Original Article

Reactive oxygen species induced by indomethacin enhance accumulation of heme carrier protein 1 and hematoporphyrin accumulation *in vitro* and *in vivo* in a brain tumor model

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Photodynamic therapy (PDT) is useful for various cancers such as high-grade glioma and cancers of other organs. However, the mechanism of tumor-specific accumulation of porphyrin is not clear. The authors previously reported that heme carrier protein 1 (HCP1) contributes to the transport of porphyrins; specifically, we showed that the production of cancer-specific reactive oxygen species from mitochondria (mitROS) leads in turn to enhanced HCP1 expression. Indomethacin (IND), a non-steroidal anti-inflammatory drug, increases ROS production by affecting mitochondrial electron transfer system. In the present work, the authors investigated the effect of pretreatment with IND on cancer-specific porphyrin accumulation, using both a glioma cell line and a rat brain tumor model. This work demonstrated that exposure of a rat glioma cell to IND results in increased generation of cancer-specific mitROS and accumulation of HCP1 expression and porphyrin concentration. Additionally, systemic dosing of a brain tumor animal model with IND resulted in elevated cellular accumulation of porphyrin in tumor cell. This is an effect not seen with normal brain tissue. Thus, the administration of IND increases intracellular porphyrin concentrations in tumor cell without exerting harmful effects on normal brain tissue, and increased porphyrin concentration in tumor cell may lead to improved PDT effect.

***Key Words*: high-grade glioma, non-steroidal anti-inflammatory drug, photodynamic therapy, reactive oxygen species**

Glioblastoma has a poor prognosis: The median survival of patients with glioblastoma is 14.6 months.[1] The short survival reflects an elevated local recurrence rate due to high infiltration capacity and high treatment resistance.[2,3] Thus, additional methods that would inhibit tumor cellular infiltration and prevent local recurrence are strongly desired.

Photodynamic therapy (PDT) is a combined treatment using photosensitizers and low-powered laser beams. Hematoporphyrin derivatives (HpD) have been used as photosensitizers for neoplasms in various organs.[2,3] In recent years, PDT with talaporfin sodium has shown efficacy for local tumor control in treatment of high-grade glioma (HGG).[4] Since the laser beam intensity in neoplastic tissue gradually decreases with depth,[2,3] a novel method to strengthen this phenomenon is needed to increase the therapeutic PDT effect.

In previous work, we demonstrated [using in a rat gastric cell line (RGK1)] that heme carrier protein 1 (HCP1), originally detected as a heme transport protein, has a crucial role in tumor-specific fluorescence and PDT efficacy, a role mediated via intracellular uptake of HpD.[5] HCP1 expression is influenced by the concentration of cancer-specific reactive oxygen species derived from mitochondria (mitROS).[5,6] In separate work with gastric cancer cells, indomethacin (IND), a non-steroidal anti-inflammatory drug (NSAID), has been shown to enhance the PDT effect through increased mitROS generation and upregulation of HCP1 expression.[7] Other laboratories have reported that HCP1 is expressed in human glioma cell lines (T98G and U251) and surgical specimens of HGG.[8] We hypothesized that exposure to IND would intensify the effect of PDT in glioma cells. IND are commonly used and their properties are well recognized. This drug is known to cause gastrointestinal problems, but this side effect can be prevented with the use of medications such as proton pump inhibitors.[9,10] Therefore, the present study investigated whether IND enhances intracellular uptake of HpD in rat glioma cells.

Materials and Methods

Cell culture. The rat glioma cell line C6 was cultured in MEM (Sigma-Aldrich Japan K.K., Tokyo, Japan) containing 10% inactivated fetal bovine serum (FBS; Life Technologies Co., Tokyo, Japan) and 1% penicillin/streptomycin (Life Technologies Co.) at 37°C under 5% CO_2. This line also was used for brain tumor implantation in the animal model (see below).

