Novel nitric oxide donor, nitrated phenylbutyrate, induces cell death of human pancreatic cancer cells and suppresses tumor growth of cancer xenografts

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Abstract. Pancreatic cancer has a low response rate to chemotherapy due to the low drug transferability caused by the low blood flow around the tumor. In the present study, focusing on nitric oxide (NO) for its vasodilatory and antitumor effects, a novel NO donor, a nitrated form of phenylbutyrate (NPB) was synthesized and the antitumor effect on human pancreatic cancer cells (AsPC1 and BxPC3) and xenografts was examined. Using Annexin V, NPB was confirmed to induce cell death against AsPC1 and BxPC3 in a time- and concentration-dependent manner. In NPB-exposed cells, DAF-FM DA (a probe to detect intracellular NO) derived fluorescence was observed. Release of nitrite and nitrate from NPB in aqueous solution was very gradual until even 72 h after dissolution. Phenylbutyrate (PB) and hydroxy PB in which the nitro group of NPB was replaced with a hydroxyl group did not have the cell death-inducing effect as observed in NPB. These results suggest that the effect of NPB was dependent on NO release form NPB. Apoptosis inhibitor, Z-VAD FMK, had no effect on the cell death-inducing effect of NPB, and NPB did not show significant activation of caspase-3/7. In addition, NPB significantly decreased cellular ATP levels, suggesting that necrosis is involved in the effect of NPB. NPB also accumulated cells specifically at the S phase of the cell cycle. A single dose of NPB (10 mg/kg) into mice with established BxPC3 xenografts significantly suppressed tumor growth for at least 7 weeks without apparent toxicity. The findings of the present study indicate that NPB has potential as a novel therapeutic agent for NO-based therapy of pancreatic cancer.

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Abbreviations: NO, nitric oxide; NPB, nitrated form of phenylbutyrate; NOx, nitrite and nitrate

Key words: nitric oxide, pancreatic cancer, necrosis, NO donor, cell cycle

Introduction

Pancreatic cancer is a highly metastatic cancer with a poor prognosis, causing >300,000 deaths annually (1). Currently, gemcitabine and 5-fluorouracil are the standard chemotherapy regimens for pancreatic cancer, and combinations such as gemcitabine plus nanoparticle albumin-bound paclitaxel and FOLFIRINOX (5-FU, leucovorin, irinotecan, oxaliplatin) therapy are also used. However, the response rate to chemotherapy is very low, with a 5-year survival rate of <10% (2,3). This is due to the low drug transferability into the tumor, because the blood flow in the pancreas and its tumor are very low and, the formation of stroma around the tumor forms a barrier (4,5).

Nitric oxide (NO) is an important biosignaling molecule that regulates various physiological and pathological responses, and is involved in maintaining blood pressure (6), balancing thrombus and thrombolytic homeostasis (7) and suppressing inflammatory responses (8). On the other hand, high concentrations of NO act in an inhibitory manner against the growth of cancer cells. In the past two decades, various NO donor drugs have been synthesized and have attracted attention for their anti-malignant tumor effects. NO-donating nonsteroidal anti-inflammatory drugs (NO-NSAIDs), (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1,2-diolate (DETA/NONOate), and sodium nitroprusside (SNP) induce apoptotic cell death (9-11), and S-nitroso-N-acetyl-DL-penicillamine (SNAP) induces apoptotic and necrotic cell growth (12). In addition, O^2-3-aminopropyl diazeniumdiolate has been reported to inhibit tumor invasion and metastasis (13). S-nitrosylated human serum albumin has been reported to shrink peritumoral stroma (14). Thus, although various efficacies of NO donor drugs have been reported to date, none have reached the stage of clinical use. One of the reasons for this is that the half-life of NO or NO donor drugs is very short, and its effects are transient.

Phenylbutyrate (PB) is an orphan drug used for the treatment of urea cycle disorders. Previously, it was determined that PB binds to human serum albumin (HSA) with a high affinity (15,16). By binding drugs and endogenous substances such as fatty acids, HSA is able to maintain their blood retention and control tissue distribution. Recently, a drug delivery system utilizing this property was developed. Detemir and degludec are insulin analogues and liraglutide is a glucagon-like peptide 1, which are acylated with fatty acids. Binding of the fatty acid moieties of these to HSA improves their kinetic properties without affecting the affinity for their receptors (17-19). In fact, they have been reported to exhibit a significant sustainable pharmacodynamic effect due to protraction of the half-life in blood in clinical use. Moreover, in tumor tissue, vascular permeability is significantly higher than in normal tissue. In addition, because the lymphatic system is not well developed, substances that reach the tumor tissue accumulate (20). This characteristic is called the enhanced permeation and retention (EPR) effect and is an important factor for passive targeting of cancer cells. Therefore, macromolecules such as HSA are more likely to flow out from tumor blood vessels. Kinoshita *et al* reported that S-nitrosylated HSA tends to accumulate in tumor tissue (14). These findings suggest that the nitrated form of phenylbutyrate (NPB) and HSA complex selectively migrates to and accumulates in tumors.

