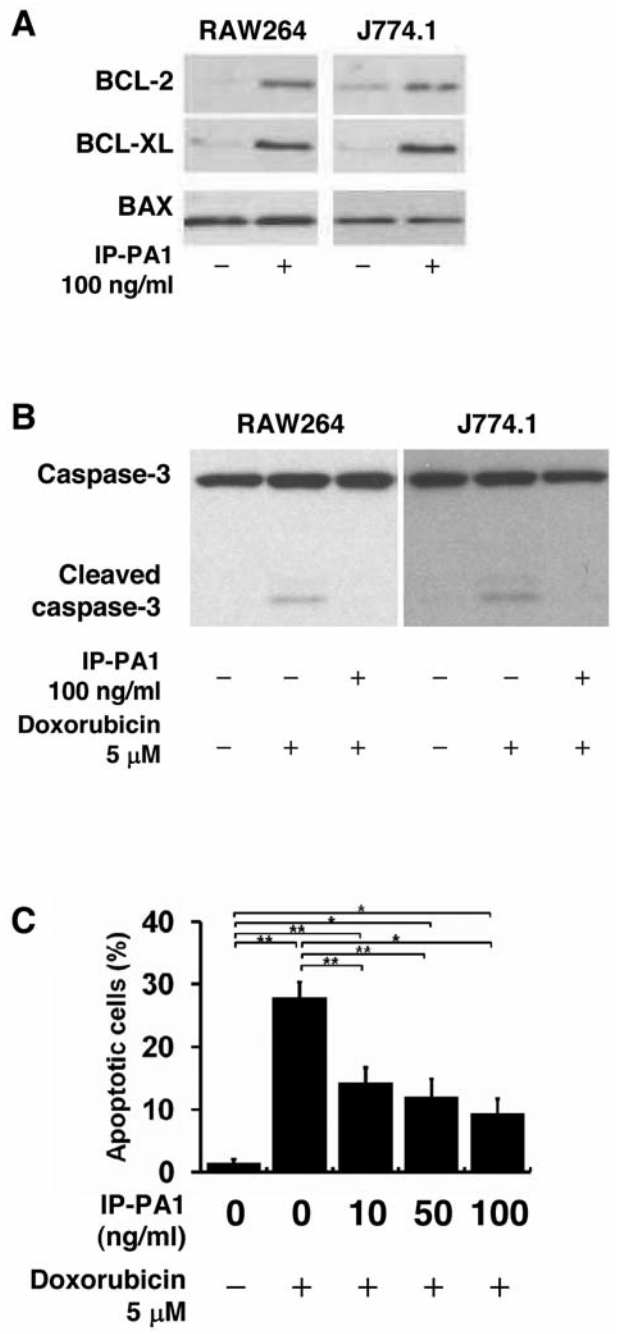

Figure 2. *IP-PA1-mediated inhibition of doxorubicin-induced apoptosis. A: RAW264 and J774.1 cells were treated with PBS or 100 ng/ml of IP-PA1. The expression of BCL-2, BCL-XL, and BAX was detected by Western blot analysis performed 1 hour after IP-PA1 treatment. B and C: RAW264 and J774.1 cells that were pretreated with the indicated doses of IP-PA1 for 1 hour were incubated with 5 μM doxorubicin for an additional 24 hours. Cleavage of caspase-3 was detected using western blot analysis (B). Apoptotic cells were examined using a flow cytometer after annexin-V staining, and the percentage of apoptotic cells (annexin-V-positive cells) was determined (C). Data are presented as the mean±SEM of 3 experiments. Significant differences between groups are indicated by *p<0.05 and **p<0.01. In (A) and (B), representative data of 3 independent experiments are shown.*

Immobilon-P membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 5% skimmed milk and blotted with primary antibodies diluted in a blocking solution. After washing, the membrane was incubated with HRP-conjugated secondary antibodies. Positive reactions were visualized using the ECL plus Western blotting detection system (Amersham Bioscience, Buckinghamshire, UK).

