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A Hydrogen Peroxide-Activatable Gemcitabine Prodrug for the Selective Treatment of Pancreatic Ductal Adenocarcinoma

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Abstract: The major concern in the use of anticancer chemotherapeutic drugs is host toxicity. Patients need to interrupt or change chemotherapy due to adverse effects. In this study, we aimed to decrease adverse events on gemcitabine (GEM) in the treatment of pancreatic ductal adenocarcinoma and focused on the difference of hydrogen peroxide (H_2O_2) levels in normal cells and cancer cells. We have designed and synthesized a novel boronate ester-caged prodrug that is activated by the high H_2O_2 concentration found in cancer cells to release GEM. An H_2O_2-activatable GEM (**A-GEM**) has higher selectivity for H_2O_2 over other ROS and cytotoxic effects corresponding to the H_2O_2 concentration in vitro. A xenograft model of immunodeficient mice indicated that the effect of **A-GEM** was not inferior to that of GEM, when administered in vivo. In particular, myelosuppression was significantly reduced following **A-GEM** treatment compared with that following GEM treatment.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive types of tumor, with a 5-year survival rate of < 5%.[1-3] PDAC is predicted to become the second leading cause of cancer-related death in the United States by 2030.[4] Surgical resection remains the only curative treatment, but only 20% of affected patients are suitable for curative resection, mainly due to rapid growth, high invasiveness, and metastasis.[5] Surgeons have developed novel and improved ways of managing PDAC.[6] Recent reports suggest that adjuvant chemotherapy following curative surgery significantly contributes to prolong the overall patient survival time after surgery to remove the pancreatic cancer.[7,8] Gemcitabine (GEM) is the first-line drug currently available for treating pancreatic cancer in clinics.[9,10] GEM is intracellularly phosphorylated by deoxycytidine kinase to GEM 5'-diphosphate and GEM 5'-triphosphate, which are incorporated into DNA, leading to apoptosis.[11] The common adverse events of GEM include nausea, vomiting, fever, reversible elevation of liver transaminases and peripheral edema.[12] Myelosuppression is the major dose-limiting toxicity[13] and the adverse event lead to therapy discontinuation.[14] GEM is effective against a variety of cancers and is used alone or in combination with other drugs.[15,16] When GEM is used in combination with other chemotherapeutic drugs, reducing the adverse events due to GEM is crucial to reduce overall adverse events. In this study, we aimed to decrease the adverse events due to GEM administration. One reported approach to reducing the toxicities of crosslinking anticancer agents in normal cells is to selectively activate the prodrug in cancer cells.[17] Several molecular-targeted drugs such as imatinib and trastuzumab exhibit promising anticancer activities and few toxic side effects by focusing on the differences between normal and cancer cells.[18,19] Cancer cells have been reported to exhibit abnormalities of metabolism and signal transduction.[20,21] These characteristics of cancer cells could be important in the development of new anticancer strategies.

A higher level of oxidative stress is observed in various cancer cells and tumors and results in the overproduction of reactive oxygen species (ROS).[22-25] H_2O_2 is a well-known ROS and has recently been shown to function as an important secondary messenger in biological systems.[26,27] H_2O_2 has high membrane permeability and is a relatively stable ROS.[28,29] In particular, the higher intrinsic H_2O_2 concentrations in cancer cells induce the expression of growth factors that lead to migration and invasion.[30] Relatively high H_2O_2 concentrations compared to normal cells are a distinctive feature of cancer cells[31,21] and several approaches have been implemented to develop H_2O_2-activatable prodrugs for targeting tumors.[33-35]

In synthetic organic chemistry, alkyl or arylboronic acids and their esters are easily dissociated by H_2O_2. The reaction between boronates and H_2O_2 is bioorthogonal and biocompatible. Indeed, boronated H_2O_2-activatable small molecules have been used for H_2O_2 detection,[36] selective gene activation,[37] and in cancer therapeutics.[33,34] Boronic acids and their esters do not appear to be intrinsically toxic and their hydrolysis end product, boric acid, is considered to be non-toxic to humans.[38] Additionally, we recently reported specific gene silencing using aryl-boronated antisense oligonucleotides.[39]

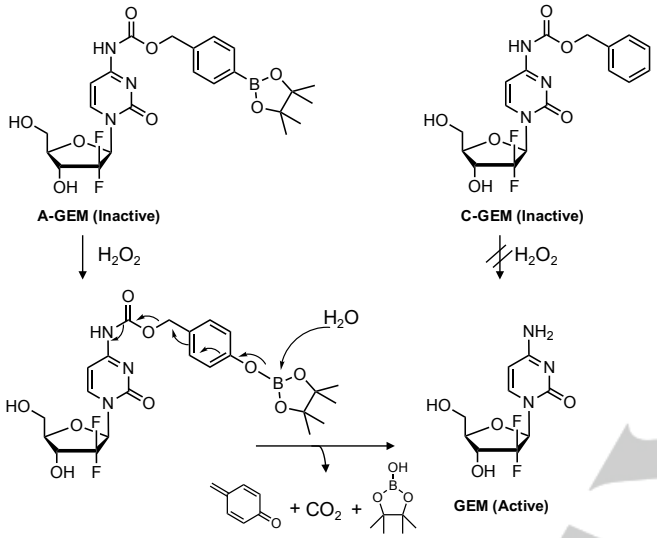
In this study, an H_2O_2-activatable GEM (**A-GEM**) was developed. Under specific conditions in vitro, this novel prodrug demonstrated anti-tumor activity comparable with that of GEM. Moreover, we found that interestingly dosed **A-GEM** exhibited a unique biodistribution and the potential for efficient drug delivery into cancer cells in vivo.

