Ibuprofen-derived nitric oxide donors with a high affinity to human serum albumin induce cell death in pancreatic cancer cells through a non-caspase 3/7-mediated pathway

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Abstract. Nitric oxide (NO) has been reported to have a cytotoxic effect on various types of cancer. However, the efficient delivery of NO donors to tumors remains challenging. The present study used ibuprofen, which has a high binding affinity to human serum albumin (HSA). A total of two types of nitrated forms of ibuprofen, 4-[(nitrooxy) methyl]benzyl 2-(4-isobutylphenyl)propanoate [nitrated ibuprofen benzyl linker (NIB)] and 2-(nitrooxy)ethyl 2-(4-isobutylphenyl) propanoate [nitrated ibuprofen ethyl linker (NIE)], were synthesized. It was demonstrated that both NIB and NIE bound to the ibuprofen-binding site of HSA. Although NOx release was observed from NIB, but not NIE, intracellular NO release was detected from both NIB and NIE, which indicated that the mechanisms of NO release may be different for NIB and NIE. Both NIB and NIE induced concentration- and time-dependent cell death in human pancreatic cancer cells, whereas this cell death was not observed with ibuprofen, which could suggest that these cell death-inducing effects may be mediated by NO. The non-specific caspase inhibitor, z-VAD-FMK, inhibited cell death induced by NIB and NIE, but activation of caspase 3/7 was not observed. These results suggested that both NIB and NIE induced cell death through a non-caspase 3/7 pathway. The findings of the present study demonstrated that both NIB and NIE, as NO donors that could be retained in blood, may potentially be useful anti-cancer agent candidates in the future.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in Japan in 2022 and the third leading cause of cancer-related death in the United States in 2021 (1,2). Patients with pancreatic cancer have a high mortality rate, with a 5-year relative survival rate of <8% in Japan in 2022 (1). Standard chemotherapy regimens for pancreatic cancer include tegafur, gimeracil and oteracil potassium, a combination of folinic acid, fluorouracil, irinotecan and oxaliplatin (3) and gemcitabine plus nab-paclitaxel (4). However, the anti-tumor effects of these treatment regimens are weaker than for other types of solid tumor, such as colon, stomach, lung and breast tumors. One of the reasons for this disparity is the inefficient drug delivery to pancreatic tumors, reflecting both the low blood flow around the pancreatic tumor and the inherent low vascularity of the pancreas (5,6). In addition, tumor-associated stroma forms a barrier to drug delivery from the blood vessels to the tumor (5,6).

Nitric oxide (NO) has broad effects on the development, progression and metastasis of cancer (7). Over the past 20 years, a number of studies have reported the cell death-inducing effects of nitro compounds, showing that these effects are mediated through intracellular signaling pathways such as Ras, extracellular signal-regulated kinase and mechanistic target of rapamycin (8,9). Modification of aspirin, a non-steroidal anti-inflammatory drug, with a nitro group has been shown to significantly enhance the induction of apoptosis in human pancreatic, colon, prostate, lung and tongue cancer cells (9). Similarly, the addition of a nitro group to doxorubicin weakens the activity of mitochondrial-related ABC transporters, which leads to decreased drug resistance to doxorubicin and increased cytotoxicity (10). A previous study by Islam *et al* (11) reported the improved efficacy of anticancer agents when they were combined with nitroglycerin treatment. Ishima *et al* (12) also reported that exposure to NO depletes stroma, which leads to enhanced extravasation and retention (EPR) effects. These findings showed that nitro compounds not only enhance the therapeutic effects of other anticancer agents, but also have therapeutic effects of their own. However,

most NO donors exhibit low blood retention and low tumor accumulation, which may be the reason for their lack of therapeutic efficacy (11).

Human serum albumin (HSA) is a type of drug-binding protein in the blood that controls tissue migration and the blood retention of bound drugs (13). Insulin and glucagon-like peptide 1 preparations that bind to HSA maintain their pharmacological effects by improving their retention in the blood (14-17). Therefore, it could be hypothesized that NO donors with a high affinity to HSA not only improve retention in the blood, but efficiently reach tumors due to the EPR effect, and the presence of HSA receptors on the tumor cell surface (SPARC and gp60) promotes the transfer of HSA-bound NO donors to tumors (18). Ibuprofen (IB), a non-steroidal anti-inflammatory drug, binds to HSA, and its binding with HSA has been previously reported (19). In the present study, two nitrated forms of ibuprofen were synthesized and their antitumor effects and mechanisms of action were investigated.

