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FAST TRACK

ENHANCEMENT OF CHEMOTHERAPEUTIC RESPONSE OF TUMOR CELLS BY A HEME OXYGENASE INHIBITOR, PEGYLATED ZINC PROTOPORPHYRIN

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Heme oxygenase-1 (HO-1), an inducible enzyme that catalyzes oxidative degradation of heme to form biliverdin, carbon monoxide and free iron, may protect tumor cells against oxidative stress, thus contributing to rapid tumor growth *in vivo*. Here, we discuss whether pegylated zinc protoporphyrin (PEG-ZnPP), a potent HO inhibitor, modulates the chemotherapeutic response of tumor cells to treatment that generates reactive oxygen species (ROS). PEG-ZnPP is a water-soluble HO inhibitor that accumulates in tumor tissues after intravenous administration. Cytotoxicity of antitumor agents *in vitro* was determined by means of MTT and annexin V assays using human colon carcinoma SW480 cells. Mice bearing sarcoma 180 tumors were used as an *in vivo* model. Pegylated D-amino acid oxidase (PEG-DAO), which behaves as an oxidative chemotherapeutic agent by generating toxic oxidants at tumor tissues, was administered with its substrate D-proline to mice with or without PEG-ZnPP pretreatment. PEG-ZnPP-treated SW480 cells became vulnerable to insults caused by various cytotoxic agents; the 50% lethal doses were reduced by 25%, 39%, 83%, and 61% for hydrogen peroxide, *t*-butyl hydroperoxide, camptothecin and doxorubicin, respectively. Cells treated with PEG-ZnPP plus cytotoxic oxidants exhibited marked production of intracellular ROS, which paralleled the incidence of apoptosis. PEG-ZnPP pretreatment significantly reduced tumor growth in mice receiving PEG-DAO/D-proline compared to no PEG-ZnPP pretreatment. These findings suggest that HO-1 may become an attractive target for chemotherapeutic intervention. Further study of the effect of PEG-ZnPP plus conventional anticancer drugs that generate ROS, such as cisplatin, camptothecin, doxorubicin, mitomycin C and etoposide, is warranted.
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Key words: *D-amino acid oxidase; zinc protoporphyrin; heme oxygenase-1; macromolecular therapeutics; tumor targeting/EPR effect*

Heme oxygenase (HO) catalyzes the initial and rate-limiting step of heme degradation in which oxidative cleavage of the porphyrin ring leads to formation of biliverdin, carbon monoxide (CO) and free iron.[1,2] Biliverdin is subsequently reduced by cytosolic biliverdin reductase to form the potent antioxidant bilirubin.[3] Three isoforms have been identified for mammalian HO: HO-1, HO-2 and HO-3.[1,2,4] Under physiological conditions, HO-1, an inducible isoform, is highly expressed in liver and spleen, whereas the constitutive HO-2 is expressed mainly in brain and testis. HO-3 is a recently identified isoform whose mRNA has been detected in many organs including spleen, liver, thymus, prostate, heart, kidney, brain and testis.[4] However, the physiological function of HO-3 remains unclear, compared to that of other 2 isoforms, because of its very low enzymatic activity.[4]

HO-1 is a member (HSP-32) of the heat shock protein family, and its expression is triggered by diverse stress-inducing stimuli including hypoxia,[5] heavy metals,[6] UV irradiation,[7] reactive oxygen species (ROS) and reactive nitrogen species such as hydrogen peroxide (H_2O_2)[7] and nitric oxide (NO).[8,9] It is believed that induction of HO-1 protects cells from these toxic stimuli by multiple mechanisms: a) decreasing the prooxidant level (heme);[10] b) increasing the antioxidant level (bilirubin);[3] c) producing the antiapoptotic molecule CO;[11] d) inducing ferritin, which removes and detoxifies free ferric ion;[12] and e) preventing overstimulation of the immune response.[13] Indeed, inhibition of HO-1 by using specific HO inhibitors such as zinc protoporphyrin (ZnPP) or tin protoporphyrin extended the pathological consequences of disorders involving these stress-inducing stimuli: graft rejection,[14] ischemia-reperfusion injury,[15] cisplatin nephrotoxicity[16] and endotoxin-induced septic shock.[13] In contrast, HO-1 inducers such as cobalt protoporphyrin had beneficial effects on certain diseases.[15,17]

NO is now known to be a potent inducer of HO-1.[8,9,18] We previously investigated the HO-1 expression in solid tumor tissues of rat hepatoma AH136B and mouse sarcoma S-180, and found extensive expression of inducible NO synthase (iNOS). [19,20] More recently, we found markedly increased HO-1 activity in these tumor tissues[18,21–23] compared to HO-1 activity in spleen. Of prime importance in these studies is that pharmacological inhibition of intratumoral HO activity by using ZnPP or its pegylated derivative (PEG-ZnPP) significantly reduced growth of these tumors *in vivo*.[18,23] These findings suggest that HO-1 expressed in tumors confers cytoprotection to the tumor cells, thus permitting rapid growth *in vivo.* This cytoprotective action may be relevant for human cancers because increased expression of HO-1 has also been observed in prostate tumors,[24] brain tumors,[25] oral squamous cell carcinomas[26] and renal cell carcinomas.[27]