Cell viability test. A cell viability test was performed using the Cell Counting Kit-8 (DOJINBO LABORATORIES, Kumamoto, Japan) according to the manufacturer's instructions. Cells were cultured overnight at 1×10^4 cells/well in a 96-well plate. The medium then was replaced with fresh medium containing 0.1, 0.2, 0.4, 0.5, 1, 2, or 5 mM IND [formulated in dimethyl sulfoxide (DMSO)]; DMSO in the medium was

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adjusted to a final concentration of 1% in all wells (and in all subsequent experiments using DMSO as the vehicle control). Following incubation for 6 h, the cells were washed two times with phosphate-buffered saline (PBS) and then resuspended in a medium containing 10% Cell Counting Kit-8 reagent. Absorbance at 450 nm was measured using a DTX880 multimode microplate reader (Beckman Coulter, Inc., Brea, CA).

Measurement of intracellular ROS by electron spin resonance. Intracellular ROS production was measured with electron spin resonance (ESR), as described previously.[11] Cells were seeded onto glass cover slides (49 mm × 5 mm × 0.2 mm) and cultured overnight. After incubation, the slides were cultured for 6 h in medium containing 0.2 mM IND. The IND concentrations in our experiments referred to previous reports using gastric mucosa cells.[7] Cells then were shifted to culture medium supplemented with respiratory buffer solution (5 mM succinate, 5 mM glutamate, and 5 mM malate), with 5 mM nicotinamide adenine dinucleotide (Sigma-Aldrich Japan K.K.), and with 10 mM 5,5-dimethyl 1-pyrroline-*N*-oxide (DOJINBO LABORATORIES). The ESR spectrum measurements were performed with a JEOL-TE X-band spectrometer (JEOL, Ltd., Tokyo, Japan) using the following settings: incident microwave power, 20 mW; frequency, 9.42 GHz; and field modulation amplitude, 0.1 mT.

HCP1 expression analysis using Western blotting. The Western blotting analysis was conducted as described previously.[11,12] Briefly, cells were seeded in three polystyrene cell culture dishes (Corning International K.K., Tokyo, Japan) at a density of 3×10^6 cells per plate. When the cells became adherent, the medium was replaced with a fresh medium, either neat, containing vehicle (DMSO), or containing 0.2 mM IND (formulated in DMSO). The cells were cultivated for 6 h and then washed twice with PBS; next, the medium was replaced with fresh standard base medium, and the cells were cultivated for another 24 h.

NuPAGE LDS Sample buffer mixed with Sample Reducing Agent (Life Technologies Co.) was used to prepare the cell lysate from each dish. Each lysate was heated at 70°C for 10 min. An aliquot of the lysate then was applied to a NuPAGE® NOVEX® 4–12% Bis-Tris gel, and the samples were electrophoresed at 120 V for 60 min. Proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Millipore Co., Billerica, MA) by electrophoresis at 4.0 A/cm^2 for 60 min. The membrane then was exposed to PVDF Blocking Reagent for Can Get Signal® and soaked overnight in Can Get Signal® Immunoreaction Enhancer Solution 1 with a 1:500 dilution of the primary antibody (anti-rabbit HCP1 antibody; Abcam, Cambridge, UK). After aspiration of the solution containing the primary antibody, the membrane was rinsed three times using Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T; obtained as TBST-10X, Cell Signaling Technology Japan, K.K., Tokyo, Japan) and exposed to a solution of horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) antibody (Cell Signaling Technology Japan, K.K.) mixed with Can Get Signal® Immunoreaction Enhancer Solution 2 for 3 h. Next, the membrane was soaked in Lumina Forte Western HRP substrate (Millipore Co.), and bands were detected using an LAS4000 (GE Health Care Japan, Tokyo, Japan). β-actin, which was as a control for protein loading, was detected with an anti-β-actin antibody (Cell Signaling Technology Japan, K.K.).

Quantification of cellular uptake of hematoporphyrin derivatives (HpD). Cells were seeded at the density of 2×10^5 cells per well in the 12-well plate. When the cells became adherent, the medium was replaced with a fresh medium neat, containing 1% DMSO, or containing 0.2 mM IND (formulated in DMSO) and incubated for another 6 h. Next, the cells then were rinsed two times using PBS, fresh medium was added, and the plates were incubated for another 24 h. The medium then was replaced with fresh medium containing 20 μM HpD (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the cells were incubated for another 6 h. The cells were lysed using 100 μl of RIPA buffer and homogenized.

