Inhibition of Heme Oxygenase-1 Enhances the Cytotoxic Effect of Gemcitabine in Urothelial Cancer Cells

MAKITO MIYAKE, KIYOHIDE FUJIMOTO, SATOSHI ANAI, SAYURI OHNISHI, YASUSHI NAKAI, TAKEKI INOUE, YOSHIKI MATSUMURA, ATSUSHI TOMIOKA, TOMOHIRO IKEDA, EIJIRO OKAJIMA, NOBUMICHI TANAKA and YOSHIHIKO HIRAO

Department of Urology, Nara Medical University, Nara, 634-8522, Japan

Abstract. Background: Elevated heme oxygenase-1 (HO-1) is associated with resistance to chemo- and radiotherapy through anti-apoptotic function. The present study evaluated whether the HO-1 inhibitor, zinc protoporphyrin IX (ZnPP), enhances the cytotoxic effect of gemcitabine in urothelial carcinoma (UC). Materials and Methods: The in vitro cytotoxic effect of combination treatment of gemcitabine and ZnPP on UC cells was examined. The in vivo growth inhibitory effects of intraperitoneal administration of gemcitabine and/or ZnPP on mouse subcutaneous tumours were examined. The apoptotic changes were analysed with the detection of DNA fragmentation and cleaved caspase-3. Results: HO-1 was up-regulated by both gemcitabine and irradiation treatment in vitro. ZnPP sensitised the UC cells to both therapies. Enhanced apoptosis was induced by the ZnPP combined with gemcitabine. ZnPP enhanced the antitumour effect of gemcitabine in vivo along with decreased numbers of proliferating cells and increased numbers of apoptotic cells. Conclusion: These findings suggest that ZnPP combined with gemcitabine or irradiation therapy may be an effective therapeutic modality for UC patients.

Bladder cancer is the fourth most common malignancy in men and the ninth in women; there were some 63,210 new cases in 2005 in the U.S.A. (1). Histologically, urothelial carcinoma (UC) constitutes more than 90% of all bladder cancers. UC is a malignant neoplasm characterised by heterogeneous cell populations. The biological behaviour, prognosis and clinical management of UC vary depending on the pathological stage and tumour grade. Approximately, 70% of newly diagnosed bladder tumors are non-muscle invasive disease (pTa-1 or pTis), and can be managed by endoscopic procedures, such as transurethral resection of bladder tumour and intravesical injection of antitumor reagents (1). In contrast, the prognosis of bladder UC patients with muscle invasion (pT2), lymphatic involvement, and/or metastasis is extremely poor. The mainstay of any therapeutic strategy for advanced bladder UC is combination chemotherapy with or without radiotherapy. At present, there are two standard chemotherapeutic regimens, namely MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) and GC (gemcitabine and cisplatin) (2). A large scale randomised control trial comparing MVAC and GC chemotherapy for patients with locally-advanced (T4b, N2 or N3) or metastatic (M1) UC confirmed that the median survival periods were 15.2 and 14.0 months, respectively, and that the overall survival was similar in both arms (hazard ratio: 1.09, 95% CI: 0.88-1.34) (2). Since GC chemotherapy demonstrated a significantly better toxicity profile in this trial, this regimen has become the standard first-line treatment for metastatic UC. However, there was a substantial fraction of patients achieving little clinical benefit due to the intrinsic or acquired chemoresistance.

Gemcitabine (GEM), a deoxycytidine analogue (2’,2’-difluorodeoxycytidine; dFdC), has exhibited a significant antitumour activity in several solid tumours in clinical trials, including non-small cell lung cancer (3), pancreatic cancer (4) and biliary tract cancer (5). Although several investigators have described molecular signatures underlying the chemoresistance against GEM in pancreatic cancer (6-9), little is known about them in UC. Identifying a key molecule associated with resistance against GEM and establishing a novel combination therapy using GEM with other active agents is required in order to achieve a stronger cytotoxic effect and improved clinical outcome.