Materials and methods

Cell culture and reagents. The human pancreatic cancer cell line, BxPC3, was obtained from the American Type Culture Collection. The cells were cultured in RPMI1640 (FUJIFILM Wako Pure Chemical Corporation), supplemented with 10% heat-inactivated fetal calf serum (Capricorn Scientific), penicillin (100 U/ml) and streptomycin (100 μg/ml) (FUJIFILM Wako Pure Chemical Corporation) and incubated at 37˚C in 95% humidified air with 5% CO_2. Ibuprofen (IB) and warfarin were purchased from FUJIFILM Wako Pure Chemical Corporation. Dansylsarcosine (DNSS) was purchased from Sigma-Aldrich (Merck KGaA). All other reagents were of analytical grade.

Synthesis of 4-[(nitrooxy)methyl]benzyl 2-(4-isobutylphenyl) propanoate [nitrated ibuprofen benzyl linker (NIB)] and 2-(nitrooxy)ethyl 2-(4-isobutylphenyl)propanoate [nitrated ibuprofen ethyl linker (NIE)]. A mixture of 4-(chloromethyl) benzyl alcohol (1.2 g; 7.66 mmol) and $AgNO_3$ (5.2 g; 30.6 mmol) in CH_3CN (12 ml) was stirred at 40˚C for 16 h. After cooling to room temperature, the white precipitate was removed by filtration and the filtrate was evaporated. The residue was dissolved in CH_2Cl_2, the resulting white precipitate was removed again by filtration and the filtrate was evaporated. The resulting residue of 4-(hydroxymethyl)benzyl nitrate (7.66 mmol) was dissolved in CH_2Cl_2 (35 ml), and ibuprofen (790 mg; 3.83 mmol), dicyclohexylcarbodiimide (1.2 g; 5.75 mmol) and 4-dimethylaminopyridne (46 mg; 0.38 mmol) were added to the aforementioned solution and stirred at room temperature for 17 h. After dilution with 50 ml of CH_2Cl_2, the mixture was washed with saturated aqueous $NaHCO_3$ and brine (saturated NaCl aqueous solution), the residual water was removed using $MgSO_4$ and subsequently evaporated. The residue was purified by flash column chromatography using silica gel (hexane/ethyl acetate=8/1 v/v) to give NIB (1.08 g; 76%). NIE was synthesized according to a previous report (20) (Fig. 1). ^1H and ^{13}C NMR spectra were recorded on a JEOL ECA 500 spectrometer operating at room temperature. Chemical shifts were reported in parts per million (δ) relative to the residual solvent peak. Multiplicities were described as singlet (s), doublet (d), doublet of doublets (dd), triplet (t) or multiplet (m). Coupling constants (J) were reported in hertz (Hz).

Binding of NIB and NIE to HSA. To obtain the binding parameters of IB to HSA, the ultrafiltration method was used. Ultrafiltration was performed using Amicon Ultra-0.5 ml Centrifugal Filters (10 kDa cutoff; 500 μl) from Merck KGaA. After centrifugation at 5,000 x g for 30 min at 25˚C, the concentration of IB in 50 μl of filtrate, also known as the concentration of unbound drug to HSA, was measured by HPLC. The HPLC system used in the present study was a Jasco model LC-2000Plus HPLC system (JASCO Corporation). YMC-PACK ODS AM-303 (5 μm particle size; 250x5 mm internal diameter; YMC Co. Ltd.) was used as the stationary phase and was maintained at 40˚C. A total of two solvents, solvent A (50 mM sodium dihydrogen phosphate) and solvent B [50 mM sodium dihydrogen phosphate and acetonitrile (30:70, v/v)] were used as mobile phases at a flow rate of 1 ml/min. Binding parameters were obtained from the Scatchard plot using the following equation: $r/[D_f]=-r \times K_a + n \times K_a$. K_a, n, $[D_f]$ and r were the binding affinity, the number of binding sites of IB, the concentration of unbound IB to HSA and the number of moles of IB bound per mole of HSA, respectively.

Fluorescent probe displacement. Warfarin (WF) and dansylsarcosine (DNSS) were used as site I and site II fluorescent probes on HSA, respectively (21). Fluorescence spectra of probes were measured using a Hitachi F-2500 fluorescence spectrophotometer at 25˚C (Hitachi, Ltd.). The concentrations of WF, DNSS and HSA used were 10 μM. The excitation wavelengths used for WF and DNSS were 320 and 350 nm, respectively.