Results

Design and synthesis of H_2O_2-activatable gemcitabine prodrug (A-GEM)

To develop a prodrug of GEM, the enzyme recognition site of GEM must be temporarily masked using a stimulus-responsive chemical structure. Intracellularly, GEM is converted to therapeutically active GEM 5'-diphosphate and 5'-triphosphate[40] metabolites by sequential phosphorylation with multiple kinases. The initial step, also the rate-limiting step, is the monophosphorylation of GEM, which is catalyzed by

deoxycytidine kinase.[11] Enzyme-substrate recognition between deoxycytidine kinase and GEM relies on hydrogen bonding between the 4-NH_2 group of the nucleobase cytosine and Asp 133, located in the active site of the enzyme.[41] Chemically and enzymatically activatable GEM prodrugs have been developed by installing a protecting group onto the 4-NH_2 group.[42-44] Thus, **A-GEM** was also designed by masking the same position in GEM with a boronate ester-based carbamate protecting group (Figure 1). This masking group was expected to be promptly removed under high H_2O_2 concentrations in tumor cells,



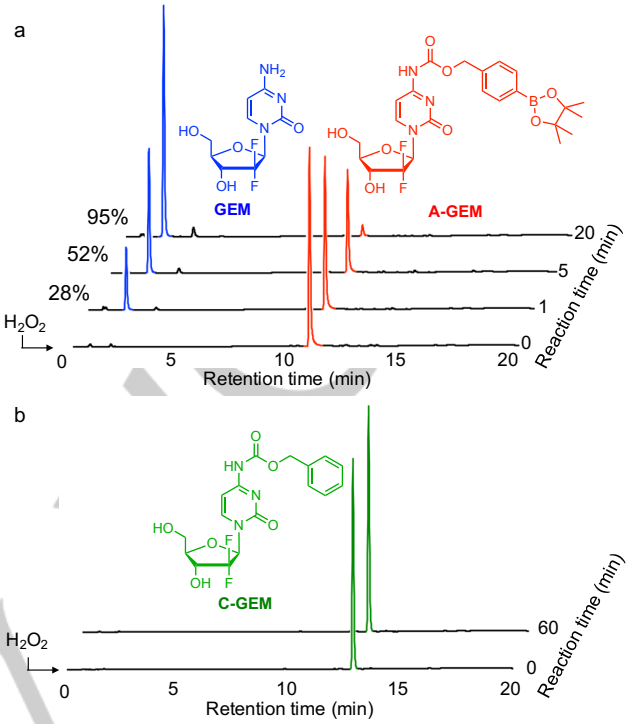
resulting in the release of the active drug. In addition, 4-*N*-Cbz-2'-deoxy-2',2'-difluorocytidine (**C-GEM**)[44] was also designed as a negative control (Figure 1).

Figure 1. Schematic representation of H_2O_2–triggered **A-GEM** activation. H_2O_2–mediated hydrolysis and release of GEM.

The prodrug **A-GEM** was synthesized according to our previous report[39] (Scheme S1). Briefly, to introduce the H_2O_2-responsive boronate ester moiety into the cytosine, a commercially available 4-(hydroxymethyl)phenylboronic acid pinacol ester was used and the benzyl alcohol derivative was converted to an imidazole derivative in two steps. The imidazole derivative was exposed to Meerwein reagent to synthesize a highly reactive imidazolium salt in situ, which was then reacted with TIPDS-protected GEM. TBAF treatment degraded the carbamate moiety, and therefore HF-pyridine for silyl deprotection, to generate **A-GEM**.

H_2O_2-decaging of A-GEM

The conversion yield of **A-GEM** to GEM following H_2O_2 treatment was determined using HPLC analysis. When exposed to an equimolar equivalent of H_2O_2 in sodium phosphate buffer (pH 7.2), virtually all of the **A-GEM** prodrug was converted to GEM within 20 min (95% yield, Figure 2a). In contrast, no conversion or degradation was observed when the negative



control **C-GEM** was exposed to H_2O_2 under similar conditions (Figure 2b).

Figure 2. HPLC chromatograms of **A-GEM** (a) and **C-GEM** (b) after H_2O_2 addition at different time points.

Since H_2O_2 is not the only ROS in human biological systems, we evaluated the activation of **A-GEM** by other ROS, such as *tert*-butylhydroperoxide (TBHP), hypochlorite (ClO^-), hydroxyl radical ($HO\cdot$), tert-butoxy radical ($^tBuO\cdot$), nitric oxide (NO), and superoxide (O_2^-). The activation of **A-GEM** was highly selective for H_2O_2 compared with the aforementioned ROS species (Figure S1). In addition to aforementioned ROS, we also evaluated the activation of **A-GEM** by peroxynitrite ($ONOO^-$), which is a potent oxidant of boronate structures.[45] However, the deprotection of **A-GEM** did not occur in the same reaction conditions. This may be due to the poor chemical stability of $ONOO^-$ in neutral buffer. An equimolecular amount of $ONOO^-$ was not enough to activate the **A-GEM**.