Heme oxygenase-1 (HO-1) catalyses the oxidation of heme to three active products, namely, carbon monoxide (CO), biliverdin and ferrous iron. The HO-1 protein is up-regulated as an adaptive response to various stress stimuli, such as...
oxidative stress, ultraviolet light, irradiation, hydrogen peroxide, hypoxia, and hyperthermia (10). HO-1 itself and its products participate in maintaining cellular homeostasis and play a protective role in reducing oxidative damage, attenuating the inflammatory response, inhibiting cellular apoptosis and regulating cellular proliferation (11). Many studies have demonstrated that elevated HO-1 levels may play a role in carcinogenesis and may potentially influence cellular growth, angiogenesis, and metastasis in several malignancies, such as renal cell carcinoma, prostate cancer and pancreatic cancer (6, 11-13). HO-1 is frequently up-regulated in tumour tissue and its expression is further increased as a cytoprotective factor in response to various therapies (11). It has been demonstrated that constitutively-expressed or up-regulated HO-1 is correlated remarkably with cellular proliferation and resistance against radiotherapy, chemotherapy and photodynamic therapy in some malignant diseases (6, 14-17). Based on this evidence, inhibition of HO-1 can be regarded as a potential therapeutic approach against cancer. In this study, experimental studies were conducted in vitro and in vivo to assess whether the inhibition of HO-1 by zinc protoporphyrin IX (ZnPP), an HO-1 inhibitor, has the potential to enhance the antitumour effect of GEM or irradiation in the UC cells.

Materials and Methods

Chemical compounds. Gemcitabine hydrochloride (GEM, molecular weight: 299.66) was purchased from Alexis Biochemicals (San Diego, CA, USA) and dissolved in sterile water at a concentration of 10 mg/ml. ZnPP was purchased from Enzo Life Sciences (Farmingdale, NY, USA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM for the experiments. The stock solution was stored at –20˚C before use.

Cell lines and cell culture. Two established UC cell lines, T24 and MGHU3 (17), maintained in RPMI-1640 (Nissui, Tokyo, Japan), were supplemented with 10% foetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO, Grand Island, NY, USA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM for the in vitro experiments. The stock solution was stored at –20°C before use.

Cell viability assay. The cells were seeded in a 96-well plate at a density of 2000 cells/well for 24 hours. They were treated with the indicated concentrations of GEM, ZnPP and a combination of these two reagents. After incubating the plates for 72 hours, a cell viability assay was performed using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer directions. Chemosensitivity against GEM was expressed as the 50% inhibitory concentration (IC50), as determined from the concentration-effect relationship using PRISM software version 4.00 (GraphPad Software, San Diego, CA, USA). The data were expressed by the relative value to the untreated cells, which was set at 100. In the analysis of the combination therapy, additive and synergistic cytotoxic effects were defined as follows (18): Additive if, (viability in presence of ZnPP)/(viability in presence of untreated cells)={viability in presence of (ZnPP+GEM)}/(viability in presence of GEM); Synergistic if, (viability in presence of ZnPP)/(viability of untreated cells)<(viability in presence of (ZnPP+GEM))/viability in presence of GEM).

Western blot analysis. Western blot analysis was performed as previously described (17). The primary antibodies used in this study were anti-HO-1 (clone 23; BD Transduction Laboratories, San Diego, CA, USA), anti-phosphorylated H2AX (Ser139) (γ-H2AX) antibody (Cell Signaling Technology, Beverly, MA, USA) and anti-actin antibody (clone AC-15; Sigma-Aldrich, St Louis, MO, USA) as an internal loading control.

Detection of apoptosis in vitro. Quantification of DNA fragmentation was performed using Cell Death Detection ELISA PLUS (Roche Molecular Biochemicals, Indianapolis, IN, USA). This is a rapid and highly-sensitive assay for detection of the early changes in apoptotic cell death and measurement of the amount of histone-associated low molecular weight DNA in the cytoplasm of cells. T24 cells were seeded in a 96-well plate at a density of 2000 cells/well in a growth medium and incubated for 24 hours. They were treated with GEM (1 ng/ml) and/or ZnPP (2.5 μM). After incubating the plates for 48 hours, the apoptotic index was assayed according to the manufacturer directions. The apoptotic index was expressed by the relative value to the untreated cells, which was set at 1.

Clonogenic assay. For clonogenic survival assays, a previously described technique was used (19). Using the T24 cell line, 2×103 cells were plated into 35-mm dishes and were allowed to grow overnight. X-ray irradiation (1.0 Gy/min) was carried out with a 150-kVp X-ray generator (Model MBR-1520R; Hitachi, Tokyo, Japan). Immediately after irradiation, the cells were trypsinised, suspended and counted using a hemocytometer. A hundred cells were plated in each well of a six-well culture plate. These cells were allowed to grow for 7 days, followed by 0.25% crystal violet staining. The colonies in each well were counted. In the experiments on combination of ZnPP and radiation therapy, the cells were treated with 2.5 μM of ZnPP for 12 hours prior to irradiation.