In the present study, to develop a sustained NO donor drug with an antitumor effect for treatment of pancreatic cancer, NPB based on chlorambucil, a PB analogue, was synthesized and the effects *in vitro* and *in vivo* were investigated.

Materials and methods

Reagents and antibodies. PB was purchased from TCI (Shanghai) Development Co., Ltd. Chlorambucil was obtained from Tokyo Chemical Industry (TCI) Co., Ltd. Dihydroxy chlorambucil was purchased from SynZeal Research Pvt Ltd. Z-VAD-FMK was obtained from Promega Corporation. Diaminofluorescein-FM diacetate (DAF-FM DA) was purchased from Goryo Chemical, Inc. Necrostatin and N-acetyl-L-cysteine (NAC) were procured from FUJIFILM Wako Pure Chemical Corporation. GSK872 was obtained from Abcam. Necrosulfonamide was purchased from Funakoshi Co., Ltd. Antibodies against caspase-3 (product no. 9662S), caspase-7 (product no. 9492S), poly (ADP-ribose) polymerase (PARP)-1 (product no. 9542S), CHOP (product no. 2895S), β-actin (cat. no. 3700S), and HRP-conjugated anti-rabbit (product no. 7074P2) were purchased from Cell Signaling Technology, Inc.

Synthesis of NPB (4-(4-(bis(2-(nitrooxy)ethyl)amino) phenyl)butanoic acid). A mixture of chlorambucil (250 mg, 0.822 mmol) and $AgNO_3$ (558 mg, 3.29 mmol) in CH_3CN (16 ml) was stirred at 70˚C overnight. After being cooled to room temperature, the suspension was filtered and the solvent was evaporated. The residue was purified by flash column chromatography on silica gel (DCM/MeOH, 99:1 to 92:8, v/v) to yield NPB (Fig. 1) (254 mg, 0.711 mmol, 86% yield) as a pale yellow oil. ^{1}H-NMR (500 MHz, $CDCl_3$): δ=7.10 (d, J=8.6 Hz, 2H), 6.66 (d, J=8.6 Hz, 2H), 4.60 (t, J=6.0 Hz, 4H), 3.71 (t, J=5.7 Hz, 4H), 2.59 (t, J=7.4 Hz, 2H), 2.37 (t, J=7.4 Hz, 2H), 1.90-1.96 (m, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$): δ=179.8, 144.1, 131.2, 129.8, 112.9, 69.9, 48.9, 33.8, 33.2, 26.3. MS (ESI): m/z calculated for $C_{14}H_20N_3O_8$ $[M+H]^+$ 358.1250, found 358.1241.

Cell culture. The human pancreatic cancer cell lines, AsPC1 (CRL-1682) and BxPC3 (CRL-1687), were obtained from the American Type Culture Collection. The cells were cultured in the recommended medium, consisting of RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10% heat-inactivated fetal calf serum (Capricorn Scientific GmbH), penicillin (100 U/ml), and streptomycin (100 μg/ml) (FUJIFILM Wako Pure Chemical Corporation), and grown at 37˚C in 95% humidified air with 5% carbon dioxide.

Cell death. Live and apoptotic cell numbers were determined using the MUSE Annexin V and Dead Cell kit (Luminex Corporation) according to the manufacturer's instructions. Briefly, AsPC1 and BxPC3 cells were seeded in a 6-well plate with $1x10^5$ cells per well, and then, incubated at 37˚C overnight. Following incubation, the cells were exposed with various concentrations of NPB (100, 300, and 500 μM) for 48 h or with 500 μM for 24, 48 and 72 h. Necrostatin, GSK872, Necrosulfonamide and NAC were used at concentrations of 20, 1 and 1 μM and 1 mM respectively. After treatment, the cells were washed twice with phosphate-buffered saline (PBS), trypsinized, and mixed well with the Muse Annexin V and Dead Cell Assay kit reagents. Reactions, which were conducted in triplicate, and analyzed using a MUSE Cell Analyzer (Luminex Corporation).