Evaluation of NO-releasing properties. NO production in aqueous solution was evaluated by measuring nitrite (NO_2^-) and nitrate (NO_3^-), the stable end-products of NO breakdown, using the NO_2/NO_3 Assay Kit-C II (Dojindo Laboratories, Inc.). Briefly, NO_2^- and NO_3^- (NOx) were measured according to the manufacturer's protocol at 1, 2, 3, 24 and 48 h after dissolving 100 μM of NIB or NIE in PBS. For the detection of NO inside cells, cells were seeded in a 6-well culture plate at $5x10^5$ cells/well and cultured overnight at 37˚C. The medium was replaced with PBS containing diaminofluorescein-FM diacetate (DAF-FM DA; 10 μM; Goryo Chemical, Inc.) and cultured at 37˚C for 1 h. After culturing, cells were washed three times with PBS, treated with NIB or NIE (200 μM) for 5 min at room temperature and imaged using a fluorescence microscope.

Annexin V and dead cell assay. Live and apoptotic cell numbers were determined using the Muse® Annexin V and Dead Cell kit (Cytek Biosciences) according to the manufacturer's instructions. Briefly, cells were seeded at a density of $2x10^5$ cells/well in 6-well plates. After 12 h, 50, 100 or 200 μM of NIE or NIB was added to each well and the plates were incubated for 6, 12 and 24 h at 37˚C. The cells were then washed twice with PBS, trypsinized (FUJIFILM Wako Pure Chemical Corporation) at 37˚C until cells were detached and mixed well with the Muse® Annexin V and Dead Cell Assay kit reagents. Samples were measured using a Muse® Cell Analyzer (MuseSoft; Version 1.5.0.0; Cytek Biosciences).

Table I. Binding constants and the number of binding sites of IB, IB + NIB and IB + NIE.

	IB	IB + NIB	IB + NIE
Ka ($x10^6 M^{-1}$)	2.11±0.65	0.49±0.12	1.32±0.17
n	0.98±0.07	1.30±0.15	1.00±0.03

NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; IB, ibuprofen; Ka, binding constant. n: the number of the binding sites on HSA

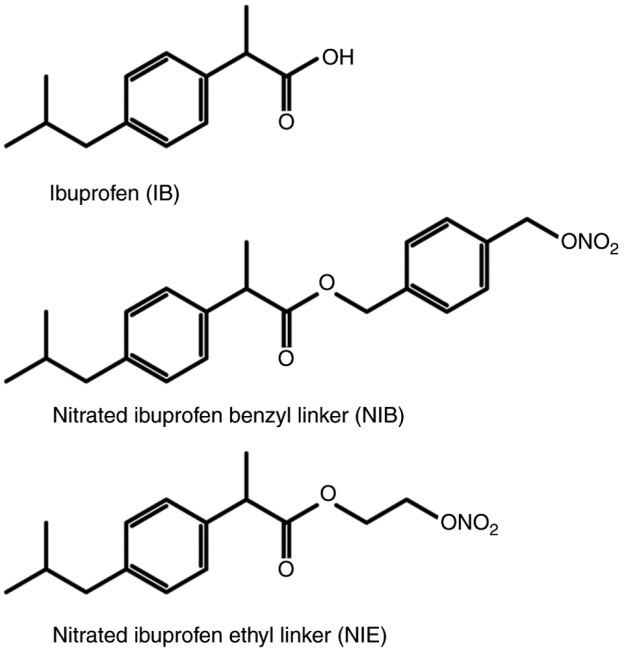

Figure 1. Chemical structures of IB, NIB, and NIE. IB, ibuprofen; NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker.