Lin and Girotti[28] observed that leukemia cells exposed to heme (an HO-1 inducer) for a long period could acquire hyperresistance to merocyanine 540, an antitumor photosensitizer, through expression of HO-1. In addition, HO-1 was recently found to protect cells

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CO, carbon monoxide; Cr, creatinine; DAO, D-amino acid oxidase; DCDHF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; EPR effect, enhanced permeability and retention effect; H_2O_2, hydrogen peroxide; Hb, hemoglobin; HO-1, heme oxygenase-1; iNOS, inducible NO synthase; LD_{50}, 50% lethal dose; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; PBS, 0.01 M phosphate-buffered 0.15 M saline; PEG, polyethylene glycol; PEG-DAO, pegylated DAO; PEG-ZnPP, pegylated ZnPP; ROS, reactive oxygen species; *t*-BuOOH, *t*-butyl hydroperoxide; RBC, red blood cells; TUNEL, terminal deoxynucleotide transferase-mediated dUTP-biotin nick end-labeling; WBC, white blood cells; ZnPP, zinc protoporphyrin.

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from apoptosis induced by doxorubicin[29] and cisplatin,[16] which are widely used anticancer agents. A characteristic that these agents have in common is that they cause oxidative injury of target cells. It is conceivable that tumor cells with up-regulated expression of HO-1 will be resistant to anticancer agents that induce oxidative injury. Therefore, disruption of this HO-1 antioxidative system by using an HO inhibitor would seem to be a logical approach to enhancing the therapeutic potential of anticancer agents. However, such an effect of an HO inhibitor in combination therapy with anticancer agents remains to be elucidated.

In our study, we investigated the effect of the HO inhibitor ZnPP on the chemotherapeutic response of tumor cells both *in vitro* and *in vivo*. To explore the effect of HO inhibition *in vivo*, we used PEG-ZnPP, a newly developed water-soluble macromolecular derivative of ZnPP.[22] Very recently, we reported that PEG-ZnPP given by intravenous injection to tumor-bearing mice could provide tumor-specific inhibition of HO activity after.[22,23] In addition, we used pegylated D-amino acid oxidase (PEG-DAO) along with its substrate D-proline in the *in vivo* experiments as an oxidative chemotherapeutic agent because of its ROS-generating and tumor-targeting characteristics.[30] Another rationale for this treatment is that amounts of various antioxidative enzymes in normal cells such as catalase, glutathione peroxidas and superoxide dismutase were known to be greatly reduced in various tumor cells,[31–34] which should increase the vulnerability of the tumor cells to ROS, and thus may result in a synergetic effect of PEG-ZnPP and PEG-DAO.

MATERIAL AND METHODS

Material

PEG-DAO and PEG-ZnPP were prepared and characterized as described previously.[22,30] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorecin diacetate (DCDHF-DA) were obtained from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes (Eugene, OR), respectively. H_2O_2 was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); *t*-butyl hydroperoxide (*t*-BuOOH) and camptothecin were from Sigma Chemical Co.; doxorubicin was a kind gift from Kyowa Hakko Kougyo, Ltd. (Tokyo, Japan). Other reagents were of reagent grade and were used without further purification.

Animals

Male ddY mice, 6 weeks old and each weighing 30–35 g, were from SLC, Inc. (Shizuoka, Japan). All experiments were carried out according to the guidelines of the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University.

Cell culture

Human colon cancer SW480 cell lines were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2-95% air. Under these conditions, SW480 cells express HO-1 mRNA, as determined by means of reverse transcriptase-polymerase chain reaction.[23]

MTT assay

In vitro cytotoxicity was determined by the MTT assay.[35] Cells were seeded in 96-well culture plates (3,000 cells/well). After an overnight preincubation, cells were exposed for 48 hr to indicated concentrations of test reagents, H_2O_2, *t*-BuOOH, camptothecin, and doxorubicin, in the absence or presence of PEG-ZnPP. The toxicity of the test reagents was quantified as the fraction of cells surviving relative to untreated controls.

Quantification of ROS level in cells treated with H_2O_2 and/or PEG-ZnPP

SW480 cells were plated in 12-well plates (10^5 cells/well). After an overnight preincubation, cells were treated with H_2O_2 and/or PEG-ZnPP for 8 hr. Then, 10 μM DCDHF-DA was added, and cells were cultured for an additional 30 min. The esterified form of DCDHF-DA can permeate cell membranes and then be deacetylated by intracellular esterases. The resulting compound, dichlorodihydrofluorescein (DCDHF), will react with ROS to give a fluorescent compound, dichlorofluorescein, which remains in the cells.[36] The amount of intracellular ROS was quantitated as a function of fluorescence intensity measured by flow cytometry (BD FACSCalibur 3A, San Jose, CA).

In vitro *apoptosis assay*

Induction of apoptosis *in vitro* by H_2O_2, PEG-ZnPP or H_2O_2 plus PEG-ZnPP was determined by a flow cytometric assay with Annexin V-FITC,[37] by using an Annexin V-FITC Apoptosis Detection Kit (BD PharMingen, San Diego, CA). In brief, SW480 cells plated in 12-well plates (10^5 cells/well) were preincubated overnight, after which cells were treated with H_2O_2 and/or PEG-ZnPP for 24 hr. After the cells were harvested by use of a rubber policeman, they were subjected to staining with the Annexin V-FITC kit and propidium iodide. The amount of apoptotic cells was analyzed by flow cytometry (BD FACSCalibur 3A).