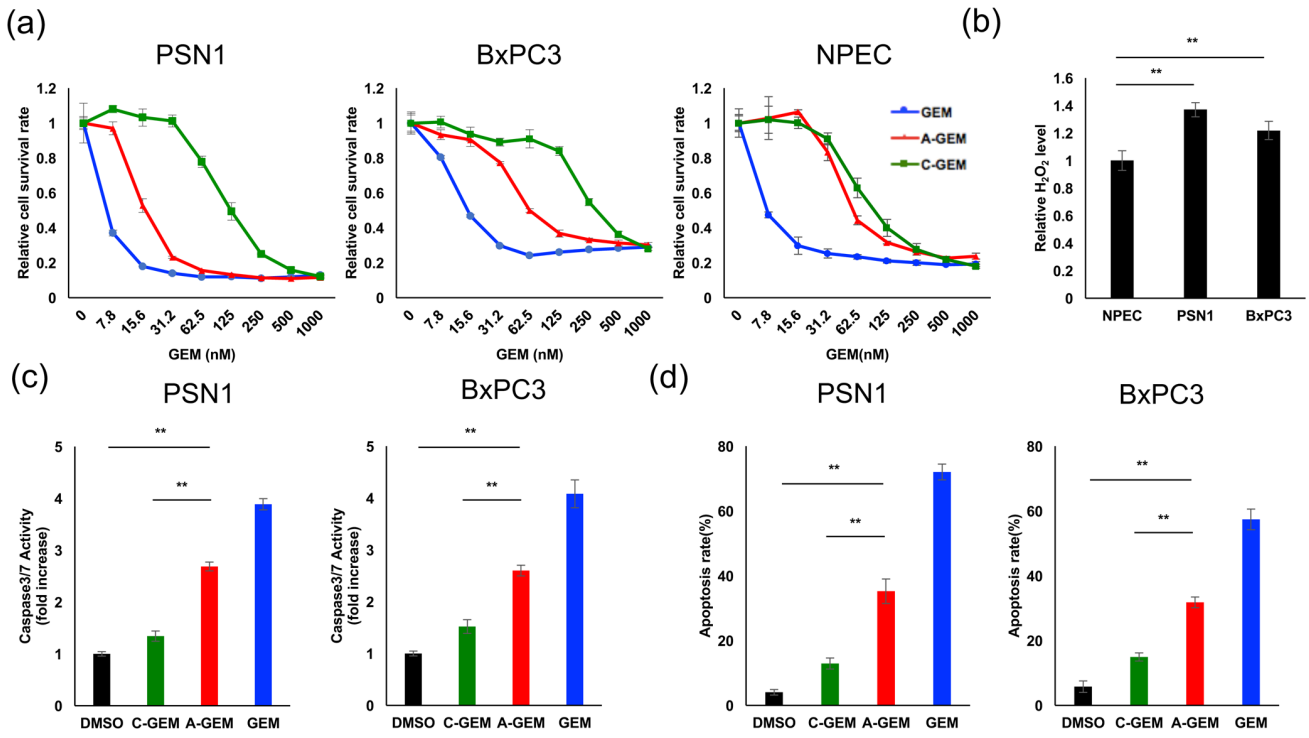


Figure 3. Evaluation of cytotoxicity effect and alteration in apoptosis of GEM, **A-GEM**, and **C-GEM** in each cell line. The mean ± standard deviation (SD) is depicted. Significant differences were observed between the values under the horizontal lines (*P < 0.05, **P < 0.01). (a) Growth-inhibitory effects of each GEM are shown. $IC_{50}$of each GEM, **A-GEM**, and **C-GEM** in each cell line as assessed by the MTT assay. (b) The H_2O_2 level of two PDAC cell lines and NPEC is shown.(c and d) Alterations in apoptosis in cell lines with each GEM for 72 hours with the IC_{50} of **A-GEM.** (c)The fold-change of Caspase3/7 activity is represented. The Caspase3/7 activity of DMSO was set as the control. (d) The ratio of apoptotic cells was determined using the Annexin V assay.