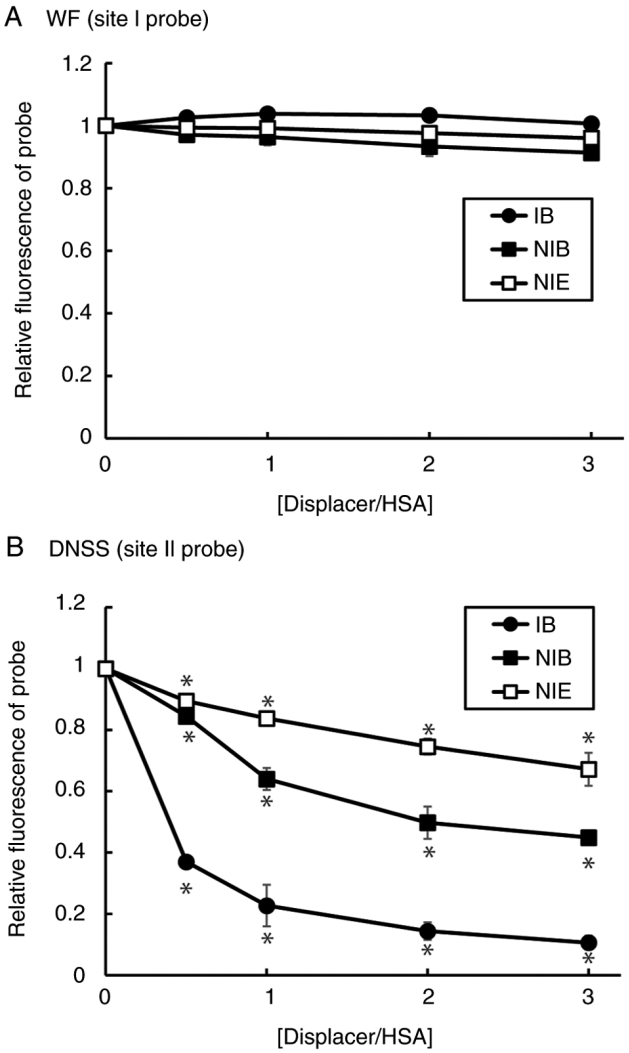

Figure 2. Effects of IB, NIB and NIE on the fluorescence intensity of (A) WF and (B) DNSS. Fluorescence intensities of 10 μM concentrations of WF and DNSS were measured in the presence of 10 μM of HSA and a number of concentrations (0-30 μM) of IB, NIB and NIE. Each value represents the ratio of fluorescence intensity in the presence or absence of a displacer (IB, NIB or NIE). Data are the mean ± SD (n=3). *P<0.05 vs. each value in the absence of displacer. IB, ibuprofen; NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; WF, warfarin; DNSS, dansylsarcosine; HSA, human serum albumin.

Lactose dehydrogenase (LDH) assay. LDH is a cytoplasmic enzyme that it is released into the cell culture medium when the cell membrane is damaged. As the released LDH is stable in cell culture medium, it can be used as an indicator of the number of dead cells or cells with damaged cell membranes (8). Cell toxicity was evaluated using the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories, Inc.) according to the manufacturer's protocol. Briefly, cells were seeded at a density of $3x10^3$ cells/well in 96-well white, flat-bottom plates. After overnight incubation at 37˚C, the cells were incubated with NIB (200 μM) or NIE (200 μM) at 37˚C for 72 h. Subsequently, the cells were incubated for 1 h at room temperature with the LDH reagent and luminescence was measured. Cytotoxicity (%) was estimated using the ratio between the concentration of LDH in the culture medium and the whole cell.

Caspase 3/7 activity assay. Cellular caspase 3/7 activity was measured using the Caspase-Glo 3/7 Reagent (Promega Corporation). Briefly, cells were seeded at a density of $1x10^4$ cells/well in 96-well plates. After incubation with NIB (200 μM), NIE (200 μM) or staurosporine (1 μM) for 6 h at 37˚C, Caspase-Glo 3/7 Reagent was added to each well. After incubation at room temperature for 1 h, the luminescence of each sample was measured using a plate-reading luminometer.

Statistical analysis. The mean values of each group were compared using a one-way ANOVA followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. Assays were conducted in triplicate.

Results

Synthesis of NIB and NIE. NIB and NIE were successfully synthesized. The chemical structure of NIB was confirmed by NMR (Fig. S1). ^{1}H-NMR results were as follows: (500 MHz, CHLOROFORM-D) δ 7.32 (d, *J*=8.0 Hz, 2H), 7.24