Animal care. Animal experiments were performed in a humane manner with the approval of the University of Tsukuba Institutional Animal Experiment Committee, in accordance with the University's Animal Experiment Regulations and the Basic Guidelines for the Appropriate Conduct of Animal Experiments at Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

***In vivo* model of brain tumor.** The method of brain tumor model and timing of sacrifices were referred to a previous paper.[13] A rat brain tumor model was generated using six-week-old female Wistar rats (Charles River Japan, Yokohama, Japan). The animals were maintained at constant humidity and temperature with a 12-h/12-h light/dark cycle. Anesthesia in rats was induced and maintained by inhalation of isoflurane (Mylan Seiyaku, Ltd., Ibaraki, Japan) at a concentration of 3.5% with oxygen as a carrier gas, followed by intraperitoneal perfusion with pentobarbital (Kyoritsu Seiyaku Corporation, Osaka, Japan) at 45 mg/kg body weight. The rats were set in a Stereotactic Instrument for Rats (NARISHIGE Group, Tokyo, Japan). A 20-mm incision was placed in the rat's head to expose the right frontal bone, and a 2-mm burr hole was drilled with an electric drill at a position 4 mm to right of the sagittal suture, just behind the coronal suture. An aliquot of a suspension of C6 cells (1×10^5 cells in 5 μl PBS) was injected slowly into the brain at a depth of 5 mm from the dural surface through the burr hole by use of a 10-μl No. 701 RN Hamilton syringe with a micromanipulator (NARISHIGE Group). Immediately following withdrawal of the needle, the burr hole was filled with bone wax to prevent backflow of the cell suspension and leakage of the CSF. The incision then was closed with sutures.

Timing of drug administration referred to previous papers. It has been reported that daily IND induced gene regulation in the rat hippocampus, IND was administered on the days 13 and 14 to evaluate the effect of IND on HCP1 expression in the normal brain.[9] HPD was administered 3 h after the second IND dose to stabilize the intracellular concentration of IND.[14] Thirteen days after intracranial injection, the animals were divided into two groups (n = 8/group). One group was injected intraperitoneally at 2 ml/kg with a solution of 10 mg/ml IND plus 1% carboxymethyl cellulose (CMC) in normal saline (for an effective IND dose of 20 mg/kg); Dosing was repeated once daily for a total of two days. The other group was injected intraperitoneally with an equivalent volume of PBS on the same schedule. At 3 h after the second IND injection (i.e., 14 days after implantation), all animals were dosed intravenously with HPD at 10 mg/kg of body weight, administered as a 4-mg/ml solution of HPD dissolved in saline. Six hours after the HpD injection, the rats were euthanized and brains were recovered. The brain tumor tissue and the brain tissue contralateral to the tumor were excised on ice and then flash-frozen on dry ice.

Measurement of HCP1 levels by Western blotting. The samples were homogenized in cold RIPA buffer using an ultrasonic homogenizer, and then subjected to centrifugation (20,000 × g, 20 min, 4°C). The soluble protein fraction was mixed with NuPAGE LDS sample buffer adjusted to 5% 2-mercaptoethanol and boiled for 10 min. HCP1 expression analysis was performed by Western blotting as described above.

Fluorescence intensity (FI) measurement of HpD using *in vitro* and *vivo* samples. For the *in vitro* study, the FI measurement of HpD was performed by transferring cell lysates to a 96-well plate, which then was placed in a Varioskan microplate reader (Thermo Fisher Scientific K.K., Tokyo, Japan). FI was measured at excitation and emission wavelengths of 415 and 625 nm, respectively.

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For the *in vivo* study, tissue samples were homogenized with DMSO, and the resulting supernatants were transferred to a 96-well plate. The Relative Fluorescence Intensity (RFI) was measured in arbitrary units (a.u.) using the Varioskan microplate reader and normalized by the weight of the respective sample. FI was measured at excitation and emission wavelengths of 415 and 625 nm, respectively. The contralateral brain tissue was used as a control in each case.