Cytotoxicity of A-GEM in human pancreatic cancer cell lines and normal pancreatic epithelial cell line

Cytotoxicity of GEM, **A-GEM**, and **C-GEM** was evaluated using human pancreatic cancer cell lines (PSN1 and BxPC3) and normal pancreatic epithelial cell line (NPEC) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after 72 h of exposure. As shown in Fig. 3a, all three drugs inhibited cell growth in a dose-dependent manner. Osmotic pressure and other such factors may have affected the cytotoxicity exhibited at high concentrations of **C-GEM**. The cytotoxicity of **C-GEM** may also be due to its partial deprotection in cells independently of H_2O_2. Compared with two pancreatic cancer cell lines, NPEC showed little difference in cytotoxic effect between **A-GEM** and **C-GEM**. The H_2O_2 level of NPEC was smaller than that of PDAC cell lines (Figure 3b). GEM is reported to exhibit cytotoxic effects via the induction of apoptosis. To quantify the level of cellular apoptosis, both caspase-3/7 activation and flow cytometry analysis of annexin V were performed. PDAC cells were exposed to GEM, **A-GEM**, and **C-GEM** for 72 h at the IC_{50} concentration of **A-GEM**. The apoptotic frequency of **A-GEM** was reduced compared to that of GEM. However, **A-GEM** had an increased cytotoxic effect compared to **C-GEM** (Fig. 3c and d). Next, the activity of **A-GEM** was evaluated in vitro in the presence of H_2O_2. The proliferation of PDAC cells alone with H_2O_2 after 72 h was evaluated to determine optimal H_2O_2 concentration required to inhibit this proliferation. Optimal H_2O_2 concentrations for PSN1 and BxPC3 were 250 and 125 μM, respectively (Figure S2a). The cytotoxicity of **C-GEM** in the presence of optimal H_2O_2 concentration was similar to that of **C-GEM** alone. In contrast, the cytotoxicity of **A-GEM** in the presence of H_2O_2 was elevated compared with that of **A-GEM** alone and was similar to that of GEM (Figure S2b).

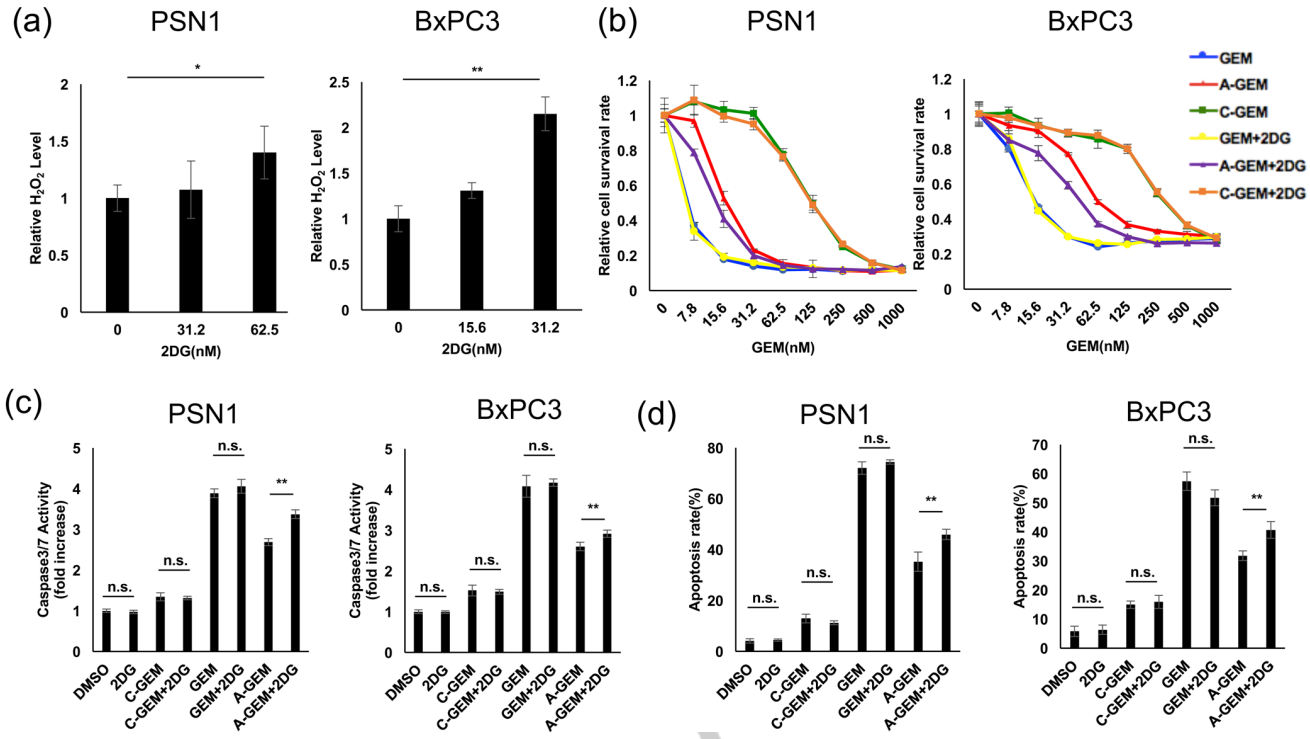


Figure 4. The synergistic effect of 2DG at the optimal concentrations with GEM, **A-GEM**, and **C-GEM** using MTT and apoptosis assays. The mean ± standard deviation (SD) is shown. Significant differences were observed between the values under the horizontal lines (*P < 0.05, **P < 0.01, n.s.; not significant). (a) The 2DG concentration that did not inhibit the proliferation of each cell was determined. (b) Growth inhibitory effects of GEM, **A-GEM**, and **C-GEM** plus 2DG at the optimal concentration are shown. (c and d) Alterations in apoptosis in cell lines with each GEM alone and each GEM plus 2DG for 72 hours with the IC_{50} of **A-GEM**. (c) The fold-change of Caspase3/7 activity is represented. The Caspase3/7 activity of DMSO was set as the control. (d) The ratio of apoptotic cells was determined using the Annexin V assay.