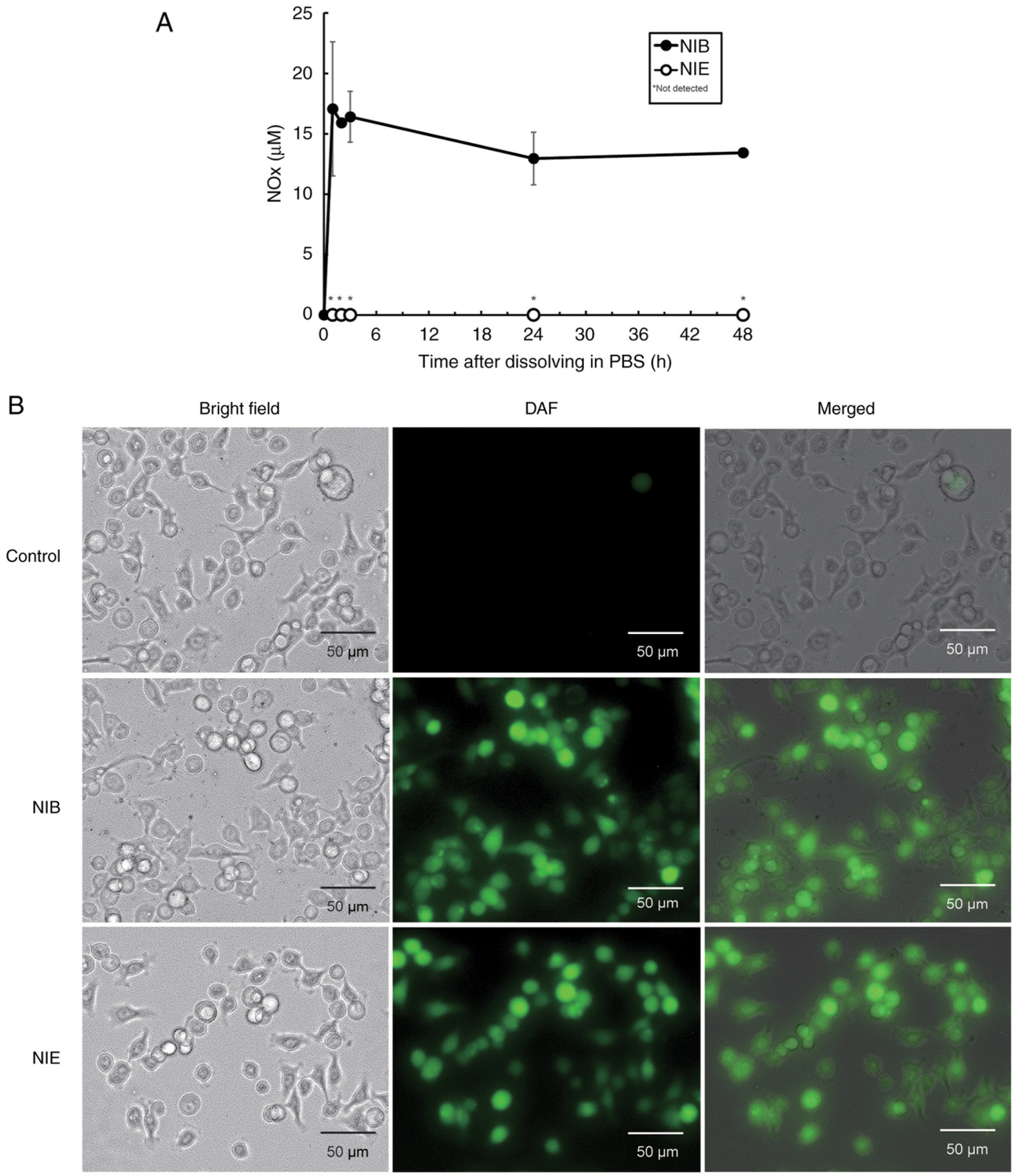

Figure 3. NOx and NO release from NIB and NIE. (A) Levels of NOx from 200 μM of NIB and of NIE were measured to 96 h after dissolution in PBS. (B) Intracellular NO was detected using DAF-FM DA and fluorescence microscopy. Data are the mean ± SD (n=3). NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; DAF-FM DA, diaminofluorescein-FM diacetate; NO, nitric oxide; NOx, nitrate and nitrite.

(d, *J*=8.0 Hz, 2H), 7.19 (d, *J*=8.0 Hz, 2H), 7.09 (d, *J*=8.0 Hz, 2H), 5.40 (s, 2H), 5.11 (s, 2H), 3.76 (q, *J*=7.3 Hz, 1H), 2.46 (d, *J*=7.4 Hz, 2H), 1.88-1.83 (m, 1H), 1.51 (d, *J*=7.4 Hz, 3H) and 0.91 (d, *J*=6.9 Hz, 6H). ^{13}C-NMR results were as follows: (126 MHz, CHLOROFORM-D) δ 174.6, 140.8, 137.7, 137.6, 132.1, 129.5, 129.3, 128.2, 127.3, 74.5, 65.8, 45.3, 45.1, 30.3, 22.5 and 18.5.