Statistical analysis. All statistical analyses were carried out using JMP software, ver. 13 (SAS Institute, Cary, NC). All data are presented as mean ± SD. Comparisons of two data sets were performed by two-tailed non-paired Student's *t* test or Wilcoxon signed-rank test. Comparison of three or more data sets was performed by two-tailed One-way Analysis of Variance (ANOVA) with post hoc Tukey–Kramer test, if appropriate. Statistical significance was set at $p<0.05$.

Results

Cytotoxicity of IND. To evaluate the cytotoxicity of IND against C6 glioma cells, a Water-Soluble Tetrazolium (WST) assay was performed. Exposure of cells to IND for 6 h resulted in a dose-dependent decrease in cell viability (Fig. 1). For instance, a 6-h exposure to 1 mM IND induced approximately 40% cell death.

Notably, exposure to IND at 2 mM or higher caused severe cytotoxicity, and almost all cells were killed at an IND concentration of 5 mM. In the following *in vitro* studies, 0.2 mM IND was used as the pretreatment IND concentration, as no severe damage was observed following exposure to IND at this low concentration.

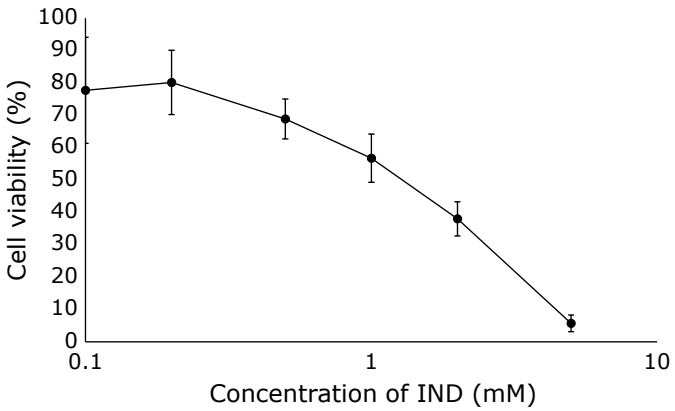

Fig. 1. Cytotoxicity of indomethacin (IND) was evaluated using a WST assay. Cells were incubated in culture medium containing IND at various concentration for 6 h. The absorbance at 450 nm was measured and viability was cultured against cells treated with medium containing 1% DMSO. $n = 6$, plot: mean, error bar: SD.

Change of ESR spectra following IND treatment. After exposure to 0.2 mM IND, the amount of ROS in the cells was detected by the ESR method. The ESR signal intensity increased approximately two-fold after IND treatment compared to that in non-treated cells (Fig. 2). These results indicated that ROS generation in C6 glioma cells is increased by exposure to 0.2 mM IND.

HCP1 protein expression in cells. The expression level of HCP1 was compared using cells treated with medium, medium containing 1% DMSO, and medium containing 0.2 mM IND. As shown in Fig. 3, cells treated with medium, medium containing 1% DMSO, and medium containing 0.2 mM IND resulted in HCP1 concentration of 1.15 ± 0.11, 1.26 ± 0.24, and 1.59 ± 0.32 (mean ± SD from 3 biological replicates: values were normalized to that of β-actin). HCP1 accumulated 1.4-fold in C6 cells following exposure to IND. On the other hand, there was no significant difference in HCP1 level between cells grown in medium with and without 1% DMSO. These results indicated that HCP1 accumulation in C6 cells is promoted by IND exposure.