An attempt was made to elevate H_2O_2 concentration in pancreatic cancer cells. Reportedly, glucose deprivation induces oxidative stress in cancer cells in humans. Oxidant production and thiol metabolism disruption consistent with metabolic oxidative stress have also been noted in cancer cells during glucose deprivation or upon treatment with the glucose analogue 2-deoxy-D-glucose (2DG).[46] Initially, the proliferation of pancreatic cancer cells in the presence of 2DG alone was assessed, and subsequently, the 2DG concentration that did not inhibit the proliferation of pancreatic cancer cells was determined. The optimal 2DG concentrations for PSN1 and BxPC3 were 62.5 and 31.2 nM, respectively (Figure S3). Intracellular H_2O_2 concentrations were elevated after administration of 2DG at these optimal concentrations (Figure 4a). We then evaluated the synergistic effect of 2DG at the optimal concentrations with GEM, **A-GEM**, and **C-GEM** using MTT and apoptosis assays. Cytotoxic effects and apoptotic frequencies in the presence of 2DG alone were similar to those in the presence of DMSO as a solvent or mock control. GEM and **C-GEM** did not exhibit a synergistic effect with 2DG; however, **A-GEM** exhibited a synergistic effect with 2DG, as identified using both MTT (Figure 4b) and apoptosis assays (Figure 4c and d).

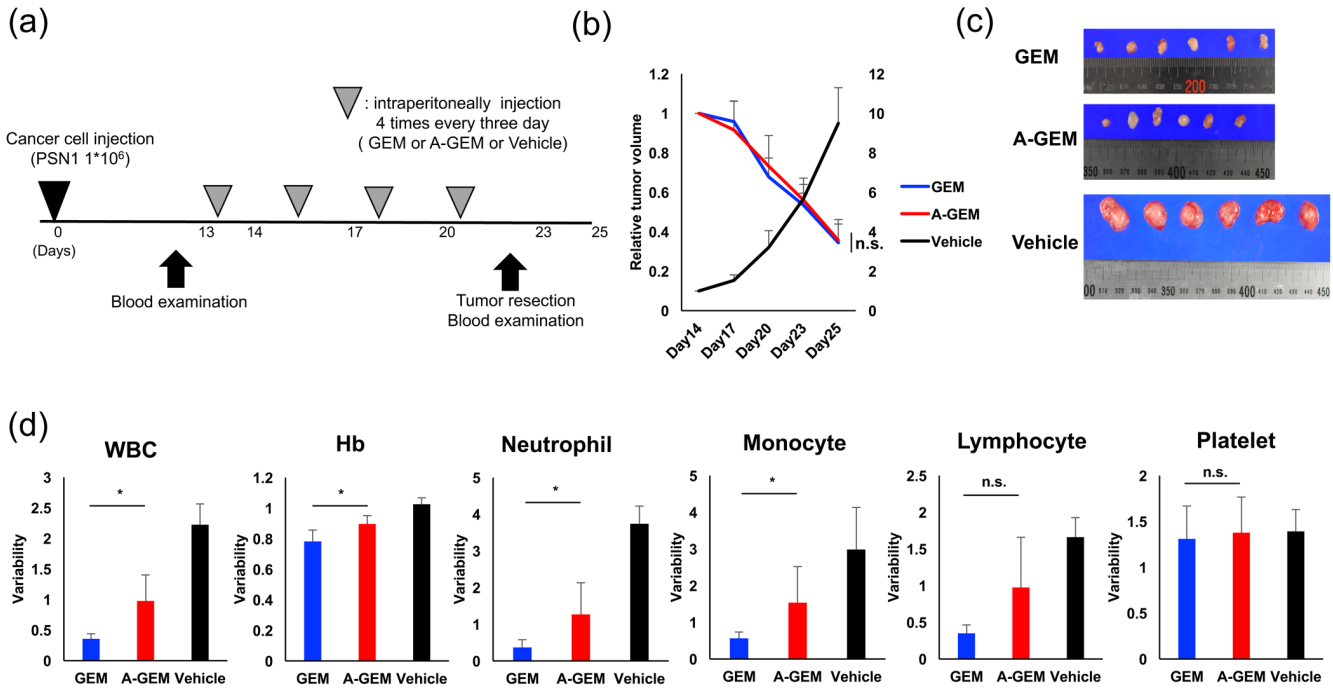


Figure 5. In vivo antitumor activity and adverse events. The mean ± standard deviation (SD) is shown. Significant differences were observed between the values under the horizontal lines (*P < 0.05, ns; not significant). (a) The time course of the animal experiment. (b and c) Tumor volume (b) and the actual tumors (c) are shown. (d) Myelosuppression was evaluated by comparing blood components before and after the treatment. The variability represents mean value ratio after/before treatment. Differences among white blood cells, neutrophil, monocyte, lymphocyte, hemoglobin, and platelet levels before and after the treatment are indicated.