Effect of NPB on growth of spheroids. AsPC1 and BxPC3 were seeded in round bottom 96-well plates with $5x10^5$ cells per well. After confirming the formation of spheroids, 500 M of NPB was added to the each well, and then incubated for 1 week at 37˚C. Spheroid structure and the spheroid area were analyzed by fluorescence microscopy.

NO release from NPB. The nitrite and nitrate (NOx) levels were quantified by the Griess method [NO_2/NO_3 Assay kit-C II (Colorimetric); Dojindo Laboratories, Inc.]. The NOx levels were assessed at 0, 3, 24, 48, 72 and 96 h after 100 μM of NPB was dissolved in PBS containing 10% MeOH. Samples were read at 540 nm in a 96-well plate using a Spectra Microplate Auto reader (Bio-Rad Model 680; Bio-Rad Laboratories, Inc.). For microscopic observation, AsPC1 and BxPC3 cells were seeded in a 6-well plate with $5x10^5$ cells per well. Following overnight incubation, the culture medium was replaced with 10 μM of DAF-FM DA, and then cells were incubated at 37˚C for 1 h. After incubation, cells were washed with PBS three times. Following washing, the cells were treated with 500 μM NPB for 5 min and observed using a fluorescence microscope. For assessment of fluorescence intensity of DAF-FM DA, cells were seeded in a black 96-well plate with $5x10^4$ cells per well. Following overnight incubation at 37˚C, the culture medium was replaced with 10 μM DAF-FM DA, followed by incubation at 37˚C for 1 h. Following incubation, the cells were washed with PBS three times. After washing, the cells were treated with 500 μM of NPB with PBS for 5 min and measured by using fluorescence plate reader (ex. 495 nm and em. 515 nm).

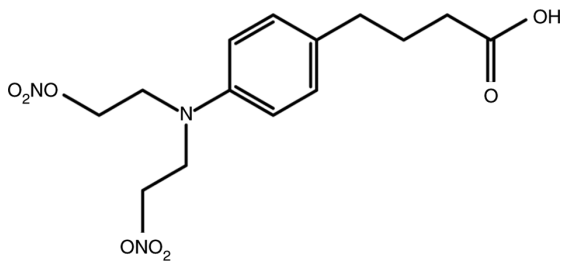

Figure 1. Chemical structure of NPB (4-(4-(bis(2-(nitrooxy)ethyl)amino) phenyl)butanoic acid). NPB, nitrated form of phenylbutyrate.

Caspase-3/7 activity. BxPC3 cells were seeded in a black 96-well plate with $1x10^4$ cells per well. Following overnight incubation at 37˚C, the cells were treated with 500 μM of NPB for 6 and 24 h. Subsequently, 100 μl of Caspase-Glo 3/7 Reagent (Promega Corporation) was added to each well. After mixing gently, the cells were incubated at room temperature for 1 h. Finally, luminescence of each sample was measured by a multifunctional microplate reader (Infinite 200 Pro; Tecan Group, Ltd.).

Western blotting. BxPC3 cells were seeded in a 6-well plate with $5x10^5$ cells per well. After overnight incubation at 37˚C, the cells were treated 500 μM of NPB for 6 and 24 h. Following treatment, the cells were lysed with RIPA buffer (Thermo Fisher Scientific, Inc.), including a Protease/Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.). Protein concentration of each lysate was determined by BCA method. Aliquots of protein (30-40 μg) were subjected to SDS-PAGE (12% of acrylamide for PARP and 10% for caspase-3/7 and β-actin), transferred to a polyvinylidene difluoride, (PVDF) membrane. After blocking with 5% skim milk in PBS including 0.1% Tween-20 for 3 h at 37˚C, the membrane was processed for incubation with caspase-3, caspase-7, PARP, CHOP or β-actin antibody (all 1:1,000) for 12 h at 4˚C, followed by anti-rabbit IgG antibodies for 1 h at 4˚C. Membranes were reacted with a chemiluminescence reagent (GE Healthcare UK Ltd.; Cytiva). Band density values were normalized to β-actin.

Assessment of the intracellular adenosine 5'-triphosphate (ATP) levels. BxPC3 cells were seeded in a white 96-well plate with $1x10^4$ cells per well. Following overnight incubation at 37˚C, cells were treated with 500 μM of NPB for 48 h, and then 100 μl of intracellular ATP Reagent (TOYO-B Net Co., Ltd.) was added to each well. After being gently mixed, luminescence of each sample was measured using a plate-reading luminometer (Infinite 200 Pro; Tecan Group, Ltd.). To correct for variations in cell number, the protein content of each sample was measured using the BCA protein assay kit (Thermo Fisher Scientific, Inc.) and the ATP content was normalized to the protein content.