Binding of NIB and NIE to HSA. To evaluate the binding properties of NIB and NIE to HSA, a quantitative displacement experiment was performed (Table I). Both NIB and NIE significantly decreased the binding constant of IB to HSA, but they had no significant effect on the number of binding sites. These results demonstrated that both NIB and NIE competitively inhibited the binding of IB to HSA. Fluorescence

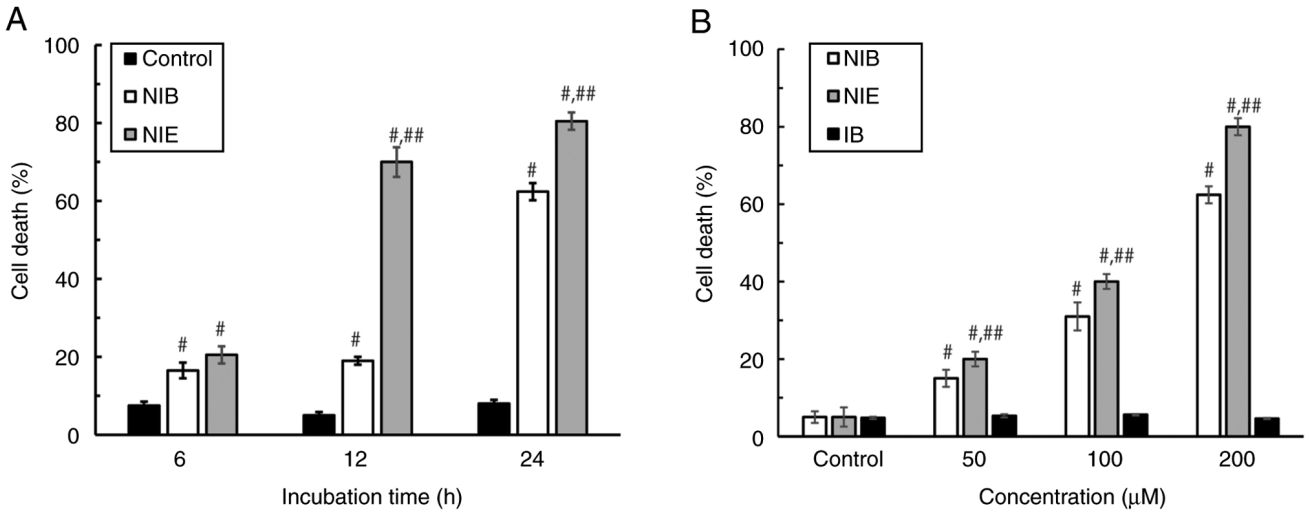

Figure 4. Cell death induction by NIB and NIE of human pancreatic cancer cells. BxPC cells were cultured with 200 μM of NIB or NIE for (A) 6, 12 and 24 h and with (B) 0, 50, 100 or 200 μM of IB, NIB or NIE for 24 h. The percentage of cell death was quantified using a Muse® Cell Analyzer (Cytek Biosciences). Data are the mean ± SD (n=3). $^{\#}$P<0.05 vs. (A) control or (B) IB; $^{\#\#}$P<0.05 vs. NIB. NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; IB, ibuprofen.

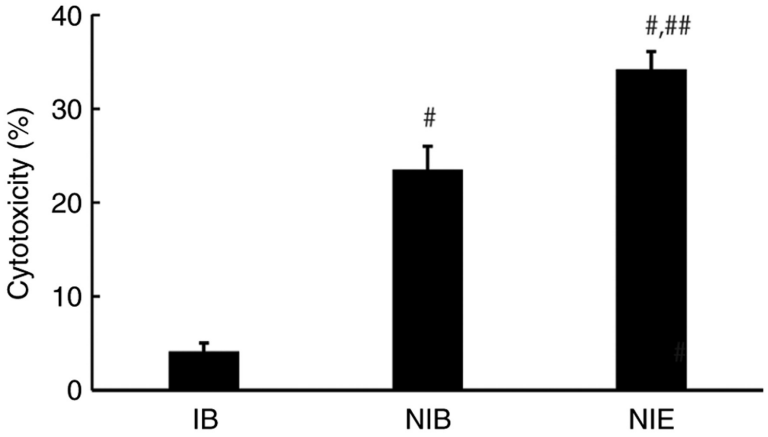

Figure 5. Cytotoxicity of NIB and NIE in human pancreatic cancer cells. Cytotoxicity was evaluated by measuring the degree of lactate dehydrogenase activity in cell culture medium at 72 h after adding 200 μM of NIB or NIE to BxPC3 cells. Data are the mean ± SD (n=3). $^{\#}$P<0.05 vs. IB; $^{\#\#}$P<0.05 vs. NIB. NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; IB, ibuprofen.

displacement experiments were performed using WF and DNSS, which are fluorescent probes for the drug binding sites I and II, respectively (Fig. 2). Although the degree of substitution was different, only DNSS was significantly substituted with both NIB and NIE. These results suggested that both NIB and NIE bind specifically to site II of HSA, as does IB.