In vivo antitumor activity of A-GEM in a murine xenograft model

Mice were randomly divided into three groups (n = 6 each): GEM, **A-GEM**, and vehicle. The molecular weight of **A-GEM** is twice the molecular weight of GEM. Thus, to equalize doses, the GEM group received 50 mg/kg of GEM and the **A-GEM** group received 100 mg/kg of **A-GEM**. The mice were administered GEM, **A-GEM**, or vehicle intraperitoneally four times on days 14, 17, 20, and 23 (Figure 5a). There was no difference in body weight loss between the GEM and **A-GEM** groups (Figure S4a). **A-GEM** had an antitumor effect compared with the vehicle. There was no difference in tumor volume between the GEM and **A-GEM** groups (Figure 5b and c). Histological findings from the TUNEL assay revealed no differences in apoptotic frequency between the GEM and **A-GEM** groups (Figure S4b).

Very interestingly, myelosuppression was reduced in the **A-GEM** group compared with the GEM group (Figure 5d). To investing the cause, GEM accumulation in tumor and bone marrow was evaluated using LC/MS. A similar amount of GEM was detected in the tumor tissue of both the GEM and **A-GEM**-treated groups. This result suggested that the majority of **A-GEM** was converted to GEM in the tumor. In contrast, in the bone marrow the detected amount of GEM in the **A-GEM** group was lesser than that in the GEM group (Figure S5). Hematopoietic stem cells are located in niches characterized by low levels of ROS, unlike cancer cells.[47,48] The difference in ROS levels, including H_2O_2, in the tissue influenced selectivity and reactivity of **A-GEM**.

Discussion

This study has two important findings. First, **A-GEM** selectively reacted to H_2O_2 among ROS species used in this study. Second, there was a difference in reactivity for **A-GEM** and **C-GEM** in PDAC cells and NPEC. Myelosuppression derived from **A-GEM** in vivo was less severe than that of original GEM. These results indicated that **A-GEM** has the potential to decrease adverse events in pancreatic ductal adenocarcinoma in the clinical setting.

Recent studies suggest that targeting unique biochemical alterations in cancer cells is a feasible approach to achieve both therapeutic activity and selectivity.[38,43] Reactive ROS homeostasis is important for the survival and progression of both normal and cancerous cells. Although certain amounts of ROS are required for proper cell function including normal metabolism and signaling, excessive amounts of them lead to oxidative

stress.[24] The level of ROS in malignant cells is higher than that in normal cells. Among ROS, H_2O_2 is comparatively stable and one of the principal ROS members. Although H_2O_2-activatable prodrugs have been reported so far, to the best of our knowledge, this is the first example for a prodrug of gemcitabine bearing the phenyl boronate moiety. The protecting group of **A-GEM** was expected to be promptly removed under high H_2O_2 concentrations in tumor cells, resulting in the release of the active drug.

In this study, we demonstrated the H_2O_2 level in PDAC cells was larger than in normal pancreatic epithelial cell. The difference of H_2O_2 level may be comparatively small between PDAC cells and NPEC because NPEC was not normal completely by being immortalized with Human Papilloma Virus. We were unable to quantify H_2O_2 in cancer cells and normal cells in vivo, but we successfully demonstrated the cytotoxicity and selectivity of **A-GEM** by evaluating apoptosis and cell viability in vitro and in vivo. It seems that **A-GEM** was converted to GEM intracellularly because its cytotoxicity was increased when the amount of H_2O_2 in the cancer cell was raised in vitro. **A-GEM** had an antitumor effect and induced apoptosis in vivo. In addition, the amount of **A-GEM** was equivalent in cancer tissues compared to GEM, whereas in the bone marrow tissue the amount was alleviated by using mass spectrometry. Hematopoietic stem cells are located in niches characterized by low levels of ROS, unlike cancer cells.[47,48] The difference in ROS levels, including H_2O_2, in the tissue influenced selectivity and reactivity of **A-GEM**. This indicated that **A-GEM** also functions as an active form in vivo. Although we have not determined the limiting tolerable dose of **A-GEM**, it is considered that there is a possibility that antitumor effect can be enhanced with the same degree of side effects as GEM by further increasing the amount of **A-GEM**.

Conclusions

A-GEM selectively reacted to H_2O_2 rather than other ROS. In vitro experiments showed **A-GEM** had less cytotoxicity in NPEC than in PDAC cells by reacting H_2O_2 level. **A-GEM** induced certain apoptosis than **C-GEM** and DMSO. **A-GEM** increased cytotoxicity in PDAC cell lines by H_2O_2 production induced by 2DG treatment, while **C-GEM** and DMSO did not increase. **A-GEM** also showed anticancer effect equivalent to that of GEM when it was administered to a xenograft model of immunodeficient mice. In addition, it exhibited reduced myelosuppression compared to GEM, suggesting that H_2O_2-targeted modifications can improve the selectivity of cytotoxic chemotherapeutic reagents. We anticipate numerous applications of **A-GEM**, given its unique combination of high efficacy and low toxicity.