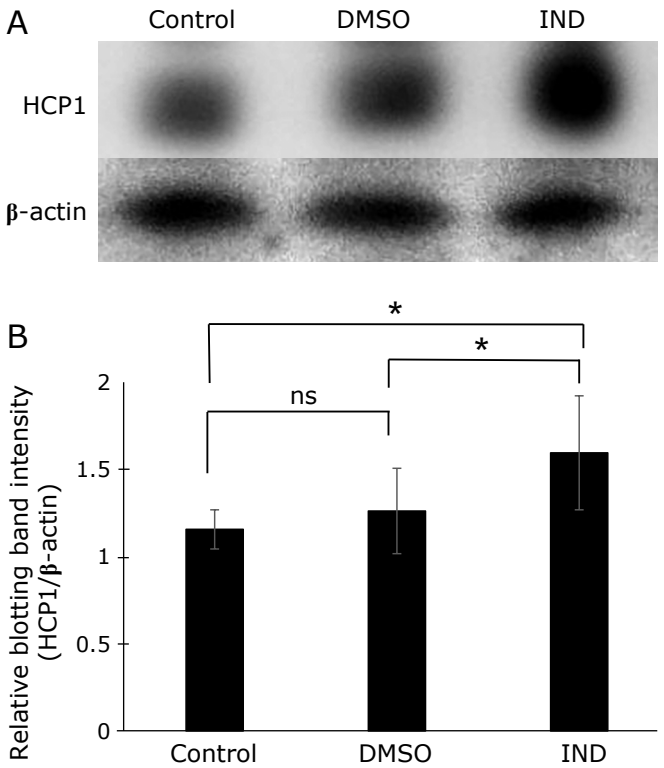

Fig. 3. Western blotting analysis of HCP-1 expression in each cell. HCP-1 protein bands of each cell (A), and expression levels were represented by use of graph (B). HCP-1 expression greater in cells exposed IND. Bar plot (mean), error bar: SD; ns: not significant, $*p<0.05$.

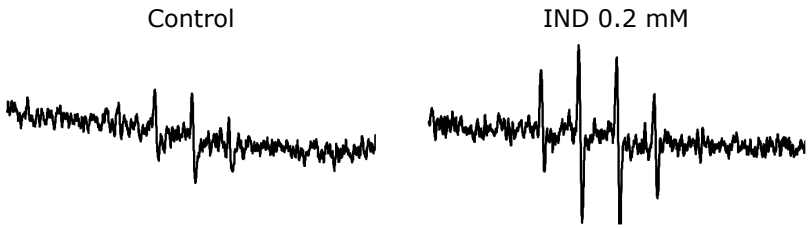

Fig. 2. Electron spin resonance spectra of respective living cells after incubation with 0.2 mM IND for 6 h were measured. Reactive oxygen species (ROS) generated by cells were trapped using 5,5-dymethyl-1-pyrroline 1-oxide (DMPO), and the signals of DMPO-adducts were detected.

Cellular uptake of HpD. The amount of HpD in IND-treated cells was compared with that in vehicle (DMSO)-treated cells. As shown in Fig. 4, HpD FI in C6 cells increased significantly following IND exposure (median FI, MEM:DMSO:IND = 54.6:74.1:85.8). These results showed that the intracellular uptake of HpD in C6 cells was enhanced by IND exposure.

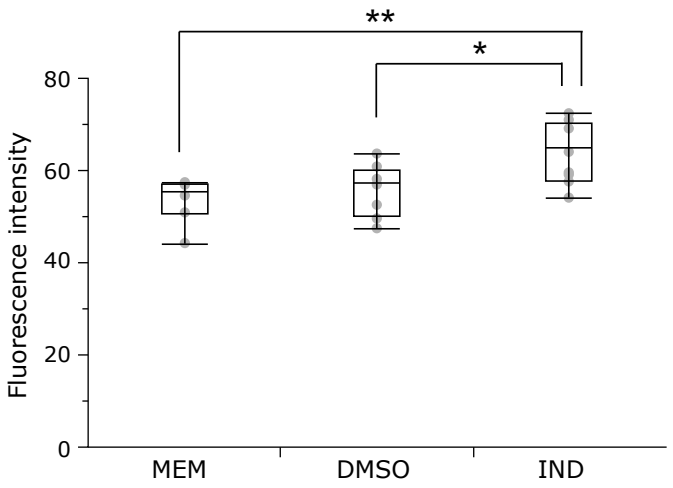

Fig. 4. The amount of hematoporphyrin derivative (HpD) incorporated in cells after treatment with 0.2 mM IND or 1% DMSO were measured by detecting the fluorescence intensity at EX. 415 nm and Em. 625 nm. $n = 8$, circles represent raw data, error bar: SD; center line, median; bottom of box, lower quartile (25%); top of box, upper quartile (75%). *p<0.05, **p<0.01.