Annexin V analysis. Apoptosis status was assessed using a TACS annexin-V-fluorescein isothiocyanate (FITC) kit (Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. In brief, RAW264 cells (2.5×10^7 cells in 5 ml) were seeded in 6-well culture plates and incubated for 4 hours. Subsequently, they were treated with 0-100 ng/ml of IP-PA1 in PBS for 1 hour. The cells were then treated with 5 μM of doxorubicin for 24 hours, collected by gentle scraping, and washed in ice-cold PBS. A total of 10^6 cells were incubated with annexin-V-FITC and propidium iodide (PI) for 15 minutes at room temperature. The percentage of apoptotic (annexin-V-positive) cells was measured on an Epics Elite flow cytometer (Coulter, Tokyo, Japan).

Statistical analysis. All data are expressed as the mean±standard error of the mean (SEM). Multiple comparisons were performed using one-way analysis of variance (ANOVA) with *post hoc* analysis followed by the Bonferroni test.

Results

NF-κB activation by IP-PA1. After IP-PA1 treatment, phosphorylation of IκB was induced in RAW264 and J774.1 cells at 1 hour, whereas the expression levels of IκB were unchanged or slightly decreased (Figure 1A). Further, IP-PA1 increased the nuclear expression and decreased the cytoplasmic expression of p65 (Figure 1B); this finding reflected the translocation of NF-κB from the cytoplasm into the nucleus. IP-PA1 induced the production of NO and TNF-α in RAW 264 cells (Figure 1C and D).

Antiapoptotic regulation by IP-PA1. The expression of the antiapoptotic proteins BCL-2 and BCL-XL was strongly enhanced after IP-PA1 treatment (Figure 2A). In contrast, the expression of the proapoptotic protein BAX was not affected by IP-PA1 (Figure 2A). The activation of caspase-3, as reflected by its cleavage 24 hours after doxorubicin treatment, was completely inhibited in RAW264 and J774.1 cells after pretreatment with 100 ng/ml IP-PA1 (Figure 2B). The percentage of apoptotic cells increased after treatment with 5 μM doxorubicin for 24 hours; however, pretreatment with IP-PA1 inhibited doxorubicin-induced apoptosis in RAW 264 cells (Figure 2C).

IP-PA1 confers protection against chemotherapeutic agent-induced inhibition of macrophage growth. In the RAW264 and J774.1 cells, IP-PA1 pretreatment significantly reduced the inhibitory effects of 1, 2.5, 5, and 10 μM of doxorubicin, cisplatin, 5-FU, and methotrexate on cell proliferation (Figure