In vivo *antitumor activity of PEG-DAO and/or PEG-ZnPP*

Mouse sarcoma S-180 cells (2×10^6 cells) were implanted subcutaneously in the dorsal skin of ddY mice as described before.[23,30] The antitumor activity of PEG-DAO/D-proline and/or PEG-ZnPP was evaluated at 6 days after tumor inoculation, when tumors had attained a diameter of 4–5 mm but had no necrotic areas. PEG-ZnPP was administrated on day 6 at 100 nmol/mouse (equivalent to 1.5 mg of ZnPP/kg, i.v.), and 24 hr later, PEG-DAO was injected once daily (0.75 unit/mouse i.v.) for 3 continuous days. Four hours after the PEG-DAO injection, D-proline was given by *i.p.* injection (0.5 mmol/mouse). Injection of D-proline continued daily for another 2 days after cessation of PEG-DAO administration. Tumor volume and body weight of the mice were measured daily during the study period. Tumor volume (V) was estimated by measuring the longitudinal cross section (L) and the transverse section (W) and applying the formula $V = (L \times W^2)/2$.

Blood cell counts and blood chemistry assays

Mice bearing S-180 tumors of about 10 mm in diameter were used for our study. Twenty-four hours after PEG-ZnPP and/or PEG-DAO/D-proline treatment as just described, mice were killed and blood was obtained from the inferior vena cava. Counts of red blood cells (RBC) and white blood cells (WBC) and hemoglobin (Hb) quantification were determined by routine clinical laboratory techniques.[38] Plasma obtained by centrifugation was used for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), blood urea nitrogen (BUN) and creatinine (Cr) values by using a sequential multiple AutoAnalyzer system (Hitachi Ltd., Tokyo, Japan).

At the same time that blood was drawn, the liver, kidney and lung were collected after reperfusion of mice with 10 mL of physiological saline containing heparin (5 units/mL). These organs were subjected to the apoptosis assay and histological examination as described below.

In situ *apoptosis detection*

In vivo induction of apoptosis by PEG-DAO/D-proline and/or PEG-ZnPP treatment was detected by using the terminal deoxynucleotide transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method,[39] with an *in situ* apoptosis detection kit (TACS; Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instruction. Tissue specimens from the mice were embedded in an embedding dish (Greiner Bio-one Co., Ltd., Tokyo, Japan) by using Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and were stored at −80°C before use. Cryosections (10 μm thick) were prepared and serial sections were used for the TUNEL assay. TUNEL-positive cells were counted in 4 different fields per sample, and counts were expressed per square millimeter of tissue section.

Histological examination

Some tissue specimens collected as described above were fixed with 10% buffered neutral formalin solution and were then embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E).

Statistical analysis

All data are expressed as means ± *SE*. Student's *t* test was used to determine the significance between experimental groups. A difference was considered statistically significant at $p < 0.05$.

RESULTS

Sensitization by PEG-ZnPP of SW480 cells to the toxic action of peroxides, camptothecin and doxorubicin in vitro

It was recently demonstrated that PEG-ZnPP itself has dose-dependent cytotoxic effects against SW480 cells.[23] Apparent cell death was observed at a PEG-ZnPP concentration higher than 25 μM.[23] Therefore, in our present experiments, we use 5 or 10 μM PEG-ZnPP as a nontoxic dose to examine the effect of HO inhibition on the cytotoxic activity of oxidative chemotherapeutics.

We first examined the effect of PEG-ZnPP on the cytotoxic action of 2 kinds of hydroperoxides, H_2O_2 and *t*-BuOOH. Both H_2O_2 and *t*-BuOOH induced death of SW480 cells in a dose-dependent manner (Fig. 1*a*,*b*). In the presence of PEG-ZnPP (5 μM), however, the dose-response curves for those oxidants shifted to a lower concentration range. The median lethal dose (LD_{50}) values for H_2O_2 and *t*-BuOOH were significantly reduced by 25% and 39%, respectively, in the presence of 5 μM PEG-ZnPP. PEG-ZnPP alone at 5 μM had no cytotoxic effect in this experimental setting (data not shown).

PEG-ZnPP had a more dramatic effect on the cytotoxicity mediated by camptothecin and doxorubicin. The LD_{50} values for camptothecin and doxorubicin were reduced in the presence of 5 μM PEG-ZnPP by 83% and 61%, respectively (Fig. 1*c*,*d*). These results suggest that PEG-ZnPP treatment makes SW480 cells clearly vulnerable to the toxic insults of peroxides and anticancer agents.