Cell cycle. For the cell cycle analysis, propidium iodide-based nuclear staining was carried out using Muse Cell Cycle Kit (Luminex Corporation) according to the manufacturer's protocol. Briefly, AxPC1 and BxPC3 cells were cultured in a 6-well plate for 24 h, and then treated with 500 μM of NPB for 24 h. After the treatment, cells were fixed in 70% ethanol and stored at -20˚C for at least 3 h. Fixed cells were washed with cold PBS and centrifuged at 300 x g for 5 min and stained using a Muse™ Cell Cycle Kit for 30 min at room temperature in dark conditions. After the staining, analysis was performed by Muse Cell Analyzer (Luminex Corporation).

Animal studies. A total of 10 male, six-week-old BALB/c nude mice (20-25 g) were obtained from Japan SLC, Inc., and raised in a laminar mouse house, with 50±5% humidity, 25˚C and a 12-h light/dark cycle. The mice were fed standard rodent food and mineral water. BxPC3 cells ($5x10^6$ cells/mouse) were s.c. injected into the right flank. When tumor volumes reached 50 mm^3, NPB (10 mg/kg) or saline were administered via the tail vein once. Following treatment, tumor formation was monitored by measuring the width and length of the mass, and the tumor volume (TV) was calculated as follows: TV (mm^3)=(L x W^2)/2, with L as the longest and W as the shortest radius of the tumor. Animals were euthanized by cervical dislocation after seven weeks from the administration of NPB. The death of the animals was confirmed by checking for cardiac arrest, decreased body temperature, and no movement. All experiments were approved by the Animal Ethics Committee of Sojo University (Kumamoto, Japan) and carried out according to the Laboratory Protocol for Animal Handling of Sojo University.

Statistical analyses. For continuous variables, unpaired Student's t-test or one-way analysis of variance (one-way ANOVA) was performed. The pairwise t-test with Holm's adjustment for post hoc test was employed after one-way ANOVA. In addition, a linear mixed model was used to analyze longitudinal data such as tumor volume and body weight. The mean ± standard deviation was used to present statistical outcome.

These analyses were performed using R version 4.0.3 (The R Foundation for Statistical Computing; https://www.r-project.org/foundation/). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell death-inducing effect of NPB. First, the effects of NPB on the cell death of human pancreatic cancer cells were examined. Human pancreatic cancer cell lines, AsPC1 and BxPC3, were exposed to 500 μM NPB for 24-72 h (Fig. 2A and B) or 100-500 μM for 48 h (Fig. 2C and D) and the number of Annexin-positive cells was determined. In both cell lines, NPB significantly induced cell death in a time- and concentration-dependent manner. Next, the effect of NPB on the growth inhibition of AsPC1 and BxPC3 spheroids was examined. Even 7 days after the addition of 500 μM of NPB, spheroid growth was significantly inhibited in both cell lines compared to their controls. In addition, disruption of spheroid surface structure was observed in AsPC1 cells (Fig. 3).

NO release from NPB. NO radical detecting agent, DAF-FM DA, was used to examine intracellular NO release from NPB. As revealed, more cells in the NPB-exposed cells had DAF-FM DA-derived fluorescence compared with the control, indicating that NO radical is generated within NPB-exposed cells

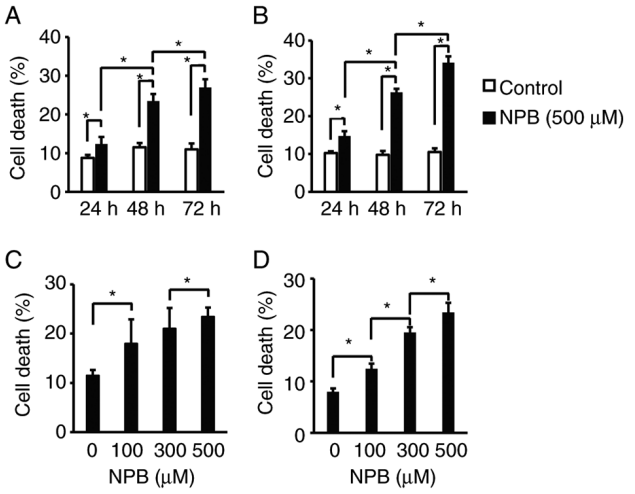

Figure 2. NPB induces death of pancreatic cancer cells *in vitro*. (A) AsPC1 and (B) BxPC3 cells were cultured with 500 μM NPB for 24-72 h. (C) AsPC1 and (D) BxPC3 cells were cultured with 100-500 μM NPB for 48 h. The percentage of cell death was determined by flow cytometry. The averages and SD of three separate experiments are shown, *P<0.05. Data were analyzed using one-way ANOVA and the pairwise t-test with Holm's adjustment. NPB, nitrated form of phenylbutyrate.