Xenograft mouse model. Female athymic BALB/C nu/nu mice (8 weeks old) were used to evaluate the therapeutic effect in vivo. The mice were maintained under specific pathogen-free conditions and provided with sterile food and water. T24 cells (2×106) were plated in 100 μl RPMI-1640 medium, together with 10 μl of Matrigel (Becton Dickson, Bedford, MA, USA), were injected subcutaneously into each mouse. When the tumours reached 5 mm in diameter, the animals were divided randomly into four groups (control, GEM monotherapy, ZnPP monotherapy and combination therapy), and treatment was initiated (day 0). For the in vivo treatment, ZnPP was dissolved in DMSO at a concentration of 100 mg/ml and further diluted in phosphate-buffered saline (PBS). GEM was dissolved in water at a concentration of 10 mg/ml and further diluted in PBS. ZnPP (12.5 mg/kg) and GEM (60 mg/kg) were administered intraperitoneally once a week for 3 weeks. In the combination therapy, GEM was administered 24 hours after ZnPP injection. In the control group, the mice received the control treatment according to the same schedule. The tumour diameters were measured twice a week with electronic calipers, and the tumour volumes were calculated using the formula: (width)2 x length)/2 (mm3). The weight of mice was measured once a week. The mice were killed on day 35, and the tumours were resected.
Immunohistochemical analysis of tumour tissues. The tumour tissues were fixed by 10% formaldehyde solution and embedded in paraffin. The sections (3-μm-thick) were deparaffinised, followed by antigen retrieval with autoclave treatment for 10 min in 0.01 M citrate buffer (pH 6.0). Immunohistochemical staining was performed with a streptavidin-biotin (SAB) complex method using Histofine SAB-PO kit (Nichirei Co., Tokyo, Japan) according to the manufacturer’s directions. The primary antibodies were mouse monoclonal anti-Ki-67 (clone MIB-1; Dako Japan, Kyoto, Japan) in ready-to-use form at room temperature for 30 min and rabbit polyclonal anti-cleaved caspase-3 (clone 5A1E; Cell Signaling Technology) in dilution of 1/200 at 4°C overnight. The sections were counterstained with Meyer’s haematoxylin and mounted with Malinol (Muto Chemical, Tokyo, Japan). For appropriate analysis of the resected tumours for Ki-67 and cleaved caspase-3, the regions of tumour cells were confirmed by haematoxylin and eosin (H&E) staining.

Statistical analysis. PRISM software version 4.00 was used for statistical analyses and drawing figures. A p-value <0.05 was considered statistically significant.

Results

ZnPP exerts direct growth inhibition of UC cells. The cell viability assay in the treatment of ZnPP demonstrated that inhibition of HO-1 caused a direct anti-tumour effect in a dose-dependent manner in both T24 and MGHU3 cells.
Significant growth inhibition was observed by dosages of ZnPP more than 2.5 μM. To avoid overdosage of GEM in the subsequent in vitro experiments, the chemosensitivity of two cell lines was examined against GEM. There was no significant difference between the IC50 of T24 and that of MGHU3 (Figure 1B).

**HO-1** is a stress-inducible protein showing cytoprotection property against GEM in UC cells. The anti-apoptotic property of HO-1 helps cells to adapt to stress and injury (10). To examine whether the up-regulation of HO-1 protein correlated with the treatment of GEM or radiotherapy in the UC cells, Western blot analysis was performed. Both cell lines presented up-regulation of HO-1 in response to stimulation by GEM and irradiation (Figure 2). The peak HO-1 expression was observed 3 to 12 hours after stimulation, whereas the expression decreased at 24 hours. The degree of inducibility of HO-1 differed between T24 and MGHU3 cells (Figure 2, T24 > MGHU3).

To determine whether the chemoresistance of UCs correlated with HO-1 up-regulation, UC cells were exposed to GEM and/or ZnPP. The cell viability assay revealed that the UC cells up-regulated the HO-1 protein in response to irradiation, suggesting that HO-1 exerts cytoprotection against irradiation (Figure 4). To determine the intrinsic radiosensitivity of T24 cells, a clonogenic assay was performed after irradiation (2.5 to 20 Gy). A dosage greater than 10 Gy was lethal for the T24 cells. Regarding the combination therapy, pre-treatment of cells with 2.5 μM of ZnPP for 12 hours rendered the cells susceptible to the cytotoxic effects of irradiation at a dosage of 1.5 Gy (Figures 4B and 4C). Western blot analysis using T24 cells showed no significant up-regulation of γ-H2AX after irradiation alone and combination of irradiation and ZnPP (data not shown).