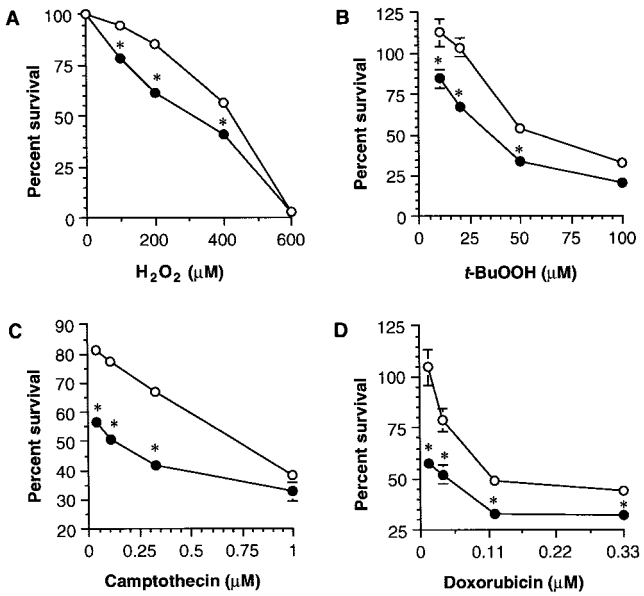

FIGURE 1 – *In vitro* cytotoxicity of ROS and ROS-generating anticancer drugs plus PEG-ZnPP. SW480 cells were exposed to increasing amounts of ROS (*a*, H_2O_2; *b*, *t*-BuOOH) or ROS-generating anticancer drugs (*c*, camptothecin; *d*, doxorubicin) for 48 hr in the presence (filled circle;) or absence (open circle) of PEG-ZnPP (5 μM). Cell viability was determined by means of the MTT assay. Values are means ± *SE* ($n = 6–8$). *$p < 0.002$.

Annexin V staining assay was used to examine the involvement of the apoptotic pathway in PEG-ZnPP-mediated enhanced cell death. Figure 2 shows the results for cells receiving different treatments. H_2O_2 (1 mM) or PEG-ZnPP (10 μM) alone induced a small but significant increase in the number of apoptotic cells compared to controls. However, we observed an extremely large increase (*i.e.*, 16.4 folds) in the fraction of apoptotic cells when cells were treated simultaneously with H_2O_2 and PEG-ZnPP.

Modulation of intracellular redox status by ROS and PEG-ZnPP

To determine whether enhanced tumor cell death induced by PEG-ZnPP correlates with the production of toxic oxidants in cells, we performed flow cytometric analysis with the oxidant-sensitive fluorescent probe DCDHF-DA.[36] Representative flow cytograms clearly show that SW480 cells receiving different treatments had distinct fluorescence intensity distributions (Fig. 3*a*). Administration of H_2O_2 (0.5 mM) or PEG-ZnPP (5 μM) alone caused a moderate but significant increase in mean fluorescence intensity of cells compared to that of untreated cells (control) (Fig. 3*b*). A maximum increase in fluorescence intensity was obtained for cells treated simultaneously with H_2O_2 and PEG-ZnPP. This result suggests that cells treated with PEG-ZnPP are less able to defend themselves against oxidative stress induced by H_2O_2 compared to cells not treated with PEG-ZnPP, the result being increased apoptosis.

Augmentation of in vivo *antitumor activity of PEG-DAO/D-proline by PEG-ZnPP*

PEG-DAO is a chemically modified oxidoreductase that generates H_2O_2 during oxidative deamination of D-amino acids.[30] We previously demonstrated that PEG-DAO administered *i.v.* generated cytotoxic H_2O_2 in solid tumor tissues when mice received the substrate D-proline and PEG-DAO separately with a time lapse interval of 2–4 hr.[30] This treatment protocol led to significant inhibition of tumor growth. To examine the effect of PEG-ZnPP on antitumor treatment *in vivo*, we again used PEG-DAO plus D-proline as the antitumor regimen. The dose and treatment period for PEG-DAO plus D-proline were reduced to $\frac{1}{4}$ of those used in the previous protocol. The total amounts of PEG-DAO administered were thus 2.25 units/mouse. As shown in Figure 4, the present treatment protocol of PEG-DAO plus D-proline (without PEG-ZnPP) in the S-180 solid tumor model showed no antitumor activity compared to the control. In addition, a single administration of PEG-ZnPP moderately suppressed tumor growth.

Although the present protocol of PEG-DAO plus D-proline exhibited no antitumor effect, tumor growth was almost completely suppressed when mice were pretreated with PEG-ZnPP followed by PEG-DAO plus D-proline. Continuous suppression of tumor growth was observed for at least 31 days after tumor inoculation, which was 22 days after the last treatment with PEG-DAO and D-proline. Three of 8 tumors in mice treated with PEG-DAO protocol plus PEG-ZnPP exhibited complete regression of growth. These findings clearly indicate the validity of using an HO inhibitor to augment the antitumor effect of ROS-generating chemotherapeutic agents, including PEG-DAO protocol. It is noteworthy that a significant antitumor effect was achieved by using a very low dose of PEG-DAO, which may attribute to the fewer side effects of this treatment protocol as described below.

The mean tumor weights (in grams) on day 31 after tumor implantation for the groups treated with the PEG-DAO protocol plus PEG-ZnPP combination, PEG-DAO protocol alone, and PEG-ZnPP alone, and for the untreated controls were 0.42 ± 0.17, 3.18 ± 0.73, 2.52 ± 0.36 and 3.34 ± 0.31, respectively ($p < 0.0003$; PEG-DAO protocol plus PEG-ZnPP group *vs.* any of the other groups). In separate experiments, PEG-conjugated protopor-