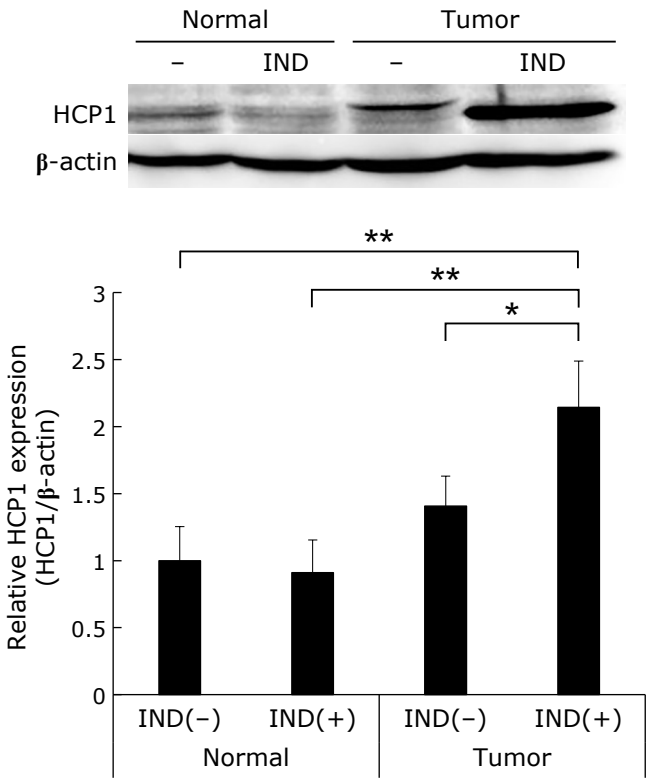

Fig. 5. Western blotting analysis of HCP-1 expression in brain and tumor with IND and without IND. HCP-1 protein bands, and expression levels were represented by use of graph. HCP-1 expression greater in tumors exposed IND. Bar plot (mean), error bar: SD; *p<0.05, **p<0.01.

HCP1 expression after IND treatment *in vivo*. We examined the expression of HCP1 in brain tumors after IND treatment using an *in vivo* rat brain tumor model. Figure 5 shows the expression of HCP1 in brain tumor and contralateral normal brain tissues. Normalized HCP1 concentrations in normal brain tissue following systemic dosing with vehicle [IND(−) and IND(+)] were 1.00 ± 0.25 and 0.93 ± 0.21. Normalized HCP1 concentration in tumor tissue with IND(−) and IND(+) were 1.47 ± 0.13 and 21.6 ± 0.36, respectively. HCP1 protein levels were significantly elevated in tumor tissues in animals administered IND compared to tumor tissues in control animals. On the other hand, there was no significant difference in HCP1 levels between the contralateral normal brain tissue from IND-dosed and control animals. These results indicated that dosing with IND promotes the accumulation of HCP1 in brain tumors, but not in normal tissues, in an *in vivo* model.

HpD accumulation after IND treatment *in vivo*. HpD accumulation in brain tumors following systemic IND treatment was investigated using a rat brain tumor model. Figure 6 shows a comparison of HpD FI in brain tumor and contralateral normal brain tissue.

FI was elevated significantly in tumor tissue from animals dosed with IND compared to tumor tissue from control animals (median FI, IND:control = 8.41:10.4). However, no significant difference was observed in the FI of contralateral normal brain tissue from mice administered IND compared to contralateral brain tissue from control animals (median FI, IND:control = 2.11:2.07). These results indicated that dosing with IND promotes HpD accumulation in tumors, but not in normal tissue, in an *in vivo* model.