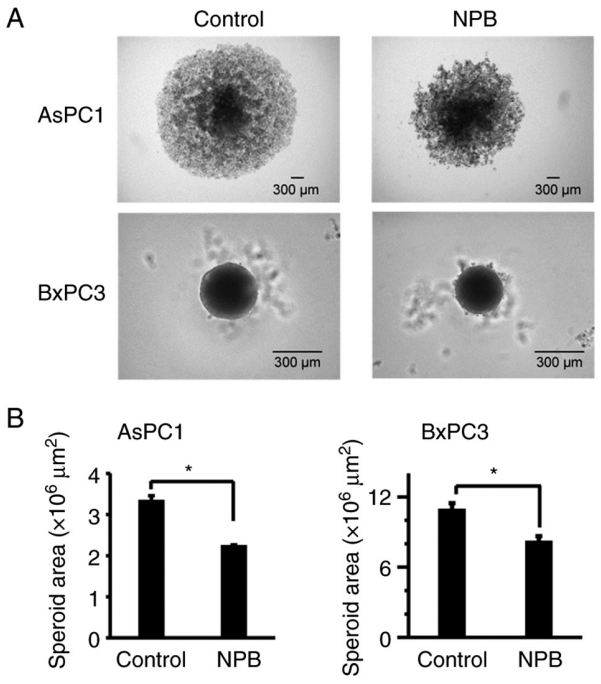

Figure 3. Effect of NPB on the growth of spheroids. AsPC1 and BxPC3 cells were cultured in U-well plates to form spheroids and treated with 500 μM NPB for 1 week. (A) Spheroid structure and (B) spheroid area were analyzed using fluorescence microscopy For B, averages and SD of three separate experiments are shown, *P<0.05 vs. Control. Data were analyzed using Student's t-test. NPB, nitrated form of phenylbutyrate.

(Fig. 4A and B). It is known that a part of nitrite ions released from the NO-donor compound are reduced to NO under anaerobic conditions in tumors but are oxidized to nitrate ions under aerobic conditions. Quantitative assessment of NOx released from NPB was performed (Fig. 4C). Notably, increased NOx was observed at least up to 96 h after dissolving NPB in PBS. Moreover, no cell death-inducing effect was observed for

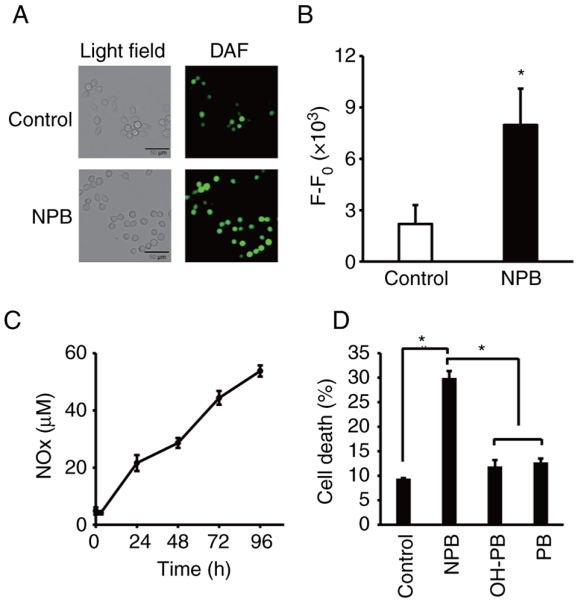

Figure 4. NO release from the NPB. (A) Levels of intracellular NO were determined by fluorescence microscopy using the NO fluorescent probe DAF-FM DA. Scale bar, 50 μm. (B) Levels of intracellular NO were determined using DAF-FM DA staining and a fluorescence plate reader (ex. 495 nm; em. 515 nm). Data were analyzed using the Student's t-test. (C) Levels of NOx produced from 50 μM NPB through 96 h after dissolution in PBS Averages and SD of three separate experiments are shown, *P<0.05. (D) BxPC3 cells were cultured in 500 μM NPB, OH-PB, or PB for 48 h. Data were analyzed using one-way ANOVA and pairwise t-test with Holm's adjustment. NO, nitrix oxide; NPB, nitrated form of phenylbutyrate; DAF-FM DA, diaminofluorescein-FM diacetate; NOx, nitrite and nitrate; PB, phenylbutyrate.