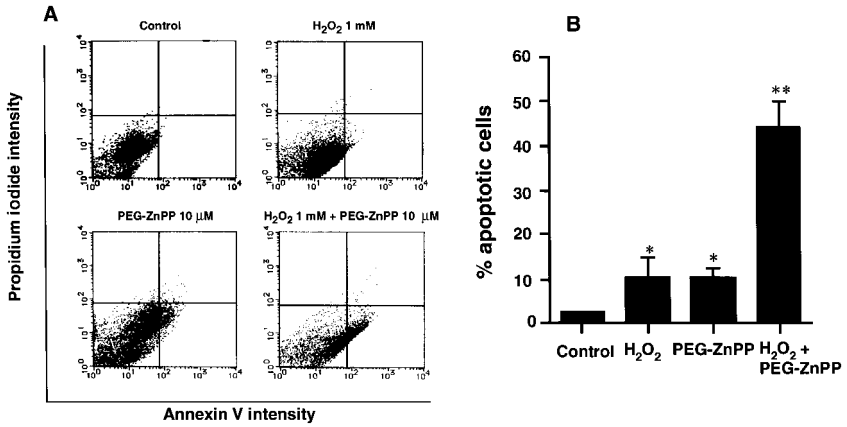

FIGURE 2 – Induction of apoptosis of SW480 cells by H_2O_2 and/or PEG-ZnPP. SW480 cells were treated with 1 mM H_2O_2 and/or 10 μM PEG-ZnPP for 24 hr. The number of apoptotic cells was measured by flow cytometry (*a*). The percentage of apoptotic cells (per the total number of calculated cells) is shown in (*b*). $*p < 0.05$, $**p < 0.001$.

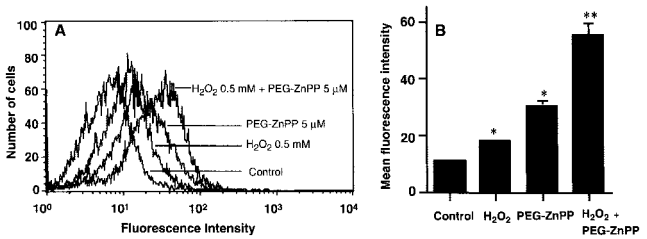

FIGURE 3 – Induction of intracellular ROS production in SW480 cells by H_2O_2 and/or PEG-ZnPP. SW480 cells were treated with 0.5 mM H_2O_2 and/or 5 μM PEG-ZnPP for 8 hr. The amount of intracellular ROS produced by these treatments was measured by flow cytometry. A representative cytogram for cells given different treatments is shown in (*a*). Mean fluorescence intensity was quantified for these treatments (*b*). $*p < 0.005$, $**p < 0.001$.

phyrin, which is a zinc-free analogue of PEG-ZnPP without HO inhibitory activity,[22] had no antitumor effect when used alone or in combination with PEG-DAO protocol, which lends further support to the importance of the HO inhibitory effect of PEG-ZnPP (data not shown).

The TUNEL assay revealed strongly positive cells in tumor tissues from mice receiving the combination of PEG-DAO protocol plus PEG-ZnPP, whereas PEG-DAO protocol or PEG-ZnPP alone induced much less apoptosis of the tumor cells (Fig. 5).

Evaluation of side effects

Body weight changes. Figure 6 shows body weight changes in mice receiving different treatments. At the early stage of observation, groups treated with PEG-DAO protocol alone or PEG-DAO protocol combined with PEG-ZnPP exhibited a slight loss of body weight. However, after cessation of the treatment, body weight recovered rapidly at a rate of growth comparable to that of the untreated control mice. At the later stage of investigation, no significant loss of body weight was observed in the group treated with PEG-DAO protocol plus PEG-ZnPP compared to the control group without tumor and given no treatment. In the group given no drugs (control) and groups given a single drug, tumor growth appeared to account for the increased body weight compared to combination treatment group.

Blood cell counts and blood chemistry assays. We also investigated the hematological toxicity of PEG-DAO protocol plus PEG-ZnPP in S-180 tumor-bearing mice, by means of blood cell counts (RBC, WBC) and blood biochemistry. No significant decreases in