Discussion

This study demonstrated that in rat brain tumor cells, both *in vitro* and *in vivo*, IND exposure caused increased ROS production and HCP1 expression, resulting in enhanced HpD accumulation. There are no reports evaluating HCP1 expression and porphyrin uptake after IND administration in a rat brain tumor model or evaluating the potentiation of PDT effects by NSAIDs in rat brain tumor cells.

Some authors have reported that ROS have an essential role in porphyrin transport.[5,15] Verschoor *et al.*[15] described tumor cells that overproduce ROS as a result of mitochondrial dysfunction, reflecting the presence of a mutation in a gene encoding a component of complex I and III of the electron transport chain. In such cells, IND causes uncoupling in the electron transport system, leading in turn to overproduction of mitROS in gastric cancer cells.[12,16] Cancer cells readily generate ROS at levels higher than those seen in normal cells, resulting in the generation of additional oncogenic mutations, and leading in turn to increased metabolism while facilitating adaptation to hypoxia.[17] Glioma cells have been shown to accumulate large gene mutation loads, not only in the nucleus but also in the mitochondria, again permitting the accumulation of additional oncogenic characteristics.[18,19] In the present study, exposure to IND resulted in a two-fold increase in the ESR signal even in the highly malignant rat glioma cell line C6, which already is known to produce elevated amounts of ROS.[7]

We previously reported that mitROS regulates the expression of HCP1, a transmembrane protein that transports heme and folate.[5,7] HCP1 is likely to be responsible for the transport of HpD in gastric cancer cells and also has been shown to be expressed in human cancer cells, including HeLa cells.[20] Among brain tumors, HGG cells have been reported to express HCP1, while normal cells in surgical specimens appear not to express this protein. Moreover, the expression of HCP1 has been shown to correlate strongly with the World Health Organization (WHO) grade of gliomas.[8] Tumor-specific HpD fluorescence also has

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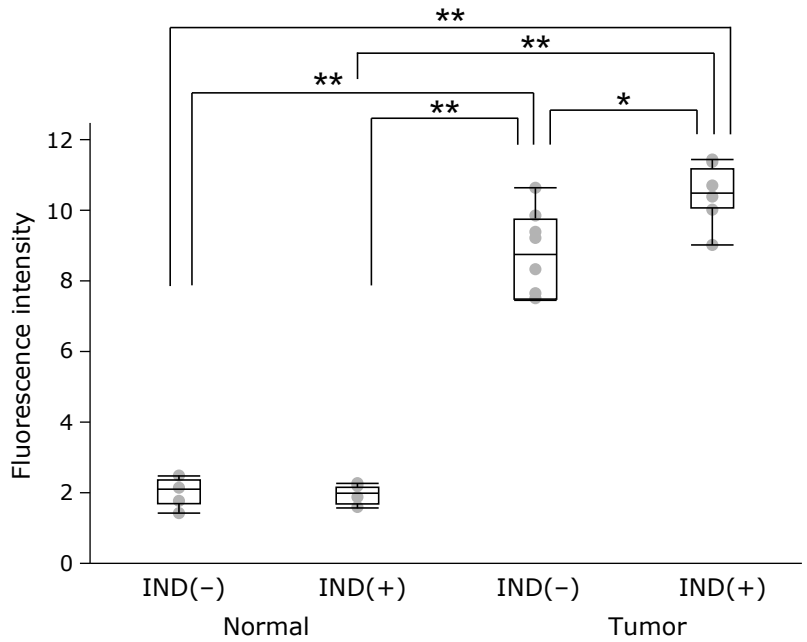

Fig. 6. The amount of hematoporphyrin derivative (HpD) incorporated in brain and tumor with IND and without IND by detecting the fluorescence intensity at EX. 415 nm and Em. 625 nm. $n = 8$, circles represent raw data, error bar: SD; center line, median; bottom of box, lower quartile (25%); top of box, upper quartile (75%). *p<0.05, **p<0.01.

been shown to be elevated in proportion to malignancy. In the present study, HCP1 expression in glioma cells was enhanced by IND exposure (Fig. 2 and 3). These phenomena indicated that ROS and HCP1 play a crucial role in porphyrin transport. Although increased HCP1 expression with IND administration may exacerbate tumor grade, this is not a crucial problem because tumor cells are killed by resection and PDT.