OH-PB, in which the NO_2 moiety of NPB was replaced by an OH group, and PB (Fig. 4D), suggesting that NO released from NPB was mainly involved in the cell death-inducing effect of NPB.

Cell death mechanism of NPB. Cell death is induced by various pathways, including apoptosis and necrosis. To date, NO donors have been reported to induce apoptosis (9-11,23-25). To investigate the involvement of caspase, which plays a central role in apoptosis, the cell death effects of NPB in the presence of a caspase inhibitor, Z-VAD-FMK (Fig. 5A) were examined. The results revealed no significant effect of Z-VAD-FMK on cell death induction by NPB. Assessment of caspase-3/7 activity after NPB exposure exhibited no activation at 6 h or 24 h after the addition of NPB (Fig. 5B). Results of western blotting also showed no degradation of PARP or caspase-3/7 (Fig. 5C). These results indicated that apoptosis was not involved in the induction of cell death by NPB. Necrosis is characterized by cell swelling and a decrease and depletion of intracellular ATP (26). Thus, the amount of ATP after the addition of NPB was determined. A total of 48 h after addition of NPB, the amount of ATP was significantly decreased compared with the control (Fig. 5D), suggesting cell death was due to necrosis. Necrostatin, GSK872 and Necrosulfonamide, which are necroptosis inhibitors, did not suppress the cell death effect by NPB (Fig. S1).

Effects of NPB on the cell cycle. Since NPB was observed to inhibit cell proliferation (Fig. S2), the effect of NPB on the cell cycle was examined and it was determined that cells exposed to

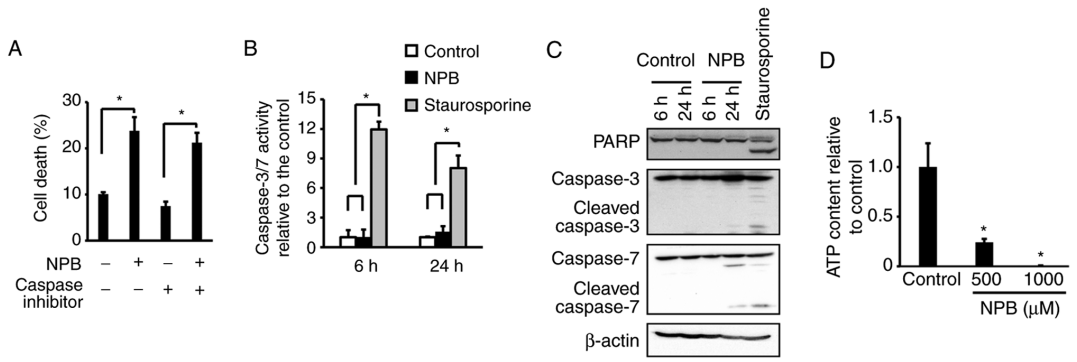

Figure 5. Mechanism of NPB-induced cell death. (A) Effect of Z-VAD-FMK, a pan-caspase inhibitor, on death of NPB-treated BxPC3 cells. The cells were treated with 10 μM Z-VAD-FMK and 500 μM NPB for 48 h. (B) Activation of caspase-3/7 in BxPC3 cells after treatment with 500 μM NPB for 6 and 24 h. (C) Western blot analysis of PARP, caspase-3, and caspase-7 in BxPC3 cells after treatment with 500 μM NPB for 6 and 24 h. (D) Content of intracellular ATP in BxPC3 cells after treatment with 500 and 1,000 μM NPB for 48 h. Averages and SD of three separate experiments are shown, *P<0.05). In A, B and D, data were analyzed using one-way ANOVA and the pairwise t-test with Holm's adjustment. NPB, nitrated form of phenylbutyrate; PARP, poly (ADP-ribose) polymerase; ATP, adenosine 5'-triphosphate.

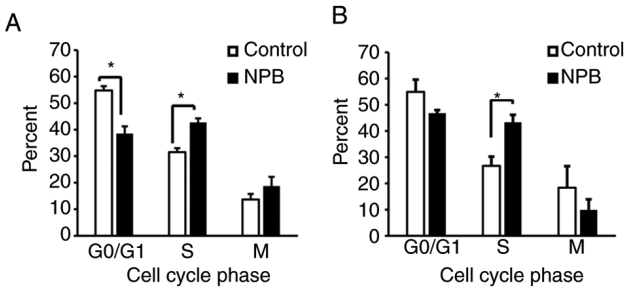

Figure 6. NPB arrests the cell cycle. Effect of NPB on the cell cycle in (A) AsPC1 and (B) BxPC3 cells. Cells were treated for 6 and 24 h with 500 μM NPB, and the cell cycle phase distribution was determined by flow cytometry. Averages and SD of three separate experiments are shown. Data were analyzed using Student's t-test. NPB, nitrated form of phenylbutyrate. *P<0.05.