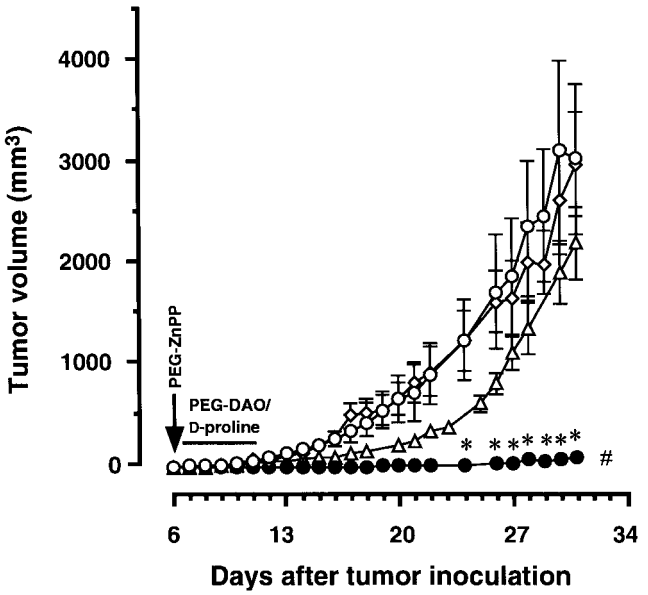

FIGURE 4 – Antitumor effect of PEG-DAO protocol plus PEG-ZnPP in the S-180 solid tumor model. S-180 cells (2×10^6 cells) were implanted *s.c.* in ddY mice. Arrowheads, administration of PEG-ZnPP (1.5 mg/kg). The PEG-DAO protocol (*i.e.*, PEG-DAO plus D-proline) was administered daily from days 7 to 11 after tumor inoculation: PEG-DAO was injected *i.v.* during the first 3 days at a dose of 0.75 unit/mouse; D-proline was injected *i.p.* 4 hr after PEG-DAO administration and, in addition, was injected daily for an extra 2 days after the cessation of PEG-DAO, at a dose of 0.5 mmol/mouse. Open circle, control, no treatment of S-180-bearing mice; closed circle, PEG-DAO protocol plus PEG-ZnPP; open diamond, PEG-DAO protocol but no PEG-ZnPP; open triangle, PEG-ZnPP alone. Data are means ± *SE* ($n = 8$–12). $*p < 0.01$ (control *vs.* each treatment). Number sign, Complete regression of tumor growth was found in 3 of 8 tumors in the PEG-DAO protocol plus PEG-ZnPP treatment group. See text for details.

RBC and WBC counts and Hb levels were found after PEG-DAO protocol plus PEG-ZnPP treatment compared to no treatment (control) (Table I). In addition, this treatment had no significant effects on major organs (liver and kidney) as evidenced by blood biochemical assays: plasma ALT, AST, LDH, BUN and Cr values (Table I).

Histological examination. Tissues from liver, kidney, lung and tumor were further subjected to H&E staining for histological

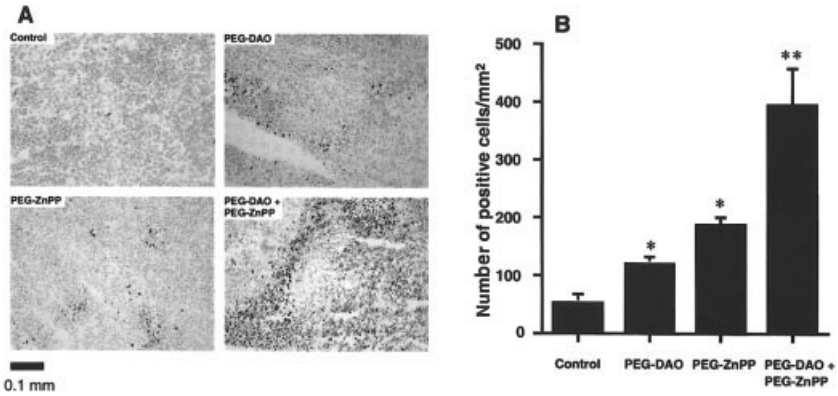

FIGURE 5 – Induction of apoptosis in S-180 solid tumors by PEG-DAO protocol plus PEG-ZnPP. Each specimen was collected and examined 24 hr after treatment. The treatment protocol was the same as that described for Figure 4. TUNEL staining of each group is shown in (*a*), with quantitative analysis of TUNEL-positive cells in each specimen shown in (*b*). TUNEL-positive cells in 4 different fields were counted and the number of positive cells per mm^2 was calculated. $*p < 0.02$ *vs.* control; $**p < 0.05$ *vs.* PEG-DAO protocol alone or PEG-ZnPP alone ($n = 4$ for each group). Data are means ± *SE*. See text for details.

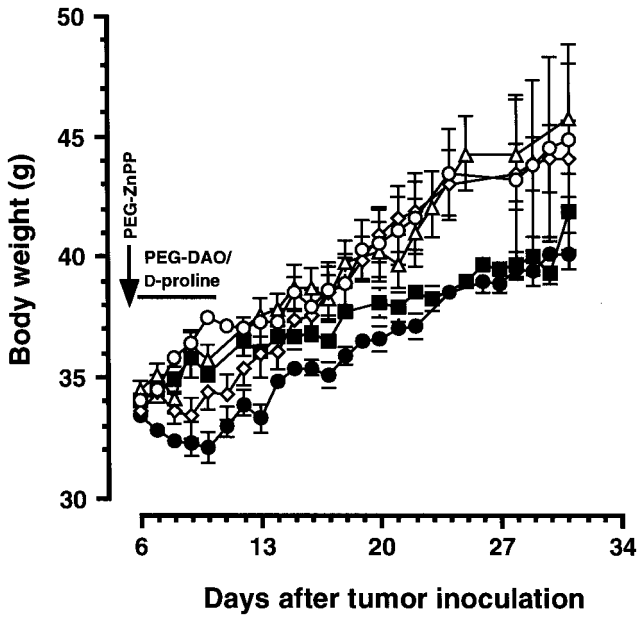

FIGURE 6 – Body weight changes in ddY mice treated with PEG-DAO protocol, PEG-ZnPP, or the combination of the 2 agents. The treatment protocol was the same as that described for Figure 4. Open circle, control mice bearing S-180 tumor that had no treatment; closed circle, PEG-DAO protocol plus PEG-ZnPP; open diamond, PEG-DAO protocol but no PEG-ZnPP; open triangle, PEG-ZnPP alone; closed square, control mice with no tumor and no treatment. Data are means ± *SE* ($n = 4–6$).

examination. Tumor tissues exhibited apparent degenerative lesions after treatment, which were most obvious in the group treated with PEG-DAO protocol plus PEG-ZnPP. However, no noteworthy pathological changes were observed in any other tissues after treated by PEG-DAO protocol plus PEG-ZnPP, compared to untreated controls (data not shown).