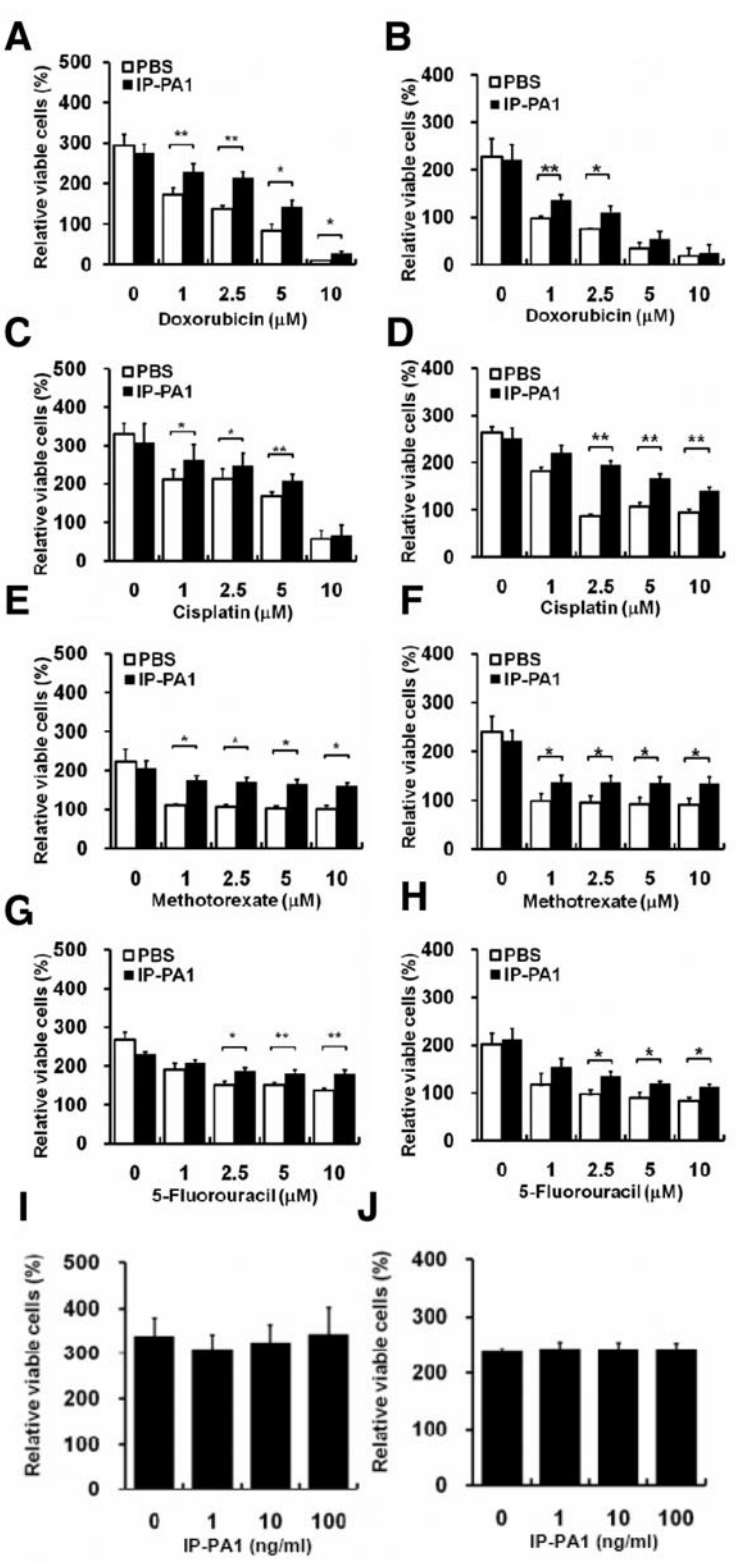

Figure 3. *Protection of RAW264 and J774.1 cells from chemotherapeutic agent-induced growth inhibition. RAW264 (A, C, E, and G) or J774.1 (B, D, F, and H) cells pretreated with PBS or 100 ng/ml of IP-PA1 were treated with doxorubicin (A and B), cisplatin (C and D), 5-fluorouracil (E and F), or methotrexate (G and H) at doses of 0, 1, 2.5, 5, and 10 μM 1 hour after the pretreatment. RAW 264 (I) or J774.1 J: cells were treated with IP-PA1 at the indicated doses. The cells were incubated for an additional 72 hours, and cell proliferation was assessed using an MTT assay. Data are presented as the mean±SEM of at least 3 experiments. Significant differences between the mean values of 2 groups are indicated by *p<0.05 and **p<0.01.*

Table I. *Protection from doxorubicin-induced growth inhibition in macrophages but not in MCF-7 and A549 cells.*

Cells	IC_{50} of doxorubicin (μM)			
	IP-PA1 (ng/ml)			
	0	1	10	100
PAW264	2.00±0.34##	2.71±0.52#	3.60±.029*	5.45±0.63**
J774.1	<1.00	1.43±0.37	1.79±0.32	1.74±0.28
MCF-7	1.69±0.33	1.82±0.20	1.98±0.22	1.72±0.24
A549	1.71±0.39	1.90±0.51	2.09±0.59	1.77±0.59

*$p<0.05$ And **$p<0.01$, compared to cells that were not pretreated with IP-PA1; #$p<0.05$ and ##$p<0.01$, compared to cells that were pretreated with 100 ng/ml of IP-PA1.

3A-H). The 50% inhibitory concentration (IC_{50}) of doxorubicin in RAW264 and J774.1 cells was increased by IP-PA1 pretreatment in a dose-dependent manner (Table I); however, pretreatment with IP-PA1 had no effect on the cytotoxic activity of doxorubicin in the human breast carcinoma cell line MCF-7 nor the human lung carcinoma cell line A549 (Table I). Further, treatment with IP-PA1 alone had no effect on the proliferation of RAW 264 and J774.1 cells (Figure 3I and J).