NPB for 24 h exhibited a significant accumulation of S-phase cells compared with the control (Fig. 6). This indicated that NPB also induced cell cycle arrest.

Antitumor effect of NPB in vivo. The antitumor effect of NPB was investigated *in vivo* (Fig. 7). Tumor-bearing mice with BxPC3 tumors (50 mm^3) implanted subcutaneously were prepared, and after a single dose of NPB (10 mg/kg) by intravenous tail injection, the tumor volumes and body weights were evaluated. There were no significant differences in body weight and no mice succumbed during the observation period. Notably, significant tumor suppression was observed even up to 7 weeks after NPB administration compared with the control group.

Discussion

Pancreatic cancer is known to have a poor response rate to chemotherapy due to low drug distribution caused by low blood flow and abundant stroma around the tumor. In addition to the vasodilation effect of NO, it has also been revealed to induce cancer cell death, suppress stromal tissue fibrosis, and shrink stromal cells themselves (21-25). Therefore, NO donors are anticipated as new anticancer drugs for the treatment of pancreatic cancer.

In vitro, NPB caused cell death in a time- and concentration-dependent manner without activation of caspase-3/7, degradation of PARP, indicating that apoptosis is not the major pathway of cell death by NPB. On the other hand, NPB caused a marked decrease in intracellular ATP, suggesting that necrosis is mainly involved in cell death by NPB. In fact, in the flow cytometric assay with Annexin, NPB increased the population of late apoptosis, which is characteristic of necrotic cells (Fig. S3). Necrosis is recognized as unregulated cell death, but recently, necroptosis, a form of necrosis regulated by receptor-interacting protein kinase (26), has emerged as another mechanism of cell death. However, significant suppressive effects of necroptosis inhibitors (Necrostatin, GSK872 and Necrosulfonamide) were not observed on the cell death effects by NPB (Fig. S1). Moreover, the factors that caused the induction of cancer cell death by NPB were investigated. Reactive oxygen oxide species (ROS) become more reactive nitrogen species (RNS), by reacting with NO. RNS oxidizes and nitrates biomolecules (27) such as nucleic acids, proteins and lipids, causing various intracellular events such as endoplasmic reticulum stress (28) and autophagy, followed by cell death. No effect was identified using ROS scavenger, NAC, on the effect of NPB, and the endoplasmic reticulum stress marker, CHOP, was not observed in BxPC3 cells exposed to NPB (Fig. S4). NPB not only causes cell death but also inhibits cell proliferation. In fact, it has been reported that nitrated aspirin causes accumulation of S-phase cells (29), and a similar tendency was also observed in NPB. The fact that NPB exerts a cancer cell death effect in a cell cycle-dependent manner suggests that the effect of NPB is marked on cancer cells which are under active cell proliferation. In addition, in the present study, toxicity to non-tumor cells was not evaluated, but the cell cycle results suggest that NPB has a selective effect on cancer cells with a fast cell cycle. In an experiment using sodium nitrite, which has low cell membrane permeability, sodium nitrite did not exhibit significant toxicity to pancreatic cancer cells. This result suggests that NO is released from NPB after NPB is uptaken into the cell membrane (data not shown).

To determine a preclinical antitumor effect, the effect of NPB *in vivo* was evaluated using a BxPC3 xenograft model. Single-dose administration of NPB significantly inhibited