NO release from NIB and NIE. Generally, NO donors liberate NOx by hydrolysis in aqueous solution (22). Therefore, the release of NOx from NIB and NIE in PBS was measured (Fig. 3A). Although the release of NOx from NIB was observed immediately after dissolution, no release of NOx from NIE was observed over the 48 h time period measured. Since the RPMI1640 medium used for culturing BxPC cells contains a large amount of nitrate ions, it is difficult to measure intracellular NO. Therefore, whether NIB and NIE released NO within cells was investigated. NO from NIB or NIE in BxPC3 cells was confirmed using DAF-FM DA, a cell membrane-permeable, NO-specific fluorescence probe. It was demonstrated that both NIB and NIE released NO within the cells (Fig. 3B). This result potentially suggests that a factor, such as an intracellular hydrolase, may be involved in the release of NO from NIE.

Effects of NIB and NIE on induction of cell death. To evaluate the cytotoxicity of NIB and NIE against the BxPC3 pancreatic cancer cell line, annexin-positive cells were detected after the addition of NIB or NIE. It was demonstrated that both NIB and NIE significantly induced cell death in both a time-(Figs. 4A, S2) and concentration-dependent manner (Figs. 4B, S2), whereas IB had no significant effect on cell death. Next, an LDH assay was performed to evaluate cell membrane damage caused by NIB or NIE (Fig. 5).

To investigate the mechanism of cell death induction by NIB and NIE, the effect of the nonspecific caspase inhibitor z-VAD FMK on cell death was investigated (Fig. 6A). Cell death

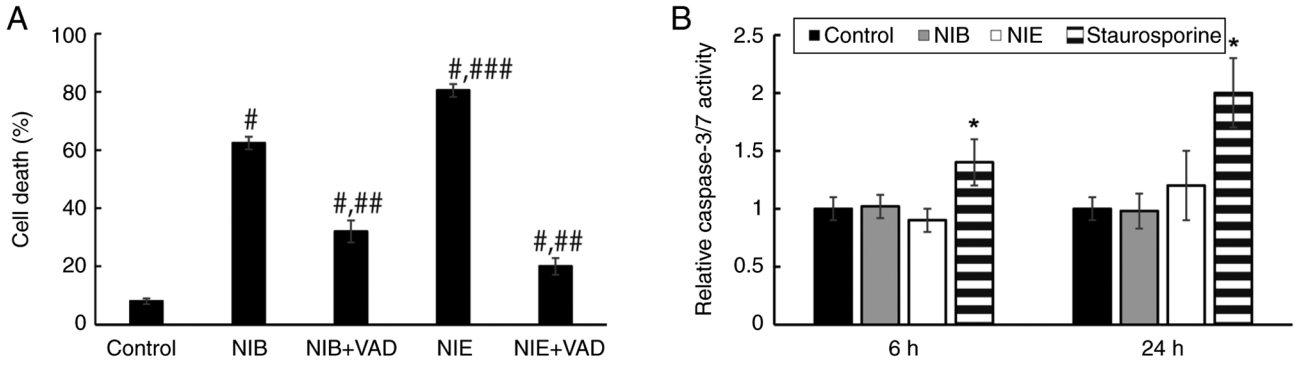

Figure 6. Involvement of caspase in cell death caused by NIB and NIE. (A) Cells were treated with 200 μM of NIB and NIE in the presence or absence of the non-specific caspase inhibitor z-VAD FMK for 24 h. The percentage of cell death (%) was quantified using a Muse® Cell Analyzer (Cytek Biosciences). (B) Activation of caspase 3/7 in BxPC3 cells was measured 6 and 24 h after treatment with 200 μM of NIB or NIE and 6 h after treatment with 1 μM of staurosporine. Data are mean ± SD (n=3). $^{\#}$P<0.05 vs. control; $^{\#\#}$P<0.05 vs. NIB or NIE alone; $^{\#\#\#}$P<0.05 vs. NIB alone; *P<0.05 vs. control. NIB, nitrated ibuprofen benzyl linker; NIE, nitrated ibuprofen ethyl linker; VAD, z-VAD FMK.

induced by both NIB and NIE was significantly suppressed in the presence of z-VAD FMK compared with cells not treated with z-VAD FMK. However, no significant activation of caspase 3 and 7, which serve important roles in apoptosis, was observed in cells treated with NIB and NIE at 6 and 24 h, despite treatment with staurosporine, a representative activator of caspase 3/7 (Figs. 6B and S1). These results suggested that NIB and NIE induced cell death through a non-caspase 3/7 pathway.