Combination of ZnPP augments the in vivo antitumour effect of GEM. To evaluate the augmented antitumour effect of GEM by ZnPP in vivo, xenograft mice bearing T24 cells were prepared and treated as described in the Materials and Methods. The mean tumour volumes (mm³) on day 35 after the initial treatment of four groups (control, GEM monotherapy, ZnPP monotherapy and combination therapy) were 754±248, 345±71, 286±48, and 67±15, respectively (p<0.01, monotherapy vs. combination) (Figure 5A). Tumour growth inhibition induced by monotherapy of ZnPP was observed, and there was no significant difference in the inhibitory effects of ZnPP and GEM monotherapies (p=0.57). From treatment initiation till tumour resection, no significant loss of body weight was observed in any of the
four groups (Figure 5B). These findings suggested that ZnPP combined with GEM is effective and tolerable as a novel therapeutic modality for the UC cells.

To investigate the cell proliferation and apoptotic status of the treated and control tumours, the expression levels of Ki-67 and cleaved caspase-3 were analysed with the immunohistochemistry method. The results showed that the monotherapy of GEM or ZnPP and their combination induced a significant decrease of the nuclear expression of Ki-67 compared to placebo (Figure 5C). An important finding was that the combination therapy induced a much larger decrease of Ki-67 expression than did either monotherapy group. Apoptotic cells were observed more remarkably in the combination group than in the other three groups (Figure 5C). These findings were comparable to the results of the in vitro studies, namely the cell viability assay (Figure 3A) and the apoptosis assay (Figure 3B).

Discussion

Since several investigators have demonstrated a synergistic or additive effect of the combination of GEM and cisplatin in preclinical experiments (20, 21), clinical evidence of GC chemotherapy for advanced bladder UC has been developed.
Figure 4. Inhibition of HO-1 sensitises T24 cells to radiation therapy. A: After irradiation at the indicated doses, 100 cells were plated on a six-well culture plate. Colony number in each well was counted after 7 days. B: In the combination therapy, the cells were exposed to 2.5 μM of ZnPP for 12 hours prior to irradiation. The colony number in each well was counted 7 days after irradiation (5 Gy). C: The colony number in each treatment group was expressed as mean±SD (n=3). **p<0.01 Comparing cells treated with monotherapy vs. combination therapy.

Figure 5. Inhibition of HO-1 by ZnPP enhances the antitumour effect of gemcitabine in vivo. A: The tumour volumes of the four groups are plotted for 35 days after the start treatment for the T24 xenografts. Intraperitoneal administration was performed on days 0, 7 and 14 for ZnPP (red arrows) and on days 1, 8, and 15 (black arrows). The data are expressed as mean±standard error of mean (SEM). B: The body weights of mice were measured once a week and plotted on the graph accordingly. C: The resected tumours of the xenograft mice were analysed by H&E staining and immunohistochemical staining for Ki-67 and cleaved caspase-3. Panels a-c: placebo; d-f: GEM monotherapy; g-i: ZnPP monotherapy; j-l: combination therapy of GEM and ZnPP. Panels a, d, g and j H&E staining; b, e, h and k: for Ki-67 staining; c, g, k, and l: cleaved caspase-3 staining. Original magnification: ×200.
EMT6) cells. In contrast, the cancer (C-26), melanoma (B16F10) and breast cancer fluorouracil using xenograft tumours of murine colon enhancement of the cytotoxic effect was observed in the pancreatic cancer cells to GEM and irradiation using short interfering RNA (siRNA) transfection sensitised previous study in which the targeted knockdown of HO-1 with HO-1 inhibitor. 

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Chemoresistance of malignant tumours can be provoked by mutations in oncopgenes, function and expression loss of tumour suppressors or dysregulation of genes involved in the cell cycle, cell proliferation, signal transduction, angiogenesis or apoptosis (28, 29). In this study, HO-1 up-regulation was identified as a key factor associated with the resistance against GEM and irradiation using T24 and MGHU3 cell lines. This is the first suggestion of the clinical potential of combining HO-1 inhibition by ZnPP with the conventional therapy for UC.