DISCUSSION

We showed here that PEG-ZnPP significantly enhances the antitumor activity of PEG-DAO plus D-proline *in vivo*. PEG-DAO with D-proline, even at a dose that was virtually inactive in the S-180 model, significantly reduced tumor growth when used with PEG-ZnPP pretreatment. This finding thus indicates a synergistic mechanism at work. To our best knowledge, this is the first demonstration that an HO inhibitor can enhance the response of tumors to a chemotherapeutic agent *in vivo*.

H_2O_2 is a cytotoxic principle needed for the antitumor action of PEG-DAO with D-proline, as we demonstrated very recently.[30] In biological systems, H_2O_2 is converted to more reactive species such as hydroxyl radical (•OH) *via* a transition metal-catalyzed reaction (the Fenton reaction).[40] Such reactive species injure vital molecules including DNA, proteins and membrane lipids, which leads to destruction of cellular integrity.[40] Several enzymes scavenge these toxic oxidants or transform them into nontoxic metabolites; *e.g.*, catalase decomposes H_2O_2 to water and molecular oxygen. Baranano *et al.*[3] recently reported that HO-1 is an important cellular component for protection against oxidative insults in that the redox cycle operates based on bilirubin reductase. They found that bilirubin, an ultimate metabolite of the HO reaction, at a physiologically relevant level (~10 nM), could protect cells from oxidative injury caused by a 10,000 times excess concentration of H_2O_2 (100 μM).[3] Our data revealed that PEG-ZnPP interferes the HO-1-dependent antioxidative effect against H_2O_2, which results in higher amounts of H_2O_2 or related oxidants. Flow cytometric analyses clearly showed that cells treated with PEG-ZnPP contained a significantly higher amount of H_2O_2-like species after exposure to exogenously added H_2O_2, compared to cells not treated with PEG-ZnPP (Fig. 3). The essential role of HO inhibition was further demonstrated by the finding that PEG-conjugated protoporphyrin, an inert analogue of PEG-ZnPP,[22] could not substitute for PEG-ZnPP.

We found that combination therapy of PEG-ZnPP with PEG-DAO protocol (or H_2O_2 as a reagent) induced apoptosis of SW480 cells *in vitro* (Fig. 2) and in S-180 solid tumors *in vivo* (Fig. 5). The degree of apoptosis varied depending on the treatment: greatest apoptosis was obtained for the combination of PEG-ZnPP and H_2O_2 (or PEG-DAO protocol and PEG-ZnPP), which was more than additive compared to apoptosis induced by PEG-ZnPP, PEG-DAO protocol or H_2O_2 alone. This tendency correlated well with oxidant-producing capacity (Fig. 3). Therefore, it is conceivable that H_2O_2-like species derived from PEG-ZnPP/PED-DAO could be a functional principle for induction of apoptosis, and hence suppress growth of tumors. This notion is consistent with previous findings that ROS including H_2O_2 participate in the activation of the apoptotic pathway through stimulation of proapoptotic pathways such as caspases or inhibition of antiapoptotic pathways such as bcl-2.[41,42]

TABLE I – CHANGES OF RBC, WBC, Hb, BUN, Cr AND PLASMA ENZYME LEVELS IN DDY MICE TREATED WITH PEG-DAO AND/OR PEG-ZnPP[1]

Treatment	RBC (10^4/μL)	WBC (/μL)	Hb (g/L)	BUN (mg/dL)	Cr (mg/dL)	AST (IU/L/)	ALT (IU/L/)	LDH (IU/L/)
Control	717.3 ± 56.6	19,188 ± 6,558	131.1 ± 5.9	21.8 ± 1.5	0.15 ± 0.01	233 ± 26.2	34.3 ± 3.2	6,046 ± 757
PEG-DAO	712.5 ± 45.6	25,613 ± 8,779	137.8 ± 4	20.1 ± 0.5	0.16 ± 0.01	241 ± 34.9	37.5 ± 8	5,165 ± 549
PEG-ZnPP	778 ± 48.2	19,700 ± 1,794	129.9 ± 7.2	17.3 ± 1.3	0.13 ± 0.01	259 ± 21.4	33.3 ± 1.5	8,078 ± 1,018
PEG-DAO plus PEG-ZnPP	754.3 ± 10.3	16,413 ± 1,783	140.7 ± 3	17.9 ± 1.4	0.16 ± 0.01	269 ± 13.8	35.5 ± 3.1	6,816 ± 534

[1]Values are presented as means ± SE. No significant difference was found between each treatment group and the control group for all indices.

Another mechanism of action of HO-1-inhibited apoptosis may involve suppressed production of CO, another product of HO enzymatic reaction that has potent antiapoptotic activity.[11] CO may also be involved in the induction of apoptosis mediated by PEG-ZnPP, in that inhibition of HO-1 by PEG-ZnPP would result in reduction of the CO level. Hence, a suppressed antiapoptotic effect would facilitate apoptosis induced by H_2O_2-like species derived from the combination therapy of PEG-ZnPP and PEG-DAO protocol.