Experimental Section

General

Purchased reagents and solvents were used without purification unless otherwise specified. All experiments involving air and/or moisture sensitive compounds were conducted under an argon atmosphere. All reactions were monitored using analytical TLC (Merck Kieselgel 60 F254; Merck, Darmstadt, Germany). Flash column chromatography was carried out using an EPCLC–W–Prep 2XY (YAMAZEN, Osaka, Japan). NMR spectra were obtained using a JNM–ECS–400 spectrometer (JEOL, Tokyo, Japan) using $CDCl_3$ or DMSO–d_6 as a solvent with tetramethylsilane as an internal standard. IR spectra were obtained using a FT/IR–4200 spectrophotometer (JASCO, Tokyo, Japan). Optical rotations were obtained on a JASCO P–2200 instrument. FAB mass spectra were obtained using a JEOL JIM–700 mass spectrometer. ESI mass spectra were obtained using a Xevo G2-XS QTof (Waters, Milford, MA, USA).

H_2O_2-decaging of A-GEM

A reaction solution comprising **A-GEM** (1 mM) and H_2O_2 (1 mM) in a DMSO-containing buffer (10 mM potassium phosphate, pH 7.2, 100 mM NaCl, and 5% (v/v) DMSO) was incubated at room temperature for the prescribed duration and immediately subjected to reverse-phase HPLC analysis using MeCN in 0.1 M triethylammonium acetate buffer (pH 7.0) as a solvent. The conversion rate of **A-GEM** was determined from the corresponding peak area monitored at 260 nm.

Other ROS-decaging of A-GEM

A reaction solution of **A-GEM** (1 mM) and reactive oxygen species (1 mM) in a DMSO-containing buffer (10 mM potassium phosphate, pH 7.2, 100 mM NaCl, and 5% (v/v) DMSO) was incubated at room temperature for 12 h and immediately subjected to reversed-phase HPLC analysis using MeCN in 0.1 M triethylammonium acetate buffer (pH 7.0). *tert*-Butylhydroperoxide (TBHP) and hypochlorite (NaOCl) were delivered from 70% and 10% aqueous solutions respectively. Hydroxyl radical (HO·) and *tert*-butoxy radical (^{t}BuO·) were generated by the reaction of 5 mM $(NH_4)_2Fe(SO_4)_2$, 10 mM EDTA with 1 mM H_2O_2 or TBHP, respectively. Nitric oxide (NO) was generated from PROLI NONOate. Superoxide (O_2^-) was produced by xanthine oxidase (4.5×10^{-3} mg/100 μL) in the presence of hypoxanthine (2 mM) and catalase (0.4 mg mL^{-1}). Peroxynitrite ($ONOO^-$) was delivered from NaOH aqueous solution and the concentration of $ONOO^-$ was determined using the absorption at 300 nm (ε = 1670 $M^{-1}cm^{-1}$). The solution was diluted with phosphate buffer and used immediately. The conversion rate of **A-GEM** was determined from the corresponding peak area monitored at 260 nm.

Cell culture

Pancreatic cancer cell lines used in this study (PSN1 and BxPC3) were procured from the Japan Cancer Resource Bank (Tokyo, Japan). Normal pancreatic epithelial cell (NPEC) was procured from Addexbio Technology (San Diego, USA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), and incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.

Cell proliferation assay

To assess the proliferation and sensitivity of pancreatic cancer cells to 2DG (FUJIFILM Wako Chemical Corporation, Osaka, Japan), they were incubated in the presence of several concentrations of 2DG for 72 h in a 96-well plate. Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan).

Growth inhibition assay and determination of cell viability

Growth inhibition was assessed using MTT assay as previously described.[49] In brief, cells were incubated for 72 h under varying concentrations of GEM and cell viability was then evaluated using MTT assay. The results were expressed as the percentage of absorbance relative to that of the untreated controls.

Apoptosis assay

To quantify the level of cellular apoptosis, both caspase-3/7 activation and flow cytometric analyses of annexin V were performed. PDAC cells were exposed for 72 h to GEM, **A-GEM**, or **C-GEM** at the 50% inhibitory concentration (IC_{50}) of **A-GEM**. Caspase-3/7 activity was evaluated using the caspase-Glo® 3/7 Assay Kit from Promega (Madison, WI, USA) and relative luminescence (RLU) was measured using the GloMax® Microplate Luminometer (Promega). Apoptotic cells that were stained with annexin V-APC (Biolegend Research Products, San Diego, CA, USA) or propidium iodide (BioVision Research Products, Milpitas, CA, USA) were enumerated using flow cytometry using a BD FACS CantoTM II system (BD Biosciences, SanJose, CA, USA).

H_2O_2 quantification assay

H_2O_2 was quantified using a ROS-Glo H_2O_2 assay kit (Promega) and relative luminescence (RLU) was measured using a GloMax® Microplate Luminometer (Promega). The H_2O_2 quantification of PDAC cell lines and NPEC was evaluated 12 hours after seeding each cell not to change the counts of cells.