Discussion

Pancreatic cancer tumors have low blood flow and abundant interstitial tissue, which makes drug delivery difficult to these tumors, resulting in a low response rate to chemotherapy (5,6). The aim of the present study was to address these problems by focusing on NO donors that had vasodilatory and cytotoxic effects on tumors and the surrounding stromal tissues. To efficiently reach the tumor environment, nitrated forms of IB, an HSA-binding drug, were synthesized to take advantage of the ability of albumin to remain in blood for a long time and accumulate in pancreatic tumors (18).

To confirm the albumin-binding properties of NIB and NIE, quantitative and qualitative substitution experiments for IB were performed. Both NIB and NIE significantly and competitively inhibited the binding of IB to HSA, reducing its binding constant, without affecting the number of binding sites. In fluorescence displacement experiments, IB, NIB and NIE significantly displaced the site II fluorescent probe DNSS. These results suggested that IB, NIB and NIE bound to site II. However, the degree of substitution with NIE was smaller compared to that with NIB. This result showed the same trend as the quantitative substitution experimental data. As the binding of HSA to IB may to involve an interaction between the carboxyl group of IB and HSA, some steric hindrance may have occurred in the case of NIE.

Release of NO from NO donors generally occurs in aqueous solutions (22). Released NO is immediately oxidized under aerobic conditions to NO_2^- and NO_3^-. In a weakly acidic and anaerobic environment, such as a tumor, released NO is not oxidized and exerts its cytotoxic effect (11). Although NIE did not release NO in aqueous solution in the present study, NO was demonstrated to be released in cells. This suggested that NO release from NIE may occur only in the intracellular environment, where the presence of hydrolytic enzymes and other factors may be involved. Similarly, NONOate prodrug is stable in solution, but releases NO following esterase activity within cells, causing apoptosis of human leukemia cell lines (23). In addition, NIE could be considered to have superior characteristics to NIB because NIE does not release NO in aqueous solutions, such as the blood. If NO is released in the blood, it will quickly be removed from the blood. However, since NIE may release NO only inside cells, NIE may be able to reach tumors without releasing NO in the blood, liberate NO after being absorbed into the cells and exert its antitumor activity.

In the present study, the cell death-inducing effects of NIB and NIE were both significantly inhibited by the caspase non-specific inhibitor, z-VAD FMK, but no activation of caspase 3/7 was observed. Necroptosis and pyroptosis are two regulated cell death pathways that exhibit morphological characteristics of necrosis, such as cell membrane pore formation, and membrane collapse. Therefore, several caspases, such as caspase 1, 4, 5, 8 and 11, which are involved in cell death events, may be involved in the induction of cell death in NIB and NIE (24). Further investigation is needed to clarify the involvement of these caspases and the mechanisms of cell death. Furthermore, the details of the mechanism of cell death induction by other NO donors have also not been clarified. It has been reported that nitrated aspirin increases oxidative stress by nitrosylating the cysteine residues of reduced glutathione in cells (25). It has also been reported that NO donors induce cell death by nitrosylating the tyrosine residues of proteins that are important for cell survival, such as p50, NF-kB, cytochrome C and ribonucleotide reductase (26,27).

In conclusion, newly synthesized NIB and NIE exhibited HSA-binding properties equivalent to IB. Although both NIB and NIE had NO-release properties, their release mechanisms were significantly different and NIE was stable in aqueous solution. Furthermore, it was shown that both NIB and NIE significantly induced the cell death of human pancreatic cancer cells in a caspase 3/7-independent manner. The present study suggested that NO could be effective in cancer therapy and both NIB and NIE may potentially be future candidate compounds for pancreatic cancer therapeutics.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KN contributed to the design of this study, data collection and interpretation and wrote the initial draft of the manuscript. YA, NS, TB, KT, AT, RK and KT contributed to data collection. SI and HM contributed to the synthesis and the structural validation of NIB and NIE. MO and KY contributed to the design of this study, interpretation and critically reviewed the manuscript. All authors approved the final version of the manuscript. KN and KY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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