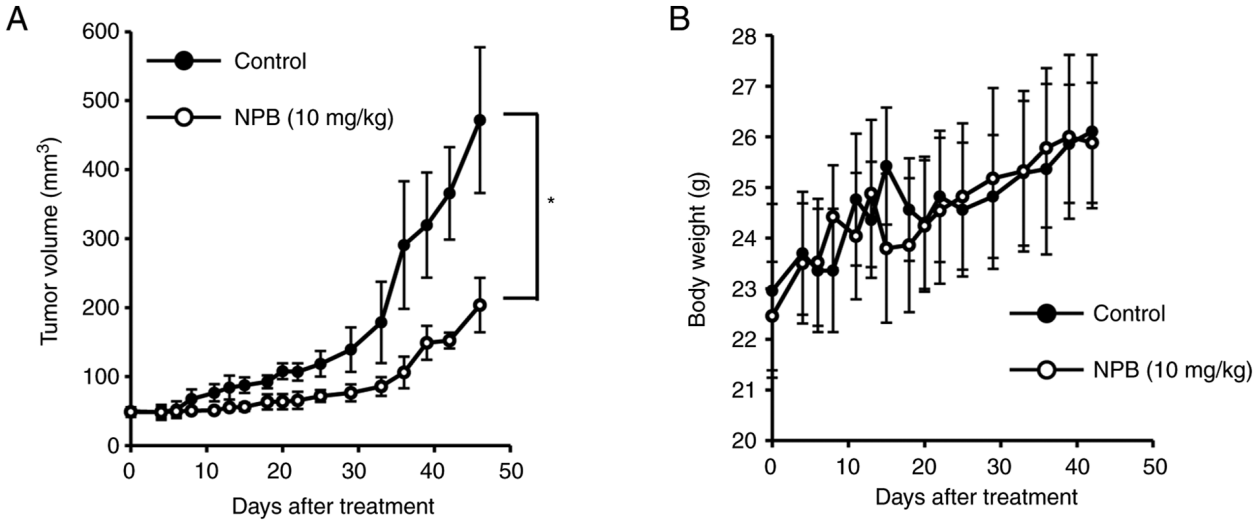

Figure 7. Antitumor effect of NPB *in vivo*. BxPC3 cells ($5x10^6$ cells/mouse) were injected s.c. into the right flank of each mouse. BALB/c nude mice received s.c. injections in the right flank with BxPC3 cells ($5x10^6$ cells/mouse). When the tumor volume reached 50 mm^3, one-time treatment with intravenous NPB (10 mg/kg) or an equal volume of saline was administered. (A) The tumor volumes and (B) body weights were measured (5 animals each for NPB and saline treatments). Averages and SD of three separate experiments are shown, *P<0.001 vs. Control. Data were analyzed using a linear mixed model. NPB, nitrated form of phenylbutyrate.

tumor growth up to 7 weeks without no significant change in body weight. In general, the retention of small molecule compounds in blood is low. In fact, numerous chemotherapeutic drugs have caused various side effects due to drug delivery to non-targeted tissues. Therefore, focusing on the high affinity of PB to HSA (15), an NO donor compound, NPB, was newly designed to bind to HSA. Notably, it was determined that NPB has an equivalent HSA binding property as PB (Table SI). The binding of NPB to HSA is also considered to be effective in selectively transporting drugs to tumors using the EPR effect. Although the binding of NPB to mouse albumin has not been confirmed, it is considered that NPB also has high binding to mouse albumin because the homology of mouse albumin with HSA is extremely high. Moreover, there is another issue in NO donor compounds, which is that the half-life of NO itself is also very short. Interestingly, NPB released NOx very gradually even up to 96 h after dissolution, whereas numerous NO compounds release most of their NOx immediately after dissolution in aqueous solution (30-34). Combined with the prolonged elimination half-life of NPB and the EPR effect by binding to HSA, gradual NOx release, and the long-term growth inhibitory effect observed in spheroid experiments, these findings demonstrated the preclinical antitumor effect as observed *in vivo*.

In the present study, a novel nitric compound, NPB, was successfully synthesized and it was revealed that NPB is a new type of chemotherapeutic agent, unlike conventional nitro compounds. The cell death-inducing effect of NPB on pancreatic cancer cells is comparable or milder than that of other nitro compounds. However, NPB is characterized by its affinity for human serum albumin and the release of NOx is extremely slow. Therefore, these are considered to be a great advantage of NPB in terms of blood retention and tumor accumulation. Indeed, it was observed that the antitumor effect of NPB on the xenograft lasted much longer despite a single dose. Further detailed antitumor mechanisms *in vitro* and *in vivo* are required for clinical application.

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Availability of data and materials

The data that support the findings of the present study are available from the corresponding author, KY, upon reasonable request.

Authors' contributions

TB contributed to the experiments, the design of this study, data collection, interpretation, and wrote the initial draft of the manuscript. KN contributed to the design of this study, and data collection and interpretation, and wrote the initial draft of the manuscript. SI contributed to the synthesis and the structural validation of NPB. WA, IS, AU, NS and YI contributed to data collection. TI contributed to the statistical analysis. MO and KY contributed to the design of this study, interpretation, and critically reviewed the manuscript. KN and KY confirm the authenticity of all the raw data. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Sojo University (Kumamoto, Japan) and was carried out according to the Laboratory Protocol for Animal Handling of Sojo University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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