In vivo experiments

This study was approved by the Animal Experiments Committee, Osaka University (approval number: 30-011-008). It was performed in accordance with the National Institute of Health guidelines for the use of experimental animals. Eight-week-old, female, congenitally athymic nude mice (nu/nu) with immunodeficiency were purchased from CLEA Japan and were maintained in a pathogen-free environment. For xenografting, PSN1 cells (1×10^6 cells) were subcutaneously transplanted in 100 µL PBS/Matrigel (BD Biosciences). Subcutaneous tumor volume was calculated as follows: (greatest diameter) × (shortest diameter)^2 × 0.5. Treatments with all GEM and vehicle were initiated when the tumor volume reached 60–100 mm^3. Each GEM was dissolved in 20 µL of ethanol, 80 µL of PEG, and 100 µL of physiological saline. Each mouse was administered a total of 200 µL of drug solution. Tumor size and body weight were measured every 3 days. Xenograft tumors were collected after sacrificing the mice on day 25, and TUNEL assay was performed on tumor tissue sections. All tumors of mice were fixed in 10% formaldehyde for 24 h, embedded in paraffin, and sectioned into 4-µm slices. We stained the tumor tissue sections of mice with hematoxylin and eosin. Then, the apoptosis assay was performed using a TdT-mediated dUTP nick end labeling (TUNEL) kit (Cat. No. G3250, Promega) according to the manufacturer's instructions. Finally, nuclei were visualized using DAPI-containing mounting medium (Cat. No. H-1200, Vector Laboratories, Burlingame, CA, USA). The images were captured using a BZ-8000 microscope (Keyence, Tokyo, Japan). Myelosuppression was evaluated by comparing blood components before and after the treatment. Myelosuppression was evaluated using a Vetscan HM2 Analyzer (ABAXIS, Union City, CA). The counts of white blood cells, neutrophils, monocytes, lymphocytes, and platelets and the level of hemoglobin before and after treatment were evaluated to observe whether any hazardous effects occurred in the examined conditions. The results represent mean value ratio after/before treatment.

Statistical analysis

Differences were considered statistically significant at $P \leq 0.05$. All statistical analyses were performed using SPSS 20.0 software (IBM Japan, Tokyo, Japan).

Sample preparation for LC/MS analysis

Snap-frozen tissues obtained at necropsy were weighed; Deionized water (100 µL) was added to homogenize the sample using a Violamo Homogenizer Mixer VH-10 (AS ONE, Osaka, Japan). Deionized water (200 µL), methanol (100 µL), and chloroform (200 µL) were added to homogenized tissue (50 mg) and the resultant sample was incubated at 70 °C for 10 min with vigorous shaking. Vortex mixing was followed by centrifugation at 19000 rpm for 15 min, and the resulting supernatant was concentrated by freeze-drying. The residue was reconstituted in 200 µL of water, of which 10-µL samples were used for LC/MS.

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Keywords: prodrugs • chemoselectivity • gemcitabine • pancreatic cancer • chemotherapy

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Contribution

The individual contributions were as follows:

KM[ab], TO[c], HI[d]: evaluation for feasibility and efficacy of the compound in vitro and in vivo.

TO[c], SO[ce]: synthesis and monitoring by mass spectrometry of the compound

SM[c], SO[ce]: synthesis of the compound and evaluation of chemical stability

MK[ab], TO[c], HI[d]: evaluation of the compound in mice.

TO[c], SM[c], SO[ce]: design and synthesis of the compound

KM[ab], MK[bd], HE[a], TS[a], YD[a], MM[a], HI[d], SO[ce]: study conception

KM[ab], TO[c], SM[c], MK[bd], YK[ce], KM[ce], SO[ce]: study design for the compound

KM[ab], HE[a], AA[bd], JK[bd], YI[a], DY[a], HA[a], TA[a], TN[a], KK[a], KG[a], SK[a], HI[d]: Study design for feasibility

KM[ab], HE[a], AA[bd], JK[bd], YI[a], DY[a], HA[a], TA[a], TN[a], KK[a], KG[a], SK[a], SO[ce]: Study design for efficacy

KM[ab], HI[d]: in vitro study

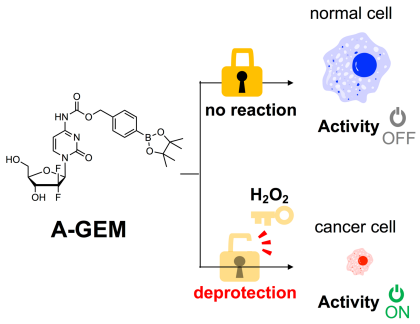
KM[ab], TO[c], MK[bd], HI[d]: In vivo study

KM[ab], TO[c], SM[c], MK[bd]: acquisition of data

All authors: analysis and interpretation of the data

The compound were continuously designed and synthesized with in vitro and in vivo feedback data, and two authors (HI and SO) were equally corresponded in this study. The synthesis, chemical quality check, in vitro efficacy check, and in vivo efficacy check were performed in parallel with data feedback and KM, TO, SM, MK were equally contributed in this study.

Entry for the Table of Contents



This article reports on the development of a new gemcitabine prodrug (**A-GEM**), which has an arylboronate moiety at the N^4-position of cytosine nucleobase. **A-GEM** was activated in the presence of hydrogen peroxide (H_2O_2) that is found in cancer cells, and it showed less myelosuppression in an immunodeficient model mouse compared to gemcitabine while maintaining the anti-tumor activity.