Here, we showed that PEG-ZnPP could enhance the cytotoxic action of 2 widely used anticancer agents, doxorubicin and camptothecin. Although these 2 agents both ultimately induce DNA damage, their putative primary molecular mechanisms of action may differ each other: doxorubicin directly intercalates with the DNA double strand or inhibits topoisomerase II and subsequently induces DNA damage,[43] whereas camptothecin induces chromatid breaks through favoring topoisomerase I stabilization, which results in arrest of replication fork.[44] Hence, it is expected that PEG-ZnPP could potentiate the cytotoxic action of these agents by modulating effects down-stream of their sites of specific pharmacological action. Simizu *et al.*[45] showed that generation of H_2O_2 is critical for apoptosis induced by various anticancer agents including doxorubicin and camptothecin. They reported that various anticancer agents activate caspase-3-like proteases, which subsequently induces activation of H_2O_2-generating enzymes such as NADPH oxidase.[45] Therefore, it is reasonable to hypothesize that PEG-ZnPP may manifest its enhancing effect of doxorubicin and camptothecin through increased oxidative stress of tumor cells. This hypothesis leads to the suggestion that PEG-ZnPP may be beneficial in combination with other antitumor agents, most of which involve ROS formation as a component of their molecular mode of action. These agents include, in addition to doxorubicin[45,46] and camptothecin,[45] vinblastine,[45] inostamycin,[45] mitomycin C,[46] etoposide,[47] neocarzinostatin,[48] 2-methoxyestradiol,[49] *N*-(4-hydroxyphenyl)retinamide[50] and pegylated xanthine oxidase,[51] as well as radiotherapy.[52] Further study along this line is warranted.

Our data showed that combination therapy of PEG-ZnPP with PEG-DAO/D-proline was well tolerated by mice. From findings with HO-1-deficient mice,[53] the liver, spleen and bone marrow are expected to be the major organs to be affected by nonspecific inhibition of HO-1 activity, which may induce destruction of hepatic and splenic structures and functions because of abnormal iron deposition, chronic anemia and marked body weight loss. However, we found no obvious signs of toxicity, including effects on those organs as evidenced by body weight change, blood chemistry assays and histological examination. This result suggests that PEG-ZnPP at the doses and schedule administered does not induce functional impairment of HO-1 in those normal and vital organs.

In fact, we recently determined that PEG-ZnPP more specifically inhibits HO activity in tumors.[23] By using radiolabeled PEG-ZnPP, we showed that PEG-ZnPP accumulated in S-180 solid tumors after *i.v.* injection and that it remained there for a long period (> 48 hr).[23] We showed that this accumulation of PEG-ZnPP in tumor could be explained by the unique characteristics of tumor vasculature and physiological behavior of PEG-ZnPP (see below for details). Accordingly, this unique *in vivo* behavior of PEG-ZnPP may explain why administration of PEG-ZnPP only once could enhance antitumor activity of PEG-DAO protocol given subsequently.

Tumor-targeted delivery of any antitumor agent is a prerequisite for achieving tumor-selective cytotoxicity without causing systemic side effects. We previously reported that biocompatible macromolecular drugs and lipids accumulate and are retained selectively in solid tumor because of the unique characteristics of the tumor vasculature and the impaired lymphatic clearance system. We named this phenomenon the EPR (enhanced permeability and retention) effect.[54–58] The EPR effect is now recognized as a general characteristic of viable and rapidly growing solid tumors, and the concept is regarded as providing one of the most rational strategies for designing drugs that selectively target solid tumors.[56–60]

This EPR effect depends on the molecular size of a drug; it operates with drugs larger than 40 kDa. The EPR effect is also inversely correlated to renal clearance.[54,55,61,62] Previous reports indicated molecular sizes of PEG-DAO and PEG-ZnPP in aqueous media of 63 kDa[30] and above 70 kDa,[22] respectively. In addition, pharmacokinetic studies revealed greatly increased plasma half-lives, area under the concentration curve and selective accumulation in tumors of these 2 compounds compared to nonpegylated agents.[23,30] These data clearly indicate that the EPR effect is relevant for PEG-DAO and PEG-ZnPP, which demonstrate the beneficial effects of polymer therapeutics.

To further establish a role for HO-1 as a molecular target for cancer therapy, it is necessary to elucidate the extent and mechanism involved in the expression of HO-1 in tumors. As mentioned in the Introduction, NO can induce the expression of HO-1 in several types of cells, as demonstrated by the use of various synthetic NO donors.[8,9] We previously showed that NOS inhibitors suppressed HO-1 induction in rat AH136B tumors, which supports the suggestion that NO biosynthesis in tumors, mostly *via* iNOS, may participate in the expression of HO-1 in this rat model.[18] It should be noted that iNOS expression was demonstrated in human cancers of the prostate,[63] and brain[64] and in oral squamous cell carcinoma,[65] where HO-1 expression was detected.[24–26] Thus, NO-mediated up-regulation of HO-1 may operate in these human cancers as well. Furthermore, iNOS expression has been found in many other cancerous sites, including colon, lung and breast in human and animal models.[66] Therefore, one would expect enhanced expression of HO-1 as well in these sites, through NO medication.

In conclusion, our present results provide the experimental evidence that HO-1 expressed in tumors holds promise as a potential molecular target for pharmacological intervention in cancer therapy. Inhibition of HO-1 activity by using PEG-ZnPP synergistically potentiated the antitumor activity of PEG-DAO/D-proline, with the added benefit of a much reduced dosage of both antitumor agents. This treatment regimen was well tolerated, with no obvious signs of severe toxicity and as yet no dose-limiting toxicity. These observations point to further applications of PEG-ZnPP in combination therapy with a variety of anticancer agents, especially those associated with oxidative injury as well as radiotherapy.

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