Discussion

Chemotherapy for cancer is well established; however, the suppression of host immunity remains a major shortcoming of this therapeutic approach. Supportive drugs that protect immune cells, especially macrophages, from chemotherapy-induced growth inhibition are required for improving the outcome of cancer. In this study, the macrophage-activating effect of IP-PA1 in the presence of chemotherapeutic agents was evaluated using the macrophage-like cell lines RAW264 and J774.1.

The inhibitory effects of IP-PA1 on doxorubicin-induced apoptosis in macrophages were evident through two typical hallmarks of apoptosis: cleavage of caspase-3 (25)(Figure 2B) and surface expression of phosphatidylserine, which is known to bind annexin-V (26)(Figure 2C). Apoptosis is an irreversible process. The initiation of apoptosis in response to apoptotic signals is regulated by the relative levels of proapoptotic proteins such as BAX (27) and anti-apoptotic proteins such as BCL-2 (28) and BCL-XL (29); the latter proteins inhibit the activation of proapoptotic proteins. Increased expression of the antiapoptotic proteins BCL-2 and BCL-XL (Figure 2A) may contribute to the inhibition of apoptosis by IP-PA1. IP-PA1 activated NF-κB, as reflected by the phosphorylation of IκB (30) (Figure 1A) and the translocation of NF-κB from the cytoplasm into the nucleus (31) (Figure 1B). The IP-PA1-mediated activation of NF-κB was functionally sufficient to produce NO (32) (Figure 1C) and TNF-α (33) (Figure 1D). Activated NF-κB enhances the expression of antiapoptotic proteins, including BCL-2 (19) and BCL-XL (20). Thus, the IP-PA1-mediated inhibition of chemotherapy-induced apoptosis observed in this study may partly be due to NF-κB activation. The expression level of the typical proapoptotic protein BAX was not affected by IP-PA1; however, BAX activity may be affected by increased levels of BCL-2 and BCL-XL, which are BAX inhibitors (28, 29).

Although IP-PA1 conferred protection against the chemotherapeutic agent-induced inhibition of macrophage growth, it did not affect the cytotoxicity of chemotherapeutic agents in MCF-7 and A549 cells (Table I). Since IP-PA1 is an LPS, it may activate the macrophage-like RAW264 and J774.1 cells *via* TLR-4 (23). MCF-7 cells are not protected by IP-PA1 because they lack functional TLR-4 on their surface (34). On the other hand, A549 cells express TLR-4 on their surface and partly respond to LPS (35). However, NF-κB expressed in A549 cells is already hyperactivated; this may be why these cells were not or only negligibly affected by IP-PA1. Therefore, the responsiveness of cancer cells to IP-PA1 should be carefully monitored for the clinical application of IP-PA1.

IP-PA1 protected RAW264 and J774.1 cells from doxorubicin-, cisplatin-, 5-FU-, and methotrexate-mediated growth inhibition (Figure 3A-H, Table I). The protective effects of 100 ng/ml IP-PA1 on growth inhibition induced by 5 μM doxorubicin were not clearly visible in J774.1 cells (Figure 3B), although this dose of IP-PA1 clearly prevented caspase-3 activation (Figure 2B); this suggests that doxorubicin exerts its cytotoxic effects not only by inducing apoptosis, but also through other mechanisms that are not necessarily NF-κB dependent.

IP-PA1, which is derived from symbiotic gram-negative bacteria found in crops, is considered safe because it has long been ingested by humans and animals (17). In addition, it has not elicited any adverse reaction in toxicity tests (15, 36). In this study, we assessed the protective effects of IP-PA1 on the chemotherapeutic agent-induced inhibition of growth in murine macrophage-like cells (Figure 3A-H, Table 1). The cells that were affected by IP-PA1 produced NO (Figure 1C) and TNF-α (Figure 1D), the most important mediators in the killing of tumour cells (37, 38). IP-PA1 is reported to enhance the cytotoxic activity of alveolar macrophages against lung carcinoma cells *in vitro* (39). Moreover, oral administration of IP-PA1 enhances immune response (15, 16, 18). IP-PA1 is an edible drug that can potentially support chemotherapy.

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