The findings of the present study demonstrated that HO-1 inhibition by ZnPP enhanced the antitumour effect of both GEM and irradiation in the UC cell lines in vitro and in vivo. These finding are consistent with the results of a previous study in which the targeted knockdown of HO-1 using short interfering RNA (siRNA) transfection sensitised the pancreatic cancer cells to GEM and irradiation in vitro (6). Nowis et al. (16) recently described that no significant enhancement of the cytotoxic effect was observed in the combination of intraperitoneal ZnPP with cisplatin or 5-fluorouracil using xenograft tumours of murine colon cancer (C-26), melanoma (B16F10) and breast cancer (EMT6) cells. In contrast, the in vitro analysis of the present study using the xenograft tumour of a UC cell line (T24) revealed that the combination therapy of ZnPP and GEM shows a remarkable enhancement of the cytotoxic effect as compared to monotherapy of ZnPP or GEM. This discrepancy could be explained by the difference in the intrinsic inducibility of HO-1. The T24 cells significantly up-regulated the HO-1 protein by exposure to GEM and irradiation to a higher level than did MGHU3 cells (Figure 2). The subsequent experiments for assessing the cytotoxic enhancement in GEM and ZnPP treatment revealed more enhancement by ZnPP in T24 cells than by MGHU3 cells (Figure 3A). Thus, inducibility of HO-1 would be a predictive marker for sensitivity to the combination therapy with HO-1 inhibitor.

ZnPP shows both direct inhibition of tumour growth and an enhanced cytotoxic effect and apoptosis in the treatment using chemotherapeutic agents (14, 15). Previous studies demonstrated that up-regulated HO-1 and degradation products from heme exhibited cytoprotective effects to various toxic stimuli by multiple mechanisms, such as: (i) decreasing the pro-oxidant level (heme), (ii) increasing the antioxidant level (bilirubin), (iii) producing the antiapoptosis molecule CO, (iv) inducing ferritin, which removes and detoxifies the free ferric ion, and (v) preventing overstimulation of the immune response (15). In this study, monotherapy of GEM or ZnPP showed a slightly-elevated apoptotic index as compared to the control, but the combination therapy of the two agents induced a remarkable enhancement of apoptosis (Figure 3B).

The major action of ZnPP treatment seems to be an increase of the intracellular oxidative stress induced by inhibition of the HO-1 enzymatic activity, leading to cellular damage and apoptosis. At the same time, the major action of GEM is inhibition of cellular DNA synthesis. After incorporation of GEM nucleotide into the DNA strand, the DNA polymerases cannot proceed further synthesis; this effect is termed “masked chain termination” (30). Incorporation of GEM into DNA is essential to induce DNA damage and fragmentation. Although the monotherapy of GEM and combination therapy of GEM and ZnPP in T24 cells induced similar levels of γ-H2AX expression and DNA damage, ZnPP did not induce expression of γ-H2AX (Figure 3C). This result confirmed that the cytotoxicity of ZnPP is not due to DNA damage. It is considered essential that the enhanced cytotoxic effect of the combination of GEM and ZnPP correlated with a decreased production of the antiapoptosis molecule CO by ZnPP.

The in vivo experiments revealed that GEM and ZnPP acted synergistically to inhibit the growth of subcutaneous xenograft tumours of T24 cells. Immunohistochemical staining for Ki-67 confirmed that the combination of intraperitoneal administration significantly decreased cell proliferation (Figure 5C). The analysis of cleaved caspase-3 revealed increased apoptotic cells as compared to monotherapy. The results are in agreement with those of the in vitro experiments, in which cellular apoptosis was detected with DNA fragmentation (Figures 3B and 5C). The dosage schedule of GEM used in this study was based on clinical usage (once a week for 3 weeks) and ZnPP was administered prior to GEM. The regimen was modified to suit the clinical needs, and this protocol could provide an effective and tolerable therapeutic modality for advanced UC patients.

In conclusion these results provide experimental evidence that HO-1 expression induced by gemcitabine or irradiation has the potential as a target molecule for chemo- or radiotherapy for patients with advanced UC. Although inhibition of HO-1 by ZnPP is a promising therapeutic modality to improve response rates and/or prolong survival, clinical trials are warranted to consolidate the true value of ZnPP-assisted therapy.
References