Previous research has shown that HCP1 is overexpressed as a result of stabilization of hypoxia inducible factor-1 (HIF-1).[21–23] ROS stabilize HIF-1a by dysfunction of prolyl hydroxylases due to ROS oxidation of Fe^{2+} to Fe^{3+}. Stabilization of HIF-1a stimulates the transcription of selected genes and the production of a few specific proteins, including HCP1.[24] Therefore, we hypothesize that mitROS generation by IND stabilizes HIF-1, leading to increased HCP1 expression and subsequent enhancement of tumor-specific accumulation of porphyrins.

As shown by the *in vivo* data presented in Fig. 6, the expression of HCP1 and the FI of contralateral brain tissue after administration of HpD were essentially unchanged when comparing tissues from mice dosed with or without IND. Mannila *et al.*[25] reported that IND has low permeability into the rat brain, but also suggested that the concomitant use of probenecid slightly increased the initial uptake of IND in the brain by increasing IND blood levels. Sathyanesan *et al.*[9] reported that administration of IND affects gene regulation in the rat hippocampus, indicating that IND may affect the normal brain. We infer that the blood-brain barrier typically inhibits IND accumulation in normal brain tissue, where IND may not affect the metabolism and transport of porphyrins.

In contrast, the present study demonstrated that IND enhances HCP1 expression and HpD accumulation in brain tumors, structures for which the blood-brain barrier may be compromised. PDT can treat neoplastic tissue within one centimeter depth of the exposed tissue, although tumor cells often broadly infiltrate the surrounding brain tissue, typically leading to tumor recurrence occurs within a 2-cm periphery of the surgical cavity. For complete remission, a deeper treatment effect is desired. Several researchers have reported that longer wavelengths of light can improve the effect of PDT in deeper areas of neoplastic tissue. However, the wavelength of the excitation light is merely a factor for PDT. Cheung *et al.*[26] demonstrated that the FI of the photosensitizer correlates with the depth of necrosis induced by PDT. Dereski *et al.*[27] showed that the depth of a PDT effect correlates with energy dose, which affects the FI. We propose that the Fl intensity, which depends on both the photosensitizer concentration and the excitation light dose, is the most important factor for the PDT effect. We conjecture that the pre-administration of IND may enhance the tumor-specific accumulation of porphyrins, facilitating the delivery of the PDT to even deeper areas.

A clinical study of the treatment of HGG tested the safety and efficacy of intraoperative PDT with talaporfin sodium,[4] a treatment that is covered by insurance in Japan. However, the detailed mechanism of cancer-specific accumulation of talaporfin sodium remains unclear. Given that HpD and talaporfin sodium have similar structural formulae, HCP1 may contribute to the intracellular accumulation of talaporfin sodium; we therefore propose that the administration of IND may enhance the PDT effect.

One limitation of our study is that the optimal blood concentration of IND is not clear, given that this parameter has not been determined. *In vitro* study, the concentration of IND was determined with reference to past experiments using gastric mucosal cells.[7] In the animal study described here, IND was administered at 20 mg/kg once daily for two consecutive days, a dose that is considered to be the limiting concentration at which the occurrence of gastric ulcers (in response to IND) can be prevented by gastric mucosal protective agents.[28] Although the accumulation of HCP1 and HpD was obtained at lower concentrations than in the *in vitro* study, the dose used in our *in vivo* study was much higher than the level that would be used clinically, leaving room to consider the dosage of IND.

In conclusion, we demonstrated that exposure to IND increases ROS and induces accumulation of HCP1 *in vitro*. We also showed that dosing with IND enhances the cancer-specific accumulation of porphyrin in a brain tumor rat model. Pretreatment with IND may be a useful method to enhance the efficacy of PDT.

Author Contributions

All authors contributed to the study. Advice for material preparation and data collection was provided HI and HK. The second draft was modified by HM and EI. All authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Conflict of Interest

EI received scholarship funds from Eisai Co., Ltd.

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