Experimental Photodynamic Therapy for Liver Cancer Cell-Implanted Nude Mice by an Indole-3-acetic Acid and Intense Pulsed Light Combination

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Recently, indole-3-acetic acid (IAA) has been introduced as a new cancer therapeutic agent through oxidative decarboxylation by horseradish peroxidase (HRP). The purpose of this study was to determine the therapeutic feasibility of IAA/light combination against liver cancer. SK-HEP-1 cells were irradiated with UVB or visible light (518 nm) in the presence of IAA. Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Then, IAA was injected in SK-HEP-1 liver cancer cell-implanted nude mice, and the tumor area was irradiated with intense pulsed light (IPL). Then, tissue was taken for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and immunohistochemical staining for 8-hydroxy-deoxyguanosine (8-OHdG), p53, caspase-3, and proliferating cell nuclear antigen (PCNA). *In vitro* experiments demonstrated that IAA alone was not cytotoxic, but activated IAA by HRP or light caused cell death. *In vivo* experiments showed that IAA/IPL treatment caused regression of tumor cells in SK-HEP-1-implanted nude mice. The TUNEL assay showed that IAA/IPL induced cancer cell apoptosis, and this was confirmed by increases in 8-OHdG, p53, and caspase-3 in IAA/IPL-treated mice. In contrast, IPL alone did not induce apoptosis, indicating that the apoptotic effect resulted from activated IAA by light. In summary, we showed that IAA/light induced tumor regression in SK-HEP-1-implanted nude mice. These results suggest the potential use of IAA/light combination in liver cancer.

Key words indole-3-acetic acid; intense pulsed light; horseradish peroxidase; apoptosis; hepatoma

Liver cancer is one of the most common malignant tumors. And surgical removal and chemotherapy has been the mainstay of liver cancer treatment. However, conventional treatments have not improved the survival rate during the past two decades. Thus, the development of new therapeutic modalities is desirable.

Indole-3-acetic acid (IAA) is a member of the group of phytohormones called auxins.[1)] Although IAA alone has little effect on cancer cells, IAA has emerged as a new cancer therapeutic agent because IAA is activated through oxidative decarboxylation by plant enzyme horseradish peroxidase (HRP) and becomes cytotoxic.[2,3)] In a recent report, we have shown that an IAA/HRP combination induces apoptosis in G361 human melanoma cells.[4)] In addition, IAA/HRP produces several kind of free radicals, including indolyl, skatolyl, and peroxyl radicals, and these radicals lead to cell death.[5,6)] Furthermore, it has been suggested that IAA is a useful enhancing agent for photodynamic therapy (PDT) using photosensitizing dyes.[7)] Recently, we reported that IAA can be activated not only by HRP but also by UV light. In addition, we showed that UV-activated IAA induces tumor cell apoptosis.[8)] Thus, we hypothesized that IAA can be developed as a new photosensitizer for PDT.

PDT uses a photosensitizing drug activated by light. Usually, PDT causes tumor cell death *via* the generation of reactive, excited oxygen molecules.[9)] For *in vivo* study, we used intense pulsed light (IPL) as a light source for PDT. IPL has a spectrum of different wavelengths (515—1200 nm).[10)]

In this study, we have investigated the effects of IAA/HRP or visible light using the human liver cancer cell line, SK-HEP-1. Moreover, we examined the effects of an IAA/IPL combination on tumor formation in nude mice implanted with SK-HEP-1 cells.

MATERIALS AND METHODS

Materials Indole-3-acetic acid, horseradish peroxidase, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). Ketamine hydrochloride was purchased from Yuhan Pharma Co., Inc. (Seoul, Korea). Antibody against 8-hydroxy-deoxyguanosine (8-OHdG) was purchased from Chemicon (Temecula, CA, U.S.A.); p53 (DO-1, sc-126) and caspase-3 (sc-7272) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), and proliferating cell nuclear antigen (PCNA) antibody was from DAKO (Glostrup, Denmark).

Cell Culture The SK-HEP-1 cell line was obtained from the Korean Cell Line Bank (Seoul, Korea). SK-HEP-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.), 50 μg/ml streptomycin, and 50 μg/ml penicillin at 37 °C in 5% CO_2.

UVB and Visible Light Irradiation Cells were irradiated with UVB (BLE-1T158; Spectronics Corp., Westbury, NY, U.S.A.). A Kodacel filter (TA401/407; Kodak, Rochester, NY, U.S.A.) was used to remove wavelengths $<$290 nm (UVC), and energy was measured using a Waldmann UV meter (model No. 585100; Waldmann Co., VS-Schwenningen, Germany). Alternatively, cells were irradiated with visible light (HL-2000 Tungsten Halogen Light Sources; Micropack GmbH, Ostfildern, Germany) for 20 min. Various band-pass filters (Tholabs Inc., Newton, NJ, U.S.A.) were used to select specific wavelengths.

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Free Radical Determination Free radical formation was determined using 2,7-dichlorofluorescin diacetate (DCFH-DA), which is oxidized by free radicals to dichlorofluorescein (DCF).[11,12] To activate DCFH-DA, 350 μl of a 1 mM stock solution of DCFH-DA in ethanol was mixed with 1.75 ml of 0.01 N NaOH and allowed to stand for 20 min before adding 17.9 ml of 25 mM sodium phosphate buffer (pH 7.2). Then, activated-DCFH-DA was treated with either of four different conditions (Fig 1C, mock, light only, IAA only, light+IAA/ 380—640 nm, 12 J/cm^2). Absorbances were determined at room temperature at 490 nm.

MTT Assay for Cell Viability Cell viability was evaluated using the MTT reduction assay. Cells (4×10^4 cells/well) were seeded into 12-well plates. After serum starvation for 24 h, the cells were treated with IAA with HRP, or UVB or visible light (518 nm). After 24 h incubation at 37 °C in 5% CO_2, 100 μl of MTT (5 mg/ml) was added to each well, and incubation was continued for further 4 h. Supernatants were removed and formazan crystals were solubilized with dimethylsulfoxide. Absorbance was measured at 540 nm using an enzyme-linked immunosorbent assay (ELISA) reader (TECAN, Salzburg, Austria).

Animal Experiments Six-week-old male nude (*nu*/*nu*) BALB/c mice (Charles River Laboratories, Wilmington, MA, U.S.A.) of 18—20 g body weight were used for all experiments. All procedures were carried out in accordance with the Guiding Principles on the Care and Use of Animals of Chung-Ang University and Seoul National University. Anesthesia was performed by intraperitoneal (i.p.) injection with a mixture of 0.35 ml ketamine (50 mg/ml) and 0.15 ml xylazine (100 mg/ml). Anesthetized animals received an intradermal injection of SK-HEP-1 cells suspended in phosphate-buffered saline (pH 7.2). After each injection, a papule was observed at the injection site.

Experimental Photodynamic Therapy The light source for experimental PDT was an Ellipse I2PL system (wavelengths, 515—1200 nm; Danish Dermatologic Development A/S, Horsholm, Denmark). Using the I2PL system, light was irradiated at 30 min after the IAA injection (50 mg/kg i.p., 10 mg/ml in 50 mM $NaHCO_3$/2% v/v ethanol/water, pH 7). Irradiance was set at 20 J/cm^2. The tumor area was irradiated with IPL twice at an interval of 15 s. The size of the irradiated area was 3.5 cm^2. IPL-treated mice were kept in darkness until the next experiment.

Histology and Immunohistochemistry Tissue samples were fixed in 10% formalin for 24 h and processed for conventional paraffin embedding. For morphologic observation, sections were stained with hematoxylin–eosin (H&E) and used for immunohistochemical analysis. Five micrometer-thick paraffin-embedded sections were used to perform assays for 8-OHdG, p53, caspase-3, and PCNA. Slides were deparaffinized in xylene, and dehydrated in a graded ethanol concentration series. The sections were then treated with proteinase K (10 μg/ml) or citrate buffer with microwave treatment (pH 6.0, DAKO). The sections were also treated with 3% H_2O_2/PBS solution for 30 min at room temperature (RT), in order to halt any endogenous peroxidase activity. Then, the sections were blocked for 30 min with DAKO® protein block serum at RT, and incubated overnight at 4 °C with anti-8-OHdG polyclonal, anti-p53 monoclonal, anti-caspase-3, or anti-PCNA monoclonal antibodies, as indicated.

Staining was performed using the avidin–biotin–peroxidase-complex technique (DAKO), followed by diaminobenzidine (DAB) and AEC, which were used as the chromogen (DAKO), and counterstained with Harris hematoxylin (Sigma). The slides were then mounted with Permount solution. Histologic assessment of the specimens was conducted using an Olysia® Soft Imaging System (Olympus, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick end Labeling (TUNEL) Assay The *in situ* TUNEL cell death detection kit (Chemicon) was used according to the manufacturer's instructions. Briefly, paraffin sections were fixed with 10% formalin for 24 h and permeabilized in 0.1% Triton X-100. DNA breaks were labeled by incubation (60 min at 37 °C) with terminal deoxynucleotidyl transferase and then anti-digoxigenin peroxidase conjugate. Color developing was performed with peroxidase substrate, and the slides were counterstained with Harris hematoxylin (Sigma), then mounted with Permount solution. Apoptosis of specimens was examined using an Olysia® Soft Imaging System (Olympus).

Statistics Differences between results were assessed for significance using Student's *t*-test.

RESULTS

IAA/Light Combination-Induced Apoptosis of Liver Cancer Cells We investigated the effects of IAA/HRP on cell viability of the liver cancer cell line, SK-HEP-1. Cell viability was measured using the MTT assay 24 h after IAA/HRP treatment. In the presence of 1.2 μg/ml HRP, IAA caused cell death of SK-HEP-1 cells in a dose-dependent manner (Fig. 1A). Nearly all cells died after treatment with 1 mM IAA and 1.2 μg/ml HRP for 24 h, whereas IAA or HRP alone was not cytotoxic. In the next set of experiments, SK-HEP-1 cells were irradiated with UVB in the presence of IAA (0—2 mM). As already shown, IAA alone was not cytotoxic up to 1 mM, and UVB did not induce cell death at 100 mJ/cm^2. However, IAA/UVB (100 mJ/cm^2) decreased cell viability in a dose-dependent manner (Fig. 1B). We further tested whether IAA was activated by visible light and induced cancer cell death. To find the wavelength for IAA activation, we tested 6 different wavelengths (380—640 nm) and observed that IAA was activated by 475 and 518 nm (Fig. 1C). Because the 518 nm wavelength was more effective, we used 518 nm light for IAA activation and found that IAA/light (518 nm) combination decreased cell viability significantly (Fig. 1D).

IAA/IPL Decreased Tumor Cell Viability IPL is used for therapeutic purposes in dermatology and has a broad wavelength spectrum (515—1200 nm). Therefore, we next examined whether IAA/IPL has anti-tumor effects using liver cancer cell-implanted nude mice. SK-HEP-1 cells (1×10^6, 1×10^5, 5×10^4) were implanted in nude mice. Twenty-four hours after implantation, IAA was injected into nude mice (50 mg/kg i.p.), and IPL (20 J/cm^2) was irradiated 30 min later. After 4 d, the mice were sacrificed and the skin tissues were examined histologically. In the control group, we observed tumor formation in a cell number-dependent manner, whereas tumor cell density was decreased in the IAA/IPL-treated group (Fig. 2). IPL alone had no effect on the tumor

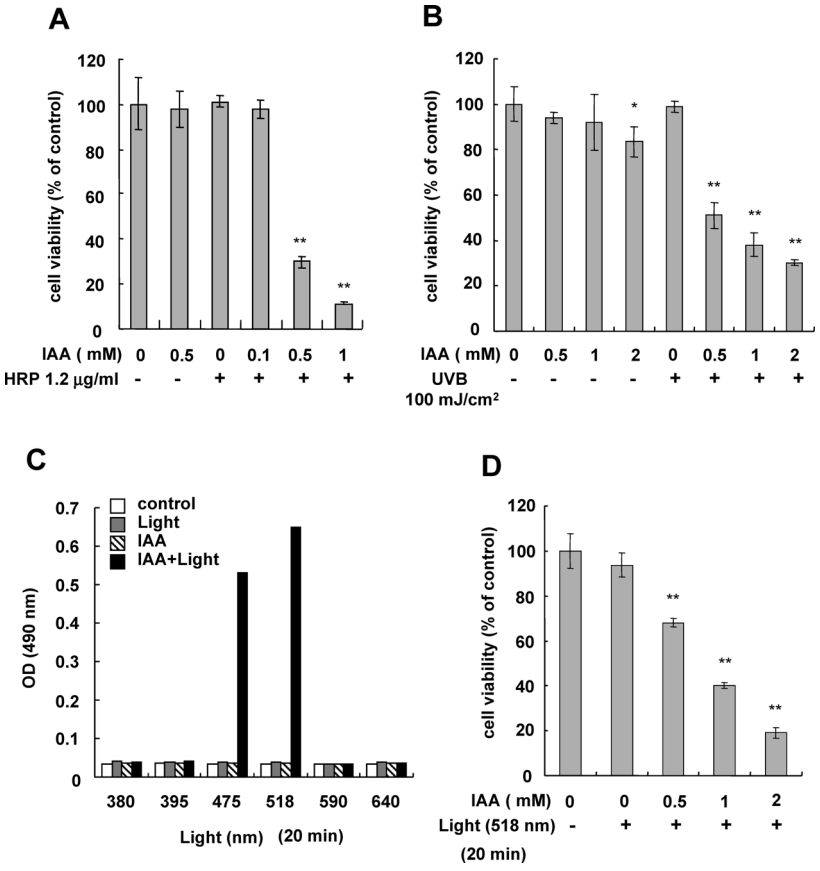

Fig. 1. Cytotoxic Effect of IAA/Light in SK-HEP-1 Cells

After serum starvation, (A) SK-HEP-1 cells were treated with varying concentrations (0.1—1 mM) of IAA in the absence and presence of HRP (1.2 μg/ml). After serum starvation, SK-HEP-1 cells were treated with varying concentrations (0.5—2 mM) of IAA and irradiated by (B) UVB (100 mJ/cm^2) or (D) visible light (518 nm, 12 J/cm^2). After 24 h, cell viability was measured by the MTT assay. Data represent the means±S.D. of triplicate assays expressed as percentages of the control. (C) IAA (1 mM) was irradiated by various wavelengths (380—640 nm). Free radical formation was measured using DCFH-DA, which is oxidized by free radicals to DCF, as described in Materials and Methods. Each experiment was repeated at least twice independently, and representative results are shown. $*p<0.05$, $**p<0.01$ compared to the untreated control.

size or cell density, indicating that IAA was activated by IPL.

IAA/IPL-Enhanced Tumor Apoptosis in Tumor-Bearing Mice We next investigated the cytotoxic effects of the IAA/IPL combination. SK-HEP-1 cells (1×10^6) were implanted into nude mice. Twenty-four hours after implantation, IAA (50 mg/kg i.p.) was injected into nude mice and IPL (20 J/cm^2) was irradiated 30 min later. After 4 d of implantation, the mice were sacrificed and H&E staining was performed. The IAA/IPL-treated groups showed a dramatically decreased tumor cell density (Fig. 3). To further investigate the effects of IAA/IPL, the TUNEL assay and immunohistochemical staining were performed (Fig. 3). The TUNEL assay indicated that IAA/IPL induced apoptotic cell death. In addition, we found that IAA/IPL induced chromatin condensation. To examine DNA damage caused by IAA/IPL, we observed the expression of 8-OHdG, which serves as a biomarker for oxidative damage in cellular DNA. Our data showed that IAA/IPL resulted in a dramatic increase in 8-OHdG expression. Moreover, we examined the tumor suppressor protein, p53, and a representative pro-apoptotic enzyme, caspase-3. IAA/IPL increased p53 and caspase-3 expression. In contrast, the PCNA positivity was dramatically decreased in the IAA/IPL-treated group.

Taken together, these data indicate that IAA/IPL treatment induced DNA damage, apoptosis, decreased tumor cell proliferation, and thus inhibited liver cancer cell growth.

DISCUSSION

Recent reports have suggested that an IAA/HRP combination can be used as a novel cancer therapeutic modality.[13,14] Furthermore, we have reported that UVB radiation can activate IAA and produce free radicals, suggesting that an IAA/UVB combination can provide a new PDT method for cancer.[8] However, adverse effects must be taken into consideration because UVB itself can cause cancer. Thus, visible light may have more merit than UVB. We tested whether IAA could be activated by visible light and induce cell death. As shown in Fig. 1C, we demonstrated that IAA was activated by visible light (475, 518 nm). Moreover, we showed that an IAA/light (518 nm) combination decreased cell viability in a liver cancer cell line.

Notably, the IAA and visible light combination yielded positive results in the DCF assay. DCF positivity suggests the formation of free radicals. Thus, it was remarkable that IAA could elicit DCF positivity by visible light because IAA is known to show no absorption at wavelengths >310 nm. Because DCFH-DA acted as a photosensitizer, we tested whether excitation of DCFH-DA was in fact induced. Therefore, as shown in Fig. 1C, we tested whether DCFH-DA could be activated only by visible light without IAA. However, excitation of DCFH-DA was not observed. Moreover, we used cell culture media without phenol red, since triple

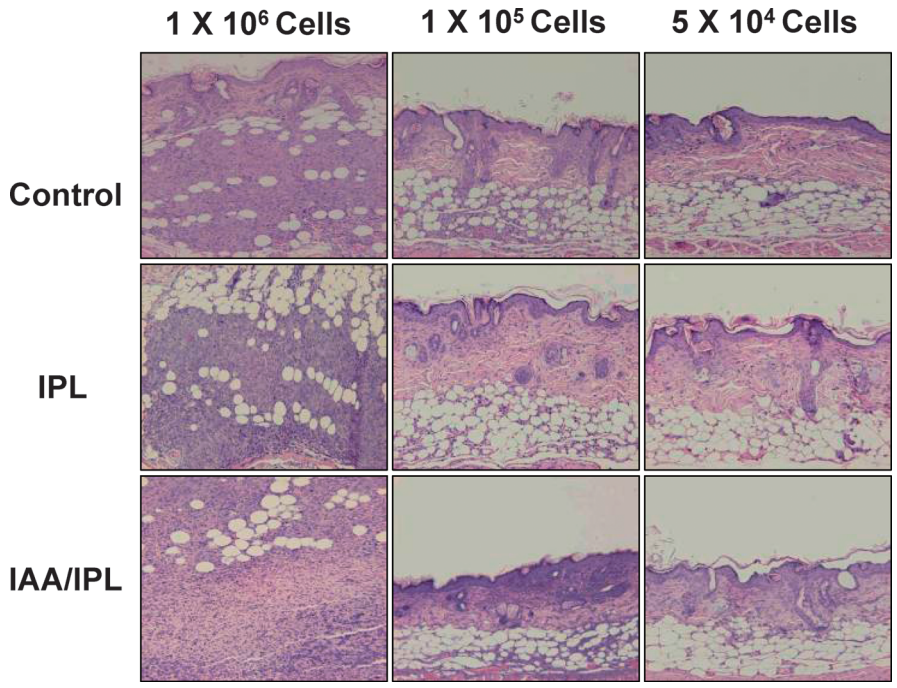

Fig. 2. Tumor Cell Viability by IAA/IPL Treatment

SK-HEP-1 cells were intradermally injected into nude mice (1×10^6, 1×10^5, or 5×10^4 cells). After 24 h, IAA was injected into nude mice (50 mg/kg i.p.) and IPL (20 J/cm^2) was irradiated 30 min later. After 4 d of implantation, the mice were sacrificed and tumor was fixed in 10% formalin. The tissue samples were prepared for H&E stain, as described in Materials and Methods. The specimens were photographed using an Olysia® Soft Imaging System (200×).

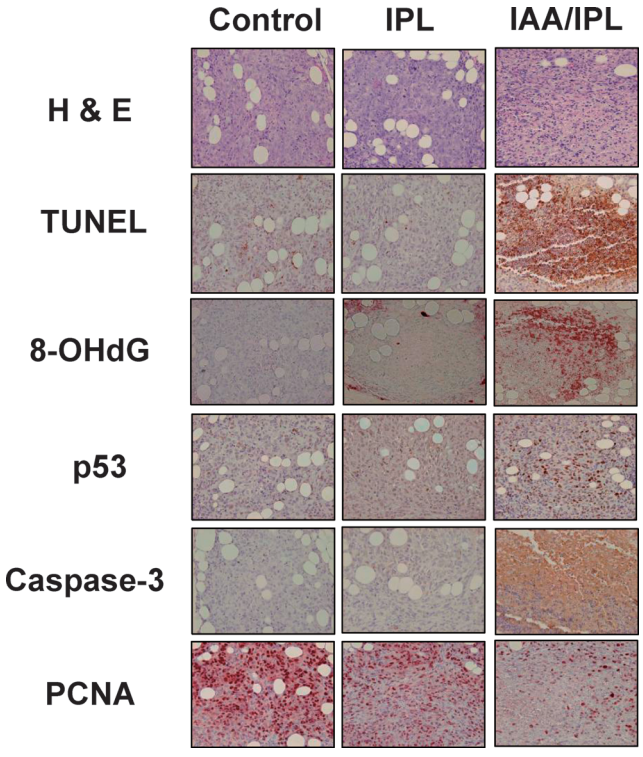

Fig. 3. Tumor Apoptosis by IAA/IPL Combination

SK-HEP-1 cells were intradermally injected to nude mice (1×10^6). After 24 h, IAA was injected into nude mice (50 mg/kg i.p.), and IPL (20 J/cm^2) was irradiated 30 min later. After 4 d of implantation, the mice were sacrificed and the injected area was fixed in 10% formalin, and then the TUNEL assay and immunohistochemical staining for 8-OHdG, p53, caspase-3, and PCNA were performed, as described in Materials and Methods. The specimens were photographed using an Olysia® Soft Imaging System (400×).

state phenol red in the cell culture media could oxidize IAA. As shown in Fig. 1D, media without IAA was not activated by light. Thus, it is unlikely that a component of the media was responsible for cell death after photoactivation.

Based on the *in vitro* results, we performed experimental PDT with a powerful and safe light source. Recently, IPL has been widely used as a useful alternative to laser systems in dermatologic treatment. IPL has a broad wavelength spectrum (515—1200 nm) and provides non-invasive treatment. Thus, in the present study we used IPL as a light source to activate IAA. Our results showed that i.p. injection of IAA and IPL irradiation induced tumor apoptosis in SK-HEP-1-implanted nude mice. Tumor apoptosis was also confirmed by histologic staining, which showed tumor cell regression, positive TUNEL staining, and increased expression of 8-OHdG, p53, and caspase-3. But, IPL alone did not induce apoptosis, indicating that the apoptotic effect resulted from IAA, which was activated by IPL. From these data, we suggest that IAA/light can be used in the treatment of liver cancer and is worthy of further development as a new cancer therapeutic method.

The activation of IAA by HRP is known to generate reactive, excited oxygen molecules, including ROS.[6,15,16] Moreover, other radicals, such as indolyl, skatolyl, and peroxyl radicals, may be involved in IAA/HRP-induced apoptosis.[17,18] We also propose that H_2O_2 is a major mediator of IAA/HRP-induced apoptotic cell death.[6] On the other hand, we recently reported that UVB-activated IAA induced apoptotic cell death of cancer cells.[8] In that study, we proposed that UVB-activated IAA may induce peroxyl radicals and/or the products of lipid peroxidation, which activate cell surface receptors and subsequently the death receptor-initiated apoptotic pathway. As shown in Fig. 1C, visible light-activated

IAA also generated free radicals, which induced SK-HEP-1 cell death. Thus, free radicals may play an important role in IAA/light-induced tumor apoptosis. However, to fully elucidate the mechanism of IAA/visible light-induced apoptosis, further studies are needed.

In the current study, 50 mg/kg of IAA was administered *via* i.p. injection. It is known that IAA and 5-hydroxy-IAA are catabolites of tryptophan in humans, and that these catabolites exist in human plasma with a range of 0.61—3.32 μmol/l and 33.0—102.6 μmol/l, respectively.[19] Thus, the clinical use of IAA (100 mg/kg *per os* [*p.o.*]) will be safe, but some reduction of blood glucose levels has been reported.[20] It has also been reported that doses of IAA (40—150 mg/kg) could be safely administered in humans.[21] In another animal study, IAA (50 mg/kg i.p.) was administered to mice and was found to be sufficient to reach the tumor concentrations of IAA three times those used in the *in vitro* experiments.[7] Thus, we used the same dose of IAA, and IAA alone showed no toxic effects. This finding will be a great advantage of IAA as a photosensitizer for PDT. Nonetheless, to use IAA as a new cancer therapy, further safety studies are required.

In conclusion, our histologic data showed that IAA/IPL treatment inhibited tumor formation in SK-HEP-1-implanted nude mice. Furthermore, IAA/IPL treatment induced tumor cell apoptosis, showing positive TUNEL staining, and increased expression of 8-OHdG, p53, and caspase-3. Because IAA alone is not toxic in humans, it can be used safely. Moreover, liver distribution of IAA could be increased, using drug targeting technology. Then, the targeted area can be irradiated with the appropriate light source. These findings raise the possibility of a potential new liver cancer therapeutic modality using IAA